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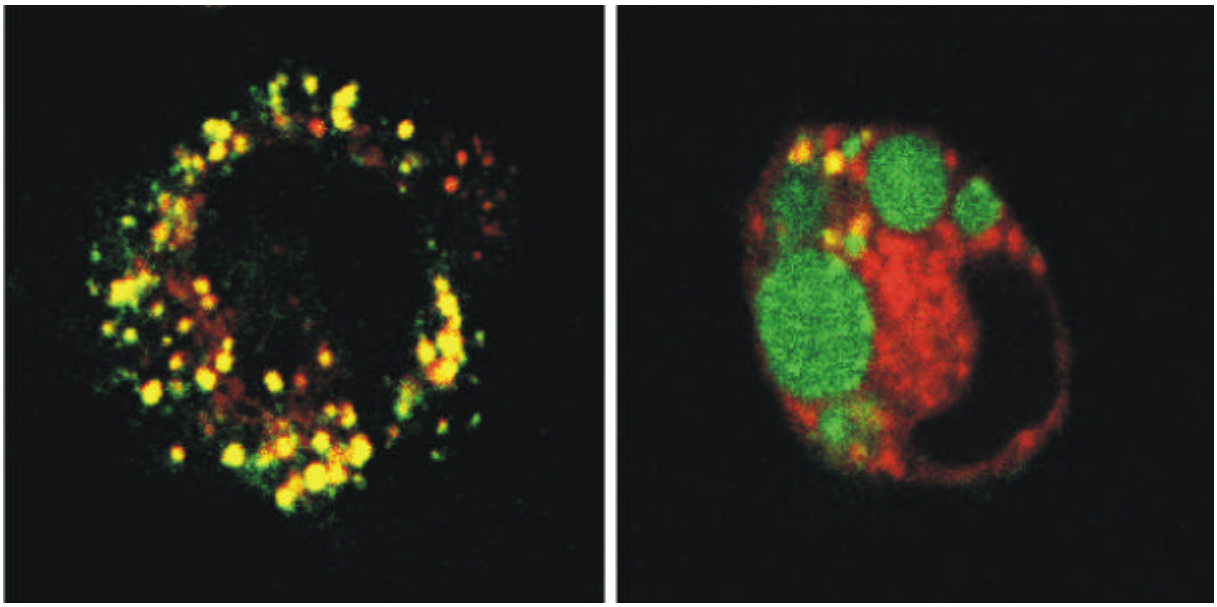
Azithromycin, a pharmacological agent which slectively impairs some pathways of endocytosis: characterization, interests and mechanism of action



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ABREVIATION LIST

AFM	atomic force microscopy
AIDS	acquired immunodeficiency syndrome
AP	adaptor protein
ARF	ADP-rybosylation factor
BHK cells	Baby Hamster Kidney cells
CALM	clathrin assembly lymphoid myeloid leukemia
CCV	clathrin-coated vesicle
CHC	clathrin heavy chain
CHO cells	Chinese Hamster Ovary cells
CI-MPR	cation-independent mannose-6-phosphate receptor
CLC	clathrin light chain
CLIP-170	cytoplasmic linker protein-170
C_6-NBD-SM	C_6-nitrobenzoxadiazol-sphingomyelin
COPI	coat protein complex I
COPII	coat protein complex II
CR	complement receptor
DOPC	dioleylphosphatidylcholine
DPPC	dipalmytoylphosphatidylcholine
ECV	endosomal carrier vesicle
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EH	Eps15 homology domain
EM	electron microscopy
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
FcR	Fc receptor
FcãR	receptor for the IgG Fc fraction
FITC	fluorescein isothyocyanate
FM	fluorescence microscopy
FYVE-finger	cysteine-rich zinc-finger like
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GED	GTPase effector domain
GEF	guanine nucleotide exchange factor
GlcCer	glucosylceramide
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GPI	glycosylphosphatidylinositol
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF2	insulin-like growth factor 2
IgG	immunoglobulin G
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor-based inhibition motif

LAP	lysosomal acid phosphatase
Lamp	lysosome-associated membrane protein
Limp	lysosomal integral membrane protein
LBPA	lysobisphosphatidic acid
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
Lgp	lysosomal membrane glycoprotein
LL	dileucine
LPS	lipopolysaccharide
LPSR	lipopolysaccharide receptor
LY	lucifer yellow
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
MLS	macrolide-lincosamide-streptogramin
MPR	mannose 6-phosphate receptor
MP	mannose 6-phosphate
MR	mannose receptor
MVB	multivesicular body
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NBD	nitrobenzoxadiazol
NEM	N-ethylmaleimide
NHE	Na^+/H^+ exchanger
NPA	Niemann-Pick syndrome type A
NPB	Niemann-Pick syndrome type B
NPC	Niemann-Pick syndrome type C
NPF	asparagine-proline-phenylalanine
NRK	Normal Rat Kidney
N-Rh-PE	*N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine
NSF	N-ethylmaleimide-sensitive factor
PA	phosphatidic acid
PAP	peroxidase-anti-peroxidase complex
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PEG-Chol	poly (ethylene glycol) cholesteryl ether
PG	phosphatidylglycerol
PH	pleckstrin homology
PI	phosphoinositides
3-PI	3-phosphoinositide
PI 3-P	phosphatidylinositol 3-phosphate
PI 3,4-P_2	phosphatidylinositol 3,4-bisphosphate
PI 3,4,5-P_3	phosphatidylinositol 3,4,5-trisphosphate
PI 4-P	phosphatidylinositol 4-phosphate
PI 4,5-P_2	phosphatidylinositol 4,5-bisphosphate
PI kinase	phosphatidylinositol kinase
PKC	protein kinase C
PLA_1	phospholipase A_1

PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PM	plasma membrane
PMN	polymorphonuclear neutrophile
PRD	proline-rich domain
PS	phosphatidylserine
PtdIns	phosphatidylinositol
Q-SNARE	SNARE providing glutamines (Q)
RBC	red blood cell
R-SNARE	SNARE providing an arginine (R)
RTK	receptor-tyrosine kinase
SAC	subapical compartment
SH3	Src homology domain
SM	sphingomyelin
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
TGN	trans-Golgi network
Tf	transferrin
TfR	transferrin receptor
TK	tyrosine kinase
TKR	tyrosine kinase receptor
TMA-DPH	1-((4-trimethylamino)phenyl)-6-phenyl-hexa-1,3,5-triene
t-SNARE	target SNARE
VAMP	vesicle associated membrane protein
v-SNARE	vesicular SNARE
Y	tyrosine

PRELIMINARY COMMENTS

Endocytosis is required for a vast number of functions that are essential for the well being of the cell. Before the advent of electron microscopy and molecular biology, endocytosis was largely defined by the nature of the material taken up by the cell, and the terms phagocytosis and pinocytosis were adopted to describe the uptake of large particles and extracellular medium respectively. However, the situation was more complex and three modes of endocytosis were described: fluid-phase, non-specific adsorptive, and receptor-mediated. The ways and modes of endocytosis are described in section 1. In addition, multiple internalization mechanisms are clearly established, and discussed in the introduction and defined as follows (see section 2): (i) clathrin-dependent endocytosis, i.e. endocytosis mediated by small vesicles that have a morphologically characteristic coat made of complex proteins that include clathrin; (ii) macropinocytosis, i.e. the formation of vesicles that internalize large volumes of fluid; (iii) caveolae, i.e. small flask-shaped invaginations of the plasma membrane seen in many but not all cell types and which are implicated in endocytosis only under special inducing conditions; (iv) dynamin- and clathrin-independent endocytosis; and (v) phagocytosis, i.e. the uptake of large particular ligands including bacteria and apoptotic cells.

After their internalization, the material passes through the endocytic compartments. The main compartmental boundaries of the endocytic pathway discussed in the introduction are defined as follows (see section 3): (i) early endosomes, i.e. the first station on the endocytic pathway which receives most types of vesicles coming in from the cell surface; (ii) late endosomes, which receive internalized material en route to lysosomes, from early endosomes or the trans-Golgi network (TGN); (iii) lysosomes, i.e. the last compartment of the endocytic pathway which is the main hydrolytic compartment of the cell; and (iv) phagosomes which receive most types of particles coming in from the cell surface.

The distinct internalization mechanisms (section 2) and the distinct compartments of the endocytic pathway (section 3), imply vesicle budding and scission processes. The driving force for membrane budding can arise from at least four different biological mechanisms: the polymerization of cytosolic coat proteins binding to integral membrane constituents, the interaction with a cytoskeletal element capable of driving budding, the adhesion of the membrane to an already curved particle, and the insertion of additional monolayer or bilayer membrane.

Membrane budding is followed by the physical separation of the bud from the donor membrane, a process termed fission. Fission can be the ultimate consequence either of the very same mechanism that mediates budding or be distinct. Section 4 first describes the mechanism of membrane budding, focusing on the driving force resulting from coat assembly. Emphasis is placed on coat assembly on the cell surface; other coats involved in the endocytic pathway are more briefly described. The second part of section 4 describes scission mechanism.

Once formed, the vesicle will be targeted to the correct membrane destination, and this involves a large set of proteins, to efficiently carry out all steps involved in delivering a newly formed transport vesicle to its target. Targeting requires: (i) the actin- and tubulin-based cytoskeleton to transport a vesicle from one part of the cell to another; (ii) proteins to collect and restrain vesicles at or near their cognate target membranes (tethering); (iii) a core layer of proteins which interact to bring vesicle membranes in close apposition (docking); and (iv) thereby driving membrane fusion. These processes are described in section 5.

Within the endocytic organelles, the internalized membranes are processed and some components such as particular types of receptors, fluid and lipids are sorted for recycling to the cell surface, while others are selected for delivery to late endocytic compartments where they may be degraded. Sorting is a key requirement of the endocytic apparatus since not all of the membrane constituents are directed at the same rate or into the same endocytic vesicle and since the constituants internalized by the same route end up into different destinations. The traffic of macromolecules within and through vesicular organelles is directed both by physical sorting, which implies the physical properties (shape and pH) and dynamics of the organelles (regulation of fusion and vesicle formation), as well as by signal sorting, which implies information contained in the structure of the macromolecule. Sorting mechanisms during endocytosis are described in section 6.

Membranes play a central role in both the structure and function of cells. Membranes basically define compartments, each membrane associated with an inside and an outside. But, membranes not only define compartments, they also determine the nature of all communication between the inside and the outside. In this context, endocytosis, one of the fundamental functions in cells, involves membranes. Indeed, several membrane properties, such as fluidity and asymmetry, are involved at distinct stages of endocytosis, including vesicle formation and sorting, and vesicle movement. In addition, lipid metabolism plays important roles in membrane

trafficking, including pinocytosis, phagocytosis, signal transduction, budding and fusion processes. All these characteristics of membranes and their roles in endocytosis are described in section 7.

Since the control of the internal pH of endosomal and phagosomal compartments plays a role in membrane traffic, section 8 discusses the acidification of these compartments and the machineries involved in this process. Since several drugs, including azithromycin, are able to accumulate in acidic compartments, we also discuss the mechanism of acidotropic accumulation. Finally, in section 9, a few comments is made on the cytoskeleton and its role in organelle distribution and motility in pinocytosis and phagocytosis.

A number of internalization mechanisms is described and several functions are ascribed to each. However, there is still a multitude of questions. In this context, pharmacological agents could be usefull to dissect pinocytic and phagocytic pathways. Agents perturbing endocytosis are numerous. In section 10, we will briefly present some inhibitors which were classified into 5 categories, based on their capacity to interfere with one or more machineries involved in pinocytic and phagocytic trafficking. These categories include: (i) agents perturbing endosome/lysosome acidification; (ii) those inducing lysosomal overloading; (iii) those interacting with membranes and/or perturbing membrane properties; (iv) enzyme modulators; and (v) agents perturbing microtubules and microfilaments.

However, a few of these agents is really specific of one pathway and/or one step of endocytosis. In view of the lack of specific inhibitors, in particular of clathrin-independent endocytosis, we were interested by the macrolide antibiotic azithromycin, based on the facts that this drug: (i) has a marked amphiphilic character and extensively accumulates in lysosomes of cultured fibroblasts (Carlier et al, 1994; Montenez et al, 1996); (ii) induces a lysosomal phospholipidosis by inhibition of the lysosomal phospholipase A_1 (PLA_1) activity (Van Bambeke et al, 1996); (iii) is able to bind to negatively charged bilayers at acidic pH (Montenez et al, 1996); and; (iv) is able to perturb the fusion of phospholipid-overloaded lysosomes with horseradish peroxidase (HRP)-containing endosomes (preliminary observation in electron microscopy; Van Bambeke et al, 1996).

CHAPTER 1. INTRODUCTION

1. Modes of endocytosis

Mammalian cells have developed a variety of mechanisms to internalize small molecules, macromolecules and particles and target them to specific sealed organelles within the cytoplasm. These mechanisms are called endocytosis, a key process found in all eukaryotes.

Endocytosis is involved in: (i) the uptake of extracellular nutrients and the cellular cholesterol homeostasis, (ii) the regulation of membrane dynamics and especially the modulation of the plasma membrane composition, (iii) the reactivity to extracellular stimuli by desensitizing, down-regulating, or recycling of receptors and membrane proteins, (iv) the maintenance of cell polarity, (v) the antigen presentation, (vi) the removal of dead cells (for reviews, see Mukherjee et al, 1997; D'Hondt et al, 2000; Marsh, 2001). Moreover, various endocytic pathways are exploited by pathogens, including viruses, microorganisms and toxins. Endocytosis is also involved in pathological processes, including atherosclerosis and Alzheimer's disease (for a review, see Mukherjee et al, 1997).

The forms of endocytosis can be classified into two general types: phagocytosis (or "cell eating"), in which large particles are internalized, and pinocytosis (or "cell drinking"), which refers to the uptake of fluid. The latter process comprises both a clathrin-dependent endocytic route and a clathrin-independent pathway (for reviews, see Mukherjee et al, 1997; D'Hondt et al, 2000; see also section 2).

Three modes of endocytosis have been described. The first mode is fluid-phase endocytosis, i.e. the entry of solutes without binding to the pericellular membrane (Steinman et al, 1974). In this non-specific process, the uptake is weak, similar for different tracers, and shows a linear uptake over a large range of concentration. Fluid-phase endocytosis is not specific of one primary endocytic pit, but tracers could rather be endocytosed via clathrin-coated and non-clathrin coated invaginations of the plasma membrane as well as by macropinocytosis (see section 2). HRP is a commonly accepted tracer of fluid-phase endocytosis. It was originally used to demonstrate fluid-phase endocytosis in various animal tissues (Straus, 1967) and has allowed to quantitate and analyse endocytosis in details in numerous types of cultured cells (Steinman et al, 1974; Draye et

al, 1988; Cupers et al, 1994; 1997; Tyteca et al, 2001). Incubation with this tracer at concentrations far above saturation of its adsorptive component provides a linear uptake over a large range of concentration (Cupers et al, 1994; Tyteca et al, 2001), which is similar to lucifer yellow (LY), another fluid-phase tracer (Swanson et al, 1987; Tyteca et al, submitted).

The second mode is the non-specific adsorptive endocytosis. This process involves the recognition by solutes or particles of non-specific sites on the plasma membrane, by hydrophobic interaction (membrane tracers), carbohydrate-protein interaction (selectins, mannose receptors [MRs]) or charge interaction (cationized ferritin which binds to the cell surface and was taken up in endocytic vesicles [Ottosen et al, 1981]). For example, a carbohydrate-protein interaction is involved in HRP endocytosis. Binding sites for HRP were detected on the surface of many cultured and isolated mammalian cells. However, these sites were saturated at low tracer concentration (Straus and Keller, 1986). Examples of membrane tracers that are endocytozed by a non-specific process also called bulk membrane endocytosis (a process which designates endocytosis of membrane tracers that are not specifically concentrated into clathrin-coated pits) are C_6-nitrobenzoxadiazol-sphingomyelin (C_6-NBD-SM), 1-((4-trimethylamino)phenyl)-6-phenyl-hexa-1,3,5-triene (TMA-DPH) and *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (*N*-Rh-PE) (Kok et al, 1990; Mayor et al, 1993; Cupers et al, 1994).

The third mode of endocytosis is receptor-mediated. In this type of endocytosis, a ligand recognizes and specifically binds to its cell surface receptor and the ligand-receptor complex is internalized. Transferrin (Tf) is a commonly accepted tracer of receptor-mediated endocytosis. Immunoglobulin G (IgG)-containing immune complexes and peroxidase-anti-peroxidase (PAP) immune complexes are also taken up by receptor-mediated endocytosis (Mellman and Plutner, 1984; Kiss and Röhlich, 1984). This process is involved in the regulation of cell-surface receptor expression. Receptor-mediated endocytosis is a saturable process, and the proteins responsible for concentrating receptors in clathrin-coated pits are obvious candidates for the saturable component. Although it was generally accepted that receptor-mediated endocytosis involves only clathrin-coated pits, this concept is under change, since some receptors, such as the interleukin-2 receptor and some G protein-coupled receptors (GPCRs), have been shown to be internalized by a clathrin-independent pathway (for a review, see Dautry-Varsat, 2001; see section 2.2.4.). Receptor-mediated endocytosis could be tightly regulated, by the level of receptor expression, by possible redistribution of an intracellular vesicular pool (Buys et al, 1984), and by the affinity of the interaction.

Table 1 shows the different pinocytic and phagocytic tracers used in this study and their modes of internalization and pathways as well as the characteristics of binding and uptake.

pinocytic and phagocytic tracers	MW/size	mode of endocytosis	plasma membrane (PM) binding	internalization mechanism (see also section 2)	destination (see also section 3)	binding and uptake in function of extracellular tracer concentration	references
HRP	44 kDa	fluid pinocytosis	MR saturated at low tracer concentration	clathrin-dependent and independent	lysosomes	weak, linear uptake	Steinman et al, 1974; Cupers et al, 1994; 1997
LY	457 Da	fluid pinocytosis	no	clathrin-dependent and independent	lysosomes	weak, linear uptake	Swanson et al, 1987
Tf	~ 100 kDa	receptor pinocytosis; constitutive process	transferrin receptor (TfR)	clathrin-dependent	recycling to the cell surface	saturable binding and uptake	Dautry-Varsat et al, 1983; Watts, 1985; Rothenberger et al, 1987
PAP immune complexes	~ 380 kDa	receptor pinocytosis; ligand-induced process	receptor for the IgG Fc fraction (FcãR)	clathrin-dependent	lysosomes	saturable binding and uptake	Mellman and Plutner, 1984; Kiss and Röhlich, 1984
latex beads	1µm 0.1µm	phagocytosis	?	phagocytosis	lysosomes	linear uptake with rate increasing with bead size	Pratten and Lloyd, 1986
C_6-NBD-SM	741 Da	bulk-membrane pinocytosis	external leaflet	clathrin-dependent and independent	recycling to the cell surface	linear PM insertion	Koval and Pagano, 1989; Mayor et al, 1993; Cupers et al, 1994
TMA-DPH	461 Da	bulk-membrane pinocytosis	deep domain of the bilayer	clathrin-dependent and independent	lysosomes and recycling	first, linear PM insertion, then selfquenching	Illinger et al, 1990; 1991; Cupers et al, 1994; Kaiser and London, 1998
***N*-Rh-PE**	1260 Da	bulk-membrane pinocytosis	in aggregates	clathrin-dependent and independent	lysosomes	linear PM insertion	Kok et al, 1990

Table 1. Pinocytic and phagocytic tracers used in this study.

2. Internalization mechanisms

In addition to the well-documented clathrin-mediated endocytic route, at least three distinct non-clathin-coated internalization mechanisms act at the surface of mammalian cells: macropinosomes, caveolae and other structures (micropinosomes). Thus, with phagocytosis, we have five types of primary endocytic pits and vesicles. Macropinocytosis and phagocytosis are generally transient features, expressed by specialized cells. In all other cells, the possible occurence of distinct constitutive pinocytic pathways could allow cells to independently internalize bulk and specific membrane constituents.

This section briefly describes, for each of these five internalization mechanisms, the structures involved, the fate of tracer internalized and the role played by the pathways. Figure 1 focuses on internalization mechanisms and pathways of endocytosis used in this study.

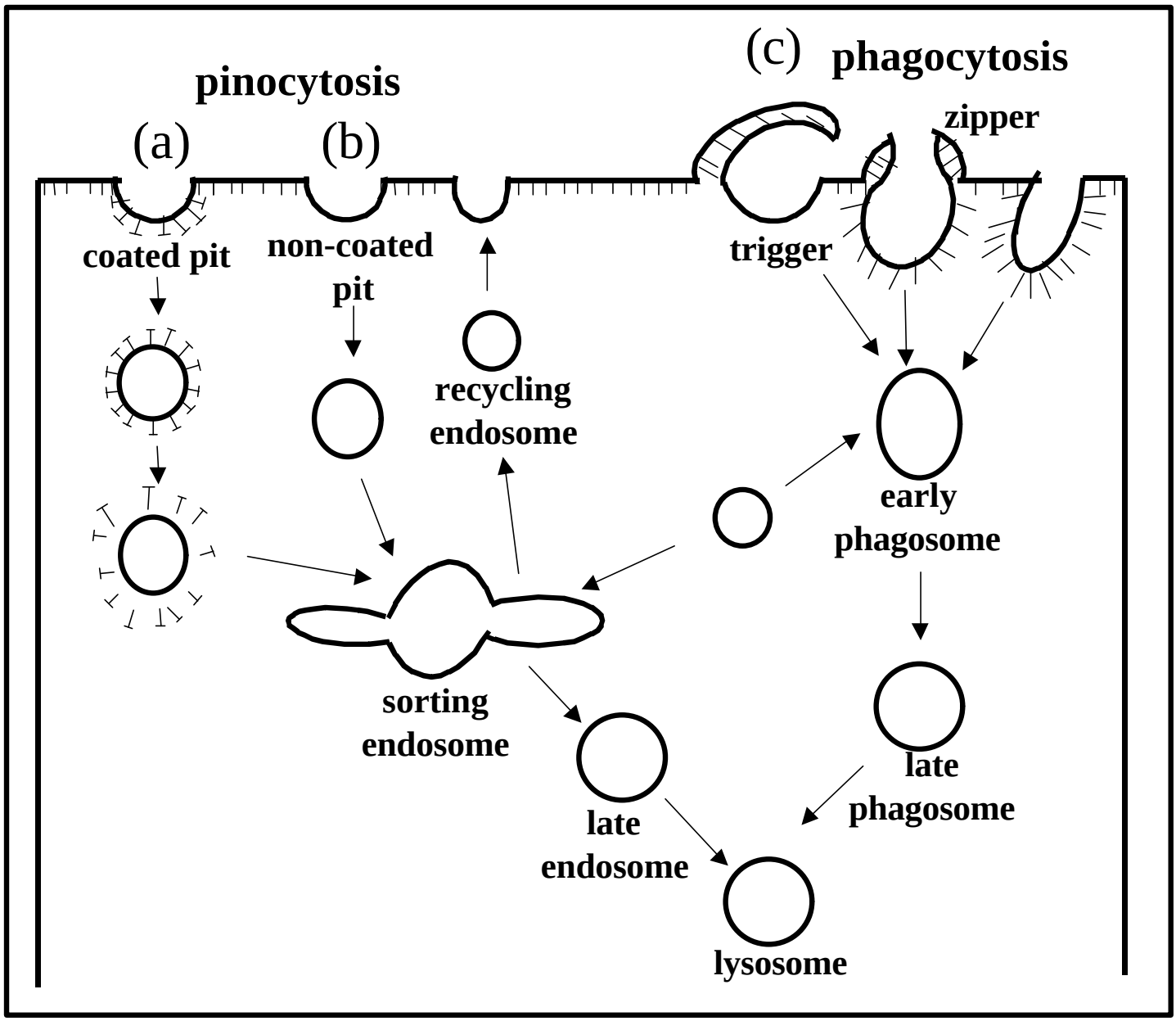


Figure 1. Pathways of endocytosis used in this study: (a) pinocytosis mediated by clathrin-coated pits (see section 2.1.); (b) pinocytosis mediated by non-clathrin-coated pits (see section 2.2.4.); (c) phagocytosis by different modes (trigger and zipper modes; see section 2.3.4.).

2.1. Clathrin-dependent endocytosis

Clathrin-mediated endocytosis allows for internalization by receptor-mediated endocytosis, which takes place in all nucleated vertebrate cells. Receptors are endocytosed by their capture in clathrin-coated pits, which are 100-150 nm invaginated structures and occupy approx. 2 % of the plasma membrane in human fibroblasts. Proteins that enter the cell by receptor-mediated endocytosis are cleared from the cell surface rapidly, due to their efficient concentration in clathrin-coated pits. These pits can concentrate a large number of receptors responsible for receptor-mediated internalization of ligands, such as Tf, low-density lipoprotein (LDL), growth factors, antibodies, and many others. For example, up to 60-70 % of all cell surface low-density lipoprotein receptors (LDLRs) are located in the coated pits in human fibroblasts (for a review, see Mukherjee et al, 1997). The interaction of clathrin, receptors and associated structural and regulatory molecules are essential in this process and will be discussed in section 4.

Clathrin-mediated endocytosis allows cells to internalize proteins and other biomolecules from their environment via corresponding specific receptors. By this process, cells take up nutrients, modulate the expression of cell surface receptors or molecules, and control their response to extracellular stimuli. For example, clathrin-mediated endocytosis plays a role in the clearance of lipoproteins from the blood stream or in endocytosis of foreign antigens and subsequent processing and presentation, which are essential for the cellular immune response. For the latter, cells are sampling the environment for soluble antigens by internalizing large amounts of fluid via macropinocytosis. During their maturation, antigens become proteolytically processed, bound to the major histocompatibility complex (MHC) class II molecules, and transported to the cell surface to stimulate cytotoxic T cells (for reviews, see Mukherjee et al, 1997; Liu et al, 2001; Maniak, 2001; see also section 2.2.2.).

The study of clathrin-coated vesicles (CCVs) comes from morphological and biochemical studies. Moreover, to dissect the process of coated-vesicle formation *in vitro*, isolated membrane fractions and semi-intact or perforated cells were used as models. To study CCV formation *in vivo*, genetic manipulation, application of dominant-negative mutants (the dynamin K44A mutant, dominant negative clathrin phenotype, mutants in Eps15 and epsin; see section 4), and fluorescently tagged molecules to trace assembly dynamics were used (for a review, see Liu et al, 2001).

The observation that LDL and many other ligands are internalized through clathrin-coated pits has led to the general view that receptor-mediated endocytosis and clathrin-mediated endocytosis being considered as one and the same. However, this is not always the case and there are several examples of receptor-mediated internalization that are not clathrin-dependent, such as phagocytosis and the internalization of the interleukin-2 receptor. Conversely, fluid-phase internalization can be mediated by CCVs (for a review, see Marsh, 2001).

2.2. Clathrin-independent endocytosis

2.2.1. Indirect arguments

Evidence for non-clathrin-coated pit endocytosis first came from morphological studies. A first approach was the examination of non-coated pits at the cell surface. However, although the characterization of clathrin-coated structures largely benefited from the characteristic coats seen by electron microscopy, the absence of a coat made it difficult to distinguish an endocytic activity from smooth areas of the plasma membrane. A second approach consisted of the study of several receptors and ligands in electron microscopy after examination of cells labelled at 4°C and of cells labelled at 4°C and then warming them to 37°C for different times. This technique has allowed to evidence that cholera and tetanus toxins were internalized in non-coated pits and vesicles. A third approach was the use of gold-labelled concanavalin A which was bound to cells at 4°C, after which cells were warmed at 37°C for a short time and fixed. Then the fixed cells were incubated with anti-concanavalin A-HRP to distinguish truly free (gold labelled) endocytic vesicles from surface-connected structures. This has revealed the presence of uncoated vesicles, about as abundant as coated vesicles, but slightly smaller. Although these approaches could not definitively demonstrate the existence of clathrin-independent endocytosis, it brought together many data in favour of its existence (for a review, see Dautry-Varsat, 2001).

Another approach is to study the internalization of fluid-phase tracers under conditions which inhibit CCV formation. This has been done through controlled perturbations that include incubation in hypertonic media (Cupers et al, 1994), potassium depletion (Cupers et al, 1994), and cytosol acidification (Sandvig et al, 1987). Potassium depletion, in combination with hypotonic shock, inhibits the assembly of coated pits, and upon exposure to hypertonic media, normal clathrin lattices underlying the plasma membrane disappeared and were replaced by accumulation of numerous microcages of clathrin. Both these treatments led to an inhibition of receptor-

mediated internalization, while neither internalization of a fluid phase tracer, nor that of two lipid tracers were affected. However, there was an accelerated regurgitation of the fluid-phase tracer. Another perturbation involves acidification of the cytosol, which causes an inhibition of the pinching off of coated vesicles. This treatment reduced the endocytic uptake of Tf and epidermal growth factor (EGF), and increased the number of Tf binding sites at the cell surface. In contrast, acidification of the cytosol had only little effect on the uptake of ricin and the fluid phase marker LY. Although several studies support the existence of clathrin-independent pathways, these methods may sometimes be difficult to integrate because their effects are variable between cell types. Moreover, the treatments are unspecific and may affect other pathways. However, these allowed to suggest that clathrin polymerization is necessary to concentrate receptor-bound ligands in primary endocytic vesicles, but superfluous to the basic endocytic machinery in rat foetal fibroblasts (Cupers et al, 1994).

More recently, dominant-negative mutants have been obtained that provide means to inhibit clathrin-mediated internalization. Among these is a dominant negative form of clathrin, but this mutant is not a specific inhibitor of clathrin-mediated internalization. Adaptor protein (AP)-2 mutant may turn out to very specifically inhibit clathrin-mediated endocytosis of receptors interacting with AP-2 and not using other adaptors such as arrestins. Mutants of proteins binding to AP-2 complex, such as Eps15, epsin, amphiphysin and arrestin, also exist (for a review, see Dautry-Varsat, 2001; see section 4). Expression of a temperature-sensitive mutant of dynamin (Damke et al, 1995) or a mutant dynamin defective in GTP binding and hydrolysis (Simpson et al, 1998) have been extensively used. Using these, endocytosis of receptors internalized by coated pits was inhibited. However, the problem is to establish how specific these methods may be. For example, dynamin mediates the budding of several different forms of nascent vesicles, such as caveolae (see section 2.2.3.). It is also involved in the clathrin- and caveolae-independent internalization of some receptors, in phagocytosis, and in later stages of endocytosis. Therefore, if endocytosis of ligands or receptors is inhibited in cells overexpressing a dynamin mutant, one cannot conclude that they are internalized via CCVs (for a review, see Dautry-Varsat, 2001; see section 4.2.).

Whereas it seems clear now that non-clathrin-coated pit uptake occurs in many cell types, the structure (s) involved and underlying machineries are poorly understood, and it is difficult to estimate the relative volume or membrane surface area taken up by these mechanisms (see Mukherjee et al, 1997). Although the initial steps taking place at the plasma membrane are

beginning to be described in a few cases, those taking place thereafter are not. No specific inhibitors are available, and specific tracers are still needed (for a review, see Dautry-Varsat, 2001).

2.2.2. Macropinocytosis

Macropinocytosis is a natural feature of professional phagocytic cells but a rare event in other cell types. It occurs constitutively in unstimulated macrophages, some antigen presenting cells such as dendritic cells, osteoclasts and many tumour cells. For example, macropinocytosis is a constitutive feature in fibroblasts transformed by oncogenes Src and Ras (Amyere et al, 2000). In many cells, macropinocytosis can be transiently induced by a variety of stimuli such as macrophage colony stimulating factor (M-CSF) or growth factors (EGF or platelet-derived growth factor [PDGF]). The growth factors known to stimulate macropinocytosis in the mammalian system act through the class of receptor-tyrosine kinases (RTKs), a signalling pathway which is particularly sensitive to mutations. One of the immediate targets of membrane-associated tyrosine kinases (TKs) is the small GTPase Ras (for a review, see Maniak, 2001).

Macropinocytosis is a non-clathrin mediated endocytic pathway. This process results into the ingestion of large volumes of fluid. It involves large membrane extensions that fuse to form vesicles, membrane ruffles and lamellipodia, therefore enables like FcγR-triggered phagocytosis. Protrusions at the plasma membrane are thought to be the consequence of rearrangements within the actin cytoskeleton and are regulated by Rho GTPase family. Macropinosomes form from membrane ruffles that close to form vesicles that can reach 500-5000 nm in diameter. This pathway can be distinguished from other pinocytic pathways by its susceptibility to agents such as cytochalasin D which depolymerize actin (see Dautry-Varsat, 2001).

In cells belonging to the immune system, such as immature dendritic cells, the biological function of macropinocytosis is obvious, since soluble material is internalized, enzymatically degraded and fragments are presented to the immune system. Another case where macropinocytosis may turn out to be central to its biological function is the osteoclast. These cells mediate bone resorption by secreting protons and enzymes to solubilize minerals and protein components of bone. This process takes place in the resorption lacuna. In order to progress deeper into the bone, the resorption products need to be removed from the lacuna by transcytosis. Interestingly, the membrane facing the lacuna has a characteristic ruffled appearance, and the

ruffles are riched in filamentous actin. The endocytic mechanism involved in bone resorption may be macropinocytosis (for a review, see Maniak, 2001).

Soon after internalization, the coat of filamentous actin dissociates from the macropinosome and makes the membrane available for fusion and fission processes. The fate of macropinosome varies between cell types. Its content can indeed either be recycled to the cell surface or be transported along the endocytic pathway. In macrophages and probably other phagocytic cells, macropinosomes acidify over about 15 min and, during this period, plasma membrane proteins like the TfR are recycled to the cell surface and the size of the macropinosome therefore decreases. Macropinosomes mature by progressive acquisition or loss of characteristic endocytic vesicle markers and finally merge with lysosomes. Thus, macropinosomes progress through macrophages showed features of both the maturation process and vesicle shuttle model of endocytosis, since macropinocytosis begins with a maturation process and ends by fusion with lysosomes (Racoosin and Swanson, 1993). In human epidermoid A431 cells, the fate of macropinosomes is different, since macropinosome content is nor acidified nor delivered to the endosomal system, but rather recycled to the cell surface. However, macropinosomes of these cells are nonetheless dynamic structures, which sometimes exhibit vesiculo-tubular morphology and are capable of homotypic fusion (Hewlett et al, 1994; for a review, see Maniak, 2001). The same observations were made in v-Src-transformed rat-1 fibroblasts, in which macropinosomes do not fuse with Tf-containing endosomes and cyclic AMP selectively promote regurgitation of macropinosomes (Veithen et al, 1998).

2.2.3. Caveolae ?

Another clathrin-independent route is that involving caveolae, which are present in many, but not all, cell types and are very abundant in smooth muscle, adipocytes and capillary endothelial cells (Dautry-Varsat, 2001). However, the existence of caveolae and their roles in endocytosis are currently controversed, and it seems that caveolae could be considered as true vesicles only under special inducing conditions.

Caveolae are small flasked-shaped membrane invaginations that can be distinguished from coated pits by their size (50-80 nm in diameter, compared to 100 nm for coated pits), their morphology and their striated coat reflecting caveolin complexes (Dautry-Varsat, 2001). Caveolae can be considered as specialized forms of raft with a specific form. Caveolin is the major

constituent of caveolae. Caveolin seems to be a cholesterol-binding protein on one hand, and it has been implicated in several signal transduction events on the other hand. Moreover, caveolae have a distinct lipid composition, since they are enriched in glycosphingolipids and cholesterol (see Dautry-Varsat, 2001). A specific glycosphingolipid, GM1, which has been shown to be localized in caveolae, allows cholera toxin to bind to the cell surface (see Mukherjee et al, 1997). Caveolae can be induced in the apical plasma membrane of Madin-Darby canine kidney (MDCK) cells (Verkade et al, 1999).

Several lines of evidence have suggested that caveolae could participate in membrane trafficking: (i) caveolae have the molecular machinery necessary for endocytosis, transcytosis, fusion (*N*-ethyl maleimide-sensitive factor [NSF], vesicular SNARE [v-SNARE]) and fission (cytosol, GTP hydrolysis and dynamin) (see Dautry-Varsat, 2001); (ii) they were proposed to play a role not only in endocytosis but also in transcytosis in endothelial cells, and it has been shown, in permeabilized cells, that GTP stimulated caveolar budding and endocytosis of the cholera toxin B chain to endosomes (Schnitzer et al, 1996); (iii) caveolae can be internalized into A431 cells, in which the glycosylphosphatidylinositol (GPI)-anchored protein, alkaline phosphatase, was clustered in caveolae after antibody-induced cross-linking (Parton et al, 1994); and (iv) they can also be alternative structures in elicited macrophages, in which, when caveolae were selectively inhibited by filipin, the rate of both fluid-phase endocytosis of HRP and receptor-mediated endocytosis of PAP immune complexes were decreased (Kiss and Geuze, 1997). However, filipin is not a selective inhibitor of internalization by caveolae, since it is also able to act in phagocytosis and non-clathrin coated vesicles (see section 10.3).

One way to adress the controversial question of whether caveolae represent structures involved in receptor-mediated endocytosis in non-endothelial cells, involves the use of cell-free systems allowing the formation of endocytic vesicles from isolated membranes. Using isolated plasma membranes from cultured fibroblasts surface-labelled with cholera toxin, it has been demonstrated that caveolae are plasma membrane domains involved in an endocytic process and resulting in the formation of caveolae-derived vesicles. In addition, caveolae- and clathrin-coated pit-mediated endocytic processes are distinct processes which can be differentiated in terms of kinetics, cytosol and nucleotide requirements but also in terms of the density and size of the endocytic vesicles formed. The fate of caveolae-derived vesicles also remains a subject of debate. Indeed, it has been evidenced that GPI-anchored folate receptor and caveolin could not be

observed within endosomal compartments, while cholera toxin was recovered in the same endosomes as those containing á-macroglobulin (Gilbert et al, 1999).

An alternative function of caveolae was proposed, in which these structures would close transiently without moving away from the plasma membrane via a process named “potocytosis”, thus different from endocytosis (no budding). By this process, cells could sequester, concentrate and internalize small molecules and ions through a receptor-mediated mechanism. Potocytosis is viewed as involving the binding of the molecule to its receptor, the closure of caveolae, a possible detachment from plasmalemma, and finally the dissociation of the ligand from its receptor by the proton gradient. An example of potocytosis is the uptake of folate. However, while caveolae are clearly involved in folate potocytosis, the endocytic internalization of folate may also exploit trafficking via an alternative vesicle pathway (for a review, see Gumbleton et al, 2000).

It is important to note that involvement of caveolae for internalization of molecules or particles has been severely questionned, and it currently seems that caveolae could be considered as true vesicles only under special inducing conditions. An example is the internalization of the SV40 virus. After associating with caveolae, SV40 leaves the plasma membrane in small, caveolin-1-containing vesicles. It then enters larger, peripheral organelles with a non-acidic pH. Although rich in caveolin-1, these organelles do not contain markers for endosomes, lysosomes, endoplasmic reticulum (ER) or Golgi, nor do they acquire ligands of clathrin-coated vesicle endocytosis. This demonstrates the existence of a two-step transport pathway from plasma membrane caveolae, through an intermediate organelle (termed the caveosome), to the ER. This pathway bypasses endosomes and the Golgi complex, and is part of the productive infectious route used by SV40 (Pelkmans et al, 2001).

At the plasma membrane, caveolae also serve to compartimentalize and integrate a wide range of signal transduction processes (for a review, see Gumbleton et al, 2000). The list of signalling molecules apparently localized to caveolae has increased in the past few years and includes Src family kinases, Ras, epidermal growth factor receptor (EGFR), phospholipase C γ (PLCγ), protein kinase C (PKC), NO synthase and many others (Kurzchalia and Parton, 1999).

2.2.4. Other internalization mechanisms ?

Several lines of evidence suggest the existence of additional pathways than macropinocytosis and caveolae. A first evidence comes from studies with the plant toxin ricin. Ricin consists of two polypeptide chains and binds by one chain (the B-chain) to both glycoproteins and glycolipids with terminal galactoses at the cell surface. The other chain (the A-chain) enters the cytosol. The toxin has proven valuable as a bulk membrane tracer in studies of endocytosis. Indeed, pharmacological inhibitors of clathrin-mediated uptake and use of dynamin and clathrin mutants revealed that ricin is taken-up by clathrin-dependent as well as clathrin-independent mechanisms. Moreover, clathrin-independent internalization of ricin can clearly be distinguished from uptake which might occur by caveolae (Sandvig and van Deurs, 1999; see Dautry-Varsat, 2001). A second evidence for the existence of non-clathrin-coated endocytosis is that the preendosomal compartment comprises distinct coated and non-coated endocytic vesicle populations, with approx. the same frequency but with distinct diameter distributions, the average non-coated vesicle being smaller (95 nm) than the average coated one (110 nm) (Hansen et al, 1991). Finally, the uptake of a GPI-linked version of the diphteria toxin, an artificial construct, was not affected by treatments which affect both clathrin-coated pit and caveolae-mediated internalization, such as overexpression of the dynamin mutation, treatment with methyl-β-cyclodextrin or nystatin (see Dautry-Varsat, 2001; see section 10.3).

What could be the characteristics of these clathrin- and caveolae-independent pathways? Except for clathrin-coated pits and caveolae, no other coated structures have been observed on the plasma membrane. It is possible that some other coats exist or that membrane microdomains could participate in sorting some membrane components that would interact with these lipids. Lipid properties could be responsible for membrane invagination and therefore vesicle formation (see Dautry-Varsat, 2001).

Physiologically important receptors enter cells by a clathrin- and caveolae-independent pathway, such as some GPCRs. Several of these receptors can be recruited by arrestins into clathrin-coated pits (see section 4.1.1.2.3.). Moreover, the angiotensin II type 1A receptor and the m2 muscarinic acetylcholine receptor do not require dynamin for their internalization and are independent of the function of arrestins. However, while the β2-adrenergic receptors are internalized via coated pits in some cell types, they may enter by a different mechanism in other cells (for a review, see Dautry-Varsat, 2001).

The interleukin-2 receptor is another interesting case of a signalling receptor. This receptor is normally expressed on activated lymphocytes and is rapidly internalized by a clathrin-independent mechanism in these cells that are devoid of caveolae. This was shown using controlled perturbations (chlorpromazine, a cationic amphiphilic drug which induces a redistribution of AP-2 to endosomes [see section 10.3.]; potassium depletion; cytosol acidification; microtubule depolymerization) as well as specific inhibition of clathrin-mediated entry (overexpression of Eps15 mutant). The involvement of dynamin was evidenced in this pathway, since internalization of the receptor was inhibited by overexpression of the dynamin K44A mutant (Subtil et al, 1994; Subtil and Dautry-Varsat, 1997; Lamaze et al, 2001; Dautry-Varsat, 2001).

2.2.5. Why several endocytic routes ?

An important question is why different pathways exist. First, this may be one way to internalize different membrane components at different rates. It is also possible that some of the intracellular pathways differ according to the structures of entry. Another potential function of clathrin-independent endocytosis may be related to signal transduction, such as membrane rafts or caveolae (see Dautry-Varsat, 2001).

2.3. Phagocytosis

2.3.1. Definition and structure involved

Phagocytosis is a receptor- and actin-dependent but clathrin-independent internalization of large particles (> 0.5 μm), including infectious agents (microorganisms, fungi), parasites, apoptotic cells and cellular debris. Phagocytosis serves two quite different functions, the removal of foreign particles, such as microorganisms and parasites, and the removal of host cells, such as apoptotic cells.

All eukaryotic cells, with the exception of yeasts, can phagocytose. In mammalian cells, phagocytosis is carried out primarily by “professional phagocytes”, which include the neutrophils, monocytes and macrophages. Other cell types also have varying phagocytic capabilities. Compared to professional phagocytes, the non-professional cells are far less efficient and are

unable to deal with as large a variety of targets. Moreover, the responses that accompany phagocytosis, such as activation of the nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase or cytokine production, seem restricted to neutrophils and macrophages.

Phagocytosis can be thought of as occuring in four basic steps. The initial event is the recognition of the target by the phagocytic cell. In the next step, receptor-ligand interaction causes specific cellular responses through signal transduction pathways. The third step is the actual process of internalization, which requires one or more membrane fusion events by the phagocytic cell to bring the phagocytic target from the extracellular milieu to the intracellular vesicular network. Fourth, the ingested particle enters the lysosomal system in the phagocyte, where it is degraded (for a review, see Jones et al, 1999).

Both pinocytosis and phagocytosis occur in macrophages. However, using polystyrene beads of different sizes, it has been shown that there is no radical discontinuity between pinocytic and phagocytic uptake, but that the contribution of phagocytosis steadily increases with increasing particle diameter. This conclusion is based on rates of clearance which increase with increasing particle diameter, and on effects of colchicine and cytochalasin B, inhibitors of microtubules and microfilaments respectively (section 10.5.). Cytochalasin B has little effect on the uptake of the smaller particles but partially inhibited the uptake of the larger, and colchicine has progressively less effect on uptake when the particle size increases (Pratten and Lloyd, 1986).

2.3.2. Receptors involved in phagocytosis

The receptors of phagocytosis have been largely described (for reviews see Mukherjee et al, 1997; Daëron, 1997; May and Machesky, 2001; Greenberg, 2001).

Particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. A particle that is endocytozed by this mechanism may be recognized directly by the receptors on the surface of the phagocyte, or it may be first “opsonized” by coating the particle with “opsonins”, molecules that render the particle they coat more susceptible to engulfment by phagocytic cells. The major opsonins are immunoglobulins (Igs) and complement components, which bind respectively to the Ig Fc fraction receptor (FcR) and the complement receptor (CR).

In mammals, binding of Igs to foreign particles leads to the prompt clearance by FcR-mediated phagocytosis. The conserved Fc domain of the Ig is recognized by FcRs present on professional phagocytes, such as neutrophils and macrophages, and the opsonized particles are rapidly internalized. This internalization is characterized by the actin-dependent extension of the plasma membrane around the particles. The major Ig opsonin is IgG which binds to FcγRs, that either contain or are associated with immunoreceptor tyrosine-based activation motif (ITAMs; FcγRI, FcγRIIA and FcγRIIIA) and those that contain immunoreceptor-based inhibition motif (ITIMs; FcγRIIB, FcγRIIIB). The former group, but not the latter, trigger phagocytosis.

CR-mediated phagocytosis is morphologically distinct from that mediated by FcRs, although both processes require actin polymerization. Complement-opsonized particles “sink” into the phagocyte. The CRs, CR1, CR3 and CR4 are expressed on macrophages and neutrophils and are all implicated in phagocytosis. CR1 is a single-pass transmembrane protein and CR3 and CR4 are both integrin heterodimers.

A growing number of cell-surface receptors other than FcRs and CRs can mediate phagocytic uptake of particles. These include non-CR integrins, lectins such as the MR, the lipopolysaccharide receptor (LPSR) CD14, and the diverse scavenger receptor group. Internalization by these receptors appears to be morphologically dynamic, as in the case of FcRs. Membrane is extended around the attached particle, and there is transient ruffling in surrounding areas of the cell. Phagocytosis mediated by these receptors is also actin dependent.

Latex beads have been used for over 30 years as a model for the phagocytic process. These inert particles, which cannot be evicted by cells, serve as a convenient model for the uptake of non-degradable particulate material. Latex beads induce a rapid and transient F-actin assembly when added to J774 macrophages. These particles, although lacking any biologically specific surface structure, can switch on the actin assembly machinery. Although the nature of the molecules that bind to the latex beads is not known, it is obvious that some receptors must be activated, such as the FcRs or CRs.

2.3.3. Mechanisms for particle entry by phagocytosis

Whereas the spectrum of phagocytic receptors is diverse and early response may differ, many of the basic processes of the cytoskeletal rearrangement and membrane trafficking that accompany phagocytosis are conserved. Three mechanisms have been described for the uptake of particles and microorganisms in mammalian cells, depending on the receptor involved.

In the "zipper mode", the particle is automatically ingested by sequential receptor-ligand interactions and does not actively participate in its uptake (Lecuit et al, 1997). Phagocytosis mediated by FcγRs is an example. Signalling is initiated by ligand binding and clustering of cell surface FcγRs. After signal transduction (src and syk activation), actin assembly and membrane trafficking are needed to trigger cytoskeletal alterations, pseudopod extension (a "zipper mechanism") and phagosomal closure (for reviews, see May and Machesky, 2001; Greenberg, 2001; see also section 2.3.5.). Another example of this type of phagocytosis is that of *Listeria monocytogenes*. This bacteria engages receptors that otherwise act in cell-cell adhesion (E-cadherin) and which are recognized by the bacterial internalin A and internalin B (Lecuit et al, 1997; for a review, see Greenberg, 2001; see also Figure 1).

In a second mode, the"sinking in mechanism" (which is another case of "zipper") that is mediated by CR, the microorganism can enter the host cell by active invasion. An example is the phagocytosis mediated by the leukocyte integrin CR3 (see Figure 1; for a review, see Greenberg, 2001).

As a third route is the "trigger mechanism" which does not require specific adhesion of the microorganism to the host cell. Phagocytosis triggered by *Salmonella* and *Shigella* are mediated by this mechanism. By injecting cytoskeleton-altering proteins and activators of Rho family GTPases into the host cell cytoplasm, these pathogens induce the formation of cell extensions that ressemble macropinosomes (a "trigger mechanism") (see Figure 1; for a review, see Greenberg, 2001).

2.3.4. Signal transduction and phagocytosis

Signal transduction processes have been largely unravelled (for a review, see May and Machesky, 2001).

The interaction between IgG and FcγR triggers rapid phosphorylation of specific tyrosine residues in the receptor motif within ITAMs. The initial ITAM phosphorylation is carried out by TKs of the src family. Following its phosphorylation, the ITAM motif acts as a docking site for syk, which leads to its phosphorylation and activation. The point at which syk acts remains controversial (see Figure 2).

Following activation of CR3 and CR4, the β-chain of these receptors becomes serine phosphorylated, which appears to be dependent on PKC activity, which is essential for internalization.

Downstream signalling involves a large range of molecules, most of these initially identified as acting downstream of FcãRs, but many now appear to be involved in signalling from other phagocytic receptors as well. Among these are phosphatidylinositol kinases (PI kinases; see section 7.2.1.2.3.), phospholipases C and D (PLC and PLD; see sections 7.2.4.3. and 7.2.5.3.), PKC, GTPases (Rho and ADP-rybosylation factor [ARF] family members; see section 4.1.1.2.4.) and the cytosolic calcium concentration. PKC are activated by the PLC product diacylglycerol, by calcium and by pharmacological agents such as phorbol esters (see also Figure 2).

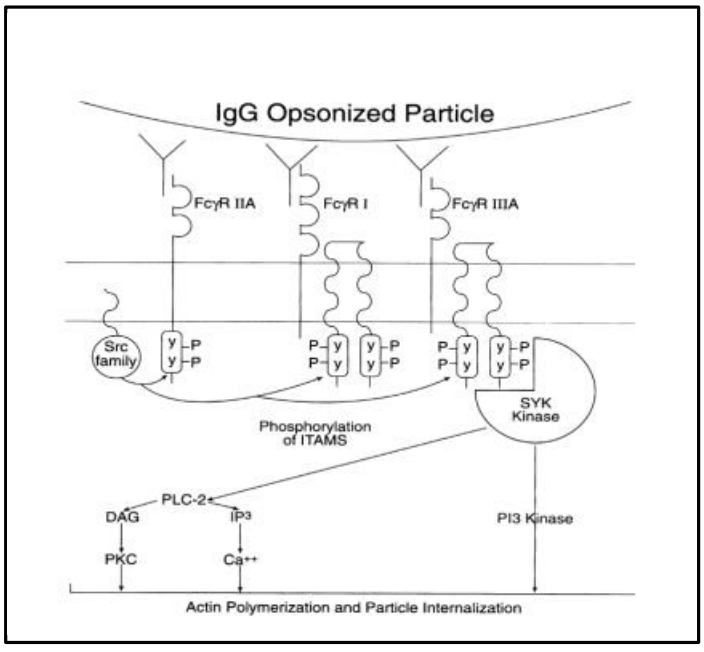


Figure 2. FcγR signal phagocytosis via their phosphorylated ITAM domains. Receptor cross-linking stimulates src family kinases to phosphorylate tyrosine residues within the ITAM domain of FcγRIIA or within the dimerized γ subunits of FcγRI or FcγRIIIA. The tyrosine kinase syk is then recruited to the phosphorylated ITAM domain, and upon its activation, it is thought to mediate particle internalization by activating PI 3-kinase and PLC. (From Aderem and Underhill, 1999).

2.3.5. Fate of particles internalized

One of the important characteristics of the maturation process during phagocytosis is that newly formed phagosomes, like early endosomes, mature to a state where they no longer fuse with early endosomes but only with lysosomes, and it has been shown that phagosome maturation seems to be similarly rapid as early endosomes which mature within about 3 min.

However, phagosome maturation and fusion with lysosomes is related to surface property and size of the phagocytic particle (de Chastellier et al, 1995; de Chastellier and Thilo, 1997). Thus, phagosomes containing *Bacillus subtilis* or particles with a hydrophilic surface, or with a hydrophobic surface but a size below 0.5 µm, mature normally and may fuse with lysosomes within less than 15 min after uptake. Phagosomes containing large hydrophobic latex beads remain immature and early endosome-like for at least 3 h after phagocytic uptake and they mature into phagolysosomes over a period of more than about 12 h, and it has been suggested that hydrophobic particles simply slow down the maturation process (de Chastellier and Thilo, 1997). Phagosomes with pathogenic *Mycobacteria* fuse with early endosomes, but they are unable to mature, and accordingly, they do not fuse with lysosomes, for extended periods (de Chastellier et al, 1995; tested up to 15 days). A more comprehensive analysis using a proteomic approach allowed to show that maturation of phagosomes of J774 macrophages containing 0.8 µm latex beads is accompanied by the sequential acquisition of hydrolases: while most of the hydrolases seem to reach their highest level at 6 h after phagosome formation, the levels of few others, such as cathepsin S, continue to increase as late as 24 h after phagosome formation (Garin et al, 2001).

3. Pathways and compartmental boundaries

3.1. Early and late endocytic compartments

3.1.1. Definition and molecular markers

Clathrin-coated pits and clathrin-independent pits lead to the appearance of cargo in the peripheral early endosomes. Early endosomes then undergo homotypic fusion. From early endosomes, cargo is recycled back to the plasma membrane, sorted to the late recycling compartment or delivered to late endosomes by means of carrier vesicles or through a maturation

process. Late endosomes either mature into lysosomes or transfer cargo to lysosomes through vesicular intermediates. The timing of delivery to early endosomes and then to late endosomes varies between cell types and between cells in culture. Early endosomes are loaded within 1-5 min of uptake, and late endosomes 4-30 min after uptake (for a review, see Seaman and Luzio, 2001).

In addition to the receptors and ligands that participate in endocytosis, other molecules provide the unique characteristics of each endocytic compartment. These include: (i) molecules that regulate internal pH; (ii) those that control organelle shape, distribution and translocation; (iii) those that regulate vesicle docking, fusion, and budding (e.g. Rabs); and (iv) the acid hydrolases and organelle membrane proteins (e.g. lysosome-associated membrane proteins [Lamps], lysosomal membrane glycoproteins [Lgps], cation-independent mannose-6-phosphate receptors [CI-MPRs]) (Mukherjee et al, 1997). The essential characteristics of the endocytic compartments have been described in Mukherjee et al (1997) and are summarized in Table 2.

Early endosomes are the first station on the endocytic pathway. They are often located in the periphery of the cell, have a characteristic tubulo-vesicular morphology and a midly acid pH. They are sorting organelles that select components for recycling to the cell surface (via tubules) or for transport to late endosomes (via the vesicular component). The latter can form multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs) that are involved in transport to late endosomes. The early endosome system can itself be divided into at least two separate compartments, sorting endosomes and recycling endosomes. Sorting endosomes have a tubulovesicular morphology with a vesicular portion of 250-400 nm diameter, and tubules which are 50-60 nm in diameter and several μm long. As a consequence, the bulk of the volume is in the vesicular portion and most of the surface area is in the tubules. Sorting endosomes receive and redistribute material coming from the cell surface and sort recycling molecules (such as Tf) from ligands that will be degraded (such as LDL). Endocytic recycling compartment (ERC) contains recycling molecules but are devoided of the molecules that will be degraded. The ERC is composed of a collection of tubules with diameters of 50-70 nm which are extensively interconnected. These tubules have varicosities and coats. Early endosome antigen 1 (EEA1) and Rab5 are located on sorting endosomes, whereas Rab11 is preferentially located in recycling endosomes (see sections 5.1.1.1. and 5.1.2.). In polarized cells, the compartment corresponding to the ERC is the subapical compartment (SAC). In HepG2 cells, a well-differentiated human hepatoma cell line, it has been shown that sorting of sphingolipids in the reverse transcytotic pathway (from the apical to the basolateral plasma membrane) is taking place in the SAC and

allows glucosylceramide (GlcCer) to remain located in the apical region and sphingomyelin (SM) to redistribute to the basolateral domain (van Ijzendoorn et al, 1997; Hoekstra et al, 1999).

Late endosomes are similar in size to sorting endosomes, but they appear to have more internal vesicular profiles, are nearer to the center of the cell. Late endosomes are acidic (approx. pH 5.5) and carry Rab7. They are often defined as organelles lacking Tf and other recycling components but containing CI-MPRs, Lgps and acid hydrolases. Their internal vesicles and membranes are enriched in lysobisphosphatidic acid (LBPA). Late endosomes receive material from early endosomes or the TGN and are thought to mediate a final set of sorting events prior to delivery to lysosomes. Sorting in late endosomes involves both the invagination of membrane vesicles into the lumen of these organelles, or the formation of transport vesicles that return mannose 6-phosphate receptor (MPR) to the Golgi.

Lysosomes are the last compartment of the endocytic pathway. These organelles are heterogeneous in morphology, approx. 200-500 nm in diameter, characterized by an amorphous electron-dense matrix, and therefore often referred to as dense-core lysosomes. They can make up to 0,5-5 % of the cell volume and are concentrated near the microtubule organizing centre. They are acidic (approx. pH 5.0-5.5), and contain the bulk of acid hydrolases, Lamps and Lgps, but no CI-MPRs (Jadot et al, 1997). Lysosomes can be regarded essentially as acid-hydrolase-storage organelles that repeatedly fuse with late endosomes to form hybrid organelles, in which digestion of endocytosed macromolecules takes place and from which lysosomes are re-formed (Luzio et al, 2000). Retrograde traffic between terminal lysosomes and the late endosomal compartment has been shown (Jahraus et al, 1994). The bulk of newly synthesized lysosomal hydrolases are delivered to the lysosome after binding to the MPRs in the TGN. The main trafficking route of MPRs appears to involve recruitment into AP-1 containing clathrin-coated pits at the TGN, vesicular traffic and fusion with early endosomes, followed by traffic to late endosomes, before return to the TGN (for a review, see Seaman and Luzio, 2001; see section 6.2.2.).

	Compartment				
	Early endocytic compartments		**Late endocytic compartments**		
Properties	**Sorting endosomes**	**Recycling endosomes**	**ECV-MVB**	**Late endosomes**	**Lysosomes**
Distribution	peripheral	throughout cytoplasm	throughout cytoplasm	mainly perinuclear	mainly perinuclear
Morphology	tubulovesicular	tubular	spherical with internal membranes	complex structure with internal membranes	variable
pH	5.9-6.0	6.4-6.5	< 6.0	5.0-6.0	5.0-5.5
Receptors	TfR and other recycling receptors; ligands destined for lysosome	TfR and other recycling receptors; no lysosomally destined molecules		CI-MPR	
Membrane proteins and internal lipids				Lgps; Lamps; LBPA	Lgps; Lamps
GTP-binding proteins	Rab4, Rab5, Rab11			Rab7, Rab9	

Table 2. Properties and characterization of endosomal compartments. (Adapted from Mukherjee et al, 1997).

3.1.2. Connections between endocytic compartments: models of endosome maturation and/or endosomal carrier vesicles

The membrane traffic pathway from early to late endosomes was the subject of some controverse with the proposal of maturation and vesicular transport models. The maturation model envisages the continuous removal and addition of material to the maturing early endosome until it becomes a late endosome. The vesicular transport model proposes the formation of an endocytic

carrier vesicle that buds from the early endosome and fuses with the late endosome thereby delivering lumenal content and membrane proteins (for a review, see Seaman and Luzio, 2001).

3.2. Phagocytic compartments

After internalization is complete, the actin-based machinery is shed from the phagosome. Phagosomes then mature, and plasma membrane proteins are recycled back to the cell surface, while phagocytic receptors are rapidly and selectively degraded. Maturation is characterized by changes in both the protein and the phospholipid composition of the phagosome in a process that ressembles the maturation of early endosomes in late endosomes. Interestingly, acquisition of hydrolases by phagosomes is sequential and accompanies maturation of phagosomes (Garin et al, 2001).

3.3. Connections between endocytosis and phagocytosis

Phagosome maturation depends on interactions (fusion events) with early and late endosomes but also with lysosomes. The endosome-phagosome fusion is biochemically very similar to endosome-endosome fusion. The introduction of early endosomal membrane and protein to early phagosomes may give early endosome functions, including protein recycling, to the early phagosome (Pitt et al, 1992). The late endosomes and the lysosomes may be involved in the degradation of phagocytozed compounds (Tjelle et al, 1998).

Maturation of phagosomes is accompanied by the sequential acquisition of hydrolases. In J774 macrophages, hydrolases are not delivered simultaneously to phagosomes containing 0.8 µm latex beads, but appear sequentially, at different time points during phagosome maturation. While most of the hydrolases seem to reach their highest level at 6 h after phagosomes formation, the levels of few others, such as cathepsin S, continue to increase as late as 24 h after phagosome formation. These results support the hypothesis that hydrolases are heterogeneously distributed along the degradative pathway (Garin et al, 2001).

The fusion processes are regulated by small GTP-binding proteins and other proteins that are also involved in fusion processes in the endocytic pathway, like annexins. Fully internalized phagosomes show enrichment of annexin I, annexin II and annexin VI and α-actinin, all of which

can interact with the actin cytoskeleton. It is therefore possible that, even after internalization, the phagosome membrane retains proteins that enable it to continue to interact with the actin cytoskeleton. A role for actin polymerization in driving vesicle movement within cells has indeed recently been demonstrated (for a review, see May and Machesky, 2001; see also sections 5.2.2. and 9.4.). Actin assembly activity of phagosomes changes drastically as they age in the cells: the youngest phagosomes (5 min) have a low *in vitro* activity, actin assembly capacity of phagosomes peaks at 2-4 h of chase, then decreases drastically at 12h, before recovery over the next 24h. In addition to its role in vesicle movement, actin assembly process has been proposed to facilitate phagosome/endosome aggregation prior to membrane fusion (Defacque et al, 2000; see also section 9.4.).

4. Vesicle budding and scission on the endocytic pathway

The distinct internalization mechanisms (section 2) and the distinct compartments of the endocytic pathway (section 3), imply vesicle budding and scission processes. The driving force for membrane budding can arise from at least four different biological mechanisms: (i) the membrane deformation that is mediated by the polymerization of cytosolic coat proteins binding to integral membrane constituents (examples: clathrin, coat protein complex I [COPI] or coat protein complex II [COPII]); (ii) the deformation of a membrane that results from its interaction with a cytoskeletal element capable of driving budding, either pushing the bud into the extracytoplasmic space or pulling it into the cytoplasm (example: the formation of actin-based plasma membrane protusions); (iii) the membrane deformation that results from enwrapping a cytoplasmic or extracytoplasmic particle, where the driving force derives from the adhesion of the membrane to an already curved particle (examples: cells eating beads or the budding of envelopped viruses from the plasma membrane); and (iv) the insertion of additional monolayer or bilayer membrane (for a review, see Huttner and Zimmerberg, 2001).

Membrane budding is followed by the physical separation of the bud from the donor membrane, a process termed fission. Fission can be the ultimate consequence of the very same mechanism that mediates budding (examples: COPI and COPII) or be distinct (example: the pinching off of CCVs by dynamin) (for a review, see Huttner and Zimmerberg, 2001).

This section first describes the mechanism of membrane budding, focusing on the driving force resulting from coat assembly. The role of lipids will be adressed at section 7. Emphasis will be placed on coat assembly on the cell surface and mechanisms of entry into the cell, i.e. clathrin and its adaptor, AP-2; other coats involved in the endocytic pathway, i.e. the adaptor-related AP-1, AP-3, AP-4 and AP-180/clathrin assembly lymphoid myeloid leukemia (CALM) complexes, and COP will be more briefly described. The second part of this section describes scission mechanism.

4.1. Vesicle budding

4.1.1. Clathrin, AP-2 and associated proteins

The best-defined internalization pathway is the CCV pathway. The cycle of coat assembly begins with coated pit formation (attachment of APs and subsequently clathrin triskelions to the membrane, leading to the formation of a polyhedral lattice), continues during recruitment of cargo proteins, coat invagination and vesicle scission (see section 4.2.), and ends with the release of the coat elements from the vesicle to the cytosol followed by their recycling back to the donor membrane.

4.1.1.1. Coated pit formation

Endocytic signals must be recognized by the endocytic machinery for rapid internalization to proceed. The clathrin coat is assembled on the cytoplasmic face of the plasma membrane, forming pits that invaginate to pinch off and become free CCVs. In cultured cells, the assembly of CCVs takes approx. 1 min, and several hundred to a thousand or more can form every minute.

The main scaffold component of CCVs is the 190 kDa protein clathrin heavy chain (CHC), which is associated with a 25 kDa protein clathrin light chain (CLC). These CHC-CLC complexes form three-legged trimers (triskelions), that can oligomerize to give the polygonal arrays seen with electron microscopy. *In vivo*, the minimum composition suggested to accomodate a vesicle is 60 triskelions assembled into 20 hexagons and 12 pentagons to achieve curvature. The triskelion is made of a triskelion hub domain, which is centered at each vertex of the cage, and three legs, which project in different directions from the hub. The legs are divided into a proximal and a distal domain by a bend midway along each. Thus, each leg contributes to two sides of a polygonal cell,

and each edge is made up of four leg domains (for a review, see Marsh and McMahon, 1999; see figure 3).

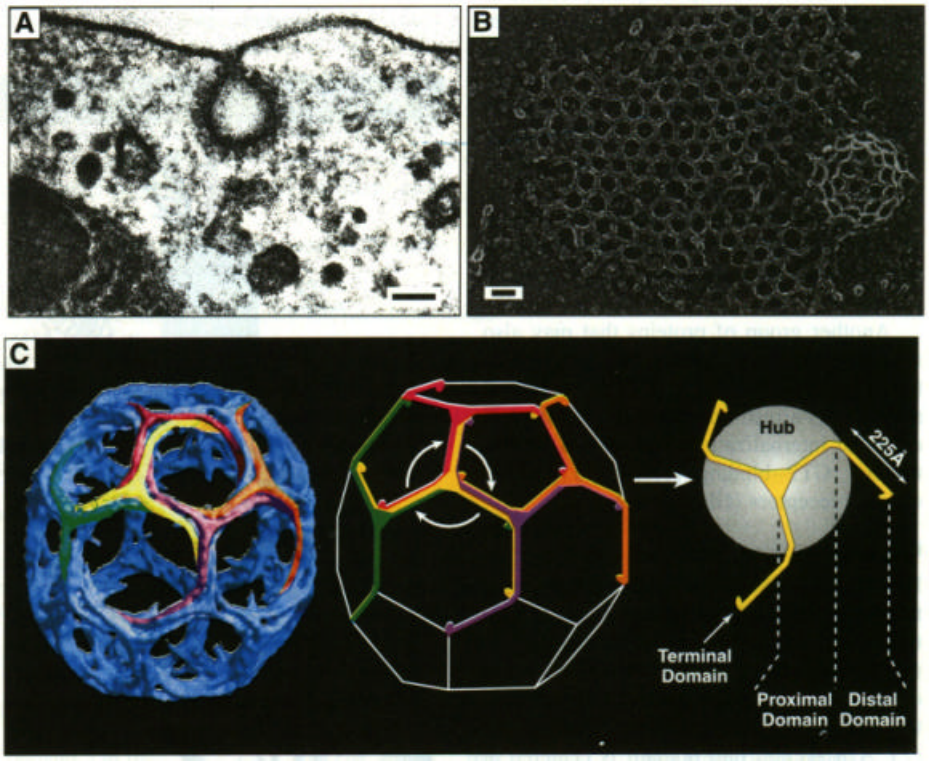


Figure 3. Clathrin structures. (A) Thin-section electron microscopy illustrating the bristle coat associated with clathrin-coated pits and coated vesicles. Scale bar, 50 nm. (B) Deep-etch image of the cytosolic side of a plasma membrane showing the polygonal lattices of a coated pit and an invaginating coated vesicle. Scale bar, 33 nm. (C) A 21 Å resolution map of a clathrin hexagonal barrel assembled from purified clathrin and AP2 complexes. The middle and right panels show schematic views of the clathrin cage and the organization of the triskelion, respectively. (From Marsh and McMahon, 1999).

Assembly is facilitated by the presence of the clathrin heterotetrameric adaptor protein AP-2. The functions of AP-2 are: (i) attachment of clathrin to the membrane; (ii) selection of the vesicle cargo; and (iii) recruitment of accessory proteins that regulate vesicle formation (for a review, see Robinson and Bonifacino, 2001). The plasma membrane AP-2 complex consists of two large chains (α- and β2-adaptin), an intermediate μ2 chain, and a small chain, σ2 (D'Hondt et al, 2000). The large subunits can be subdivided into trunk, hinge and appendage domains. The functions of the AP-2 complex have been attributed to individual domains of the various subunits: μ2 binds to the internalization signal present in the cytoplasmic domain of membrane proteins that are internalized in CCVs; the hinge domain of β2 interacts with clathrin to promote coat formation; and the appendage domain of α recruits the accessory/regulatory proteins. The role of binding to μ2 in endocytosis seems to be more important for some receptors than for others (for reviews, see Owen and Luzio, 2000; D'Hondt et al, 2000). So, AP-2 is positioned between the clathrin lattice

and the vesicle membrane and is proposed to link cargo selection to clathrin-coated pit formation. In addition to the AP-2, three other AP complexes are described (see Figure 4; see section 4.1.2.).

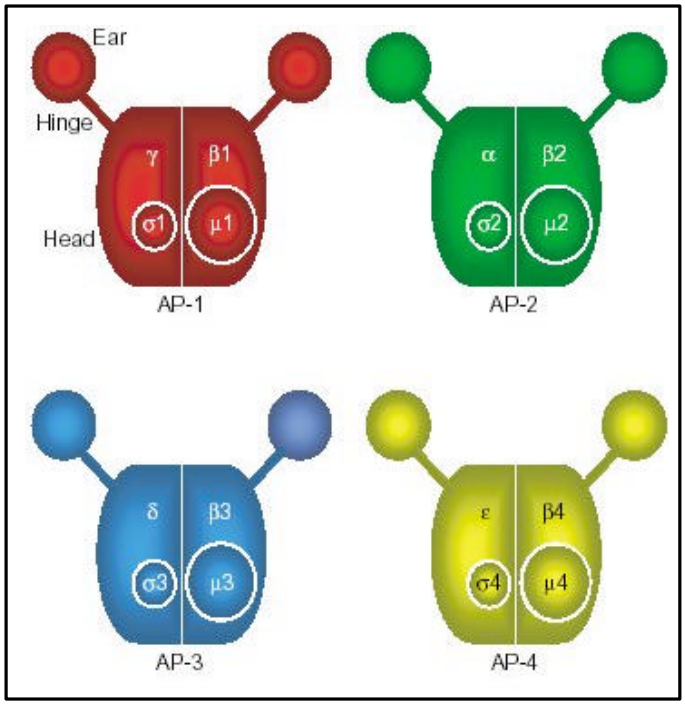


Figure 4. Schematic diagrams of the four AP complexes. All four complexes consist of two large subunits: a β subunit and a more divergent subunit, either γ, α, δ or ε; a medium (μ) subunit; and a small (σ) subunit. The carboxy-terminal domains of the two large subunits project as 'ears', connected to the 'head' of the complex by flexible hinges. (From Robinson and Bonifacino, 2001).

If clathrin and AP-2 complex are components of clathrin-coated pits at the cell surface (for a review, see Owen and Luzio, 2000), AP-2 recruitment also occurs at the late endosomes (see Riezman et al, 1997), as well as on dense lysosomes *in vitro* and this might regulate retrograde traffic out of the lysosomal compartment (Traub et al, 1996). Clathrin and AP-2 are required for both opsonic and non-opsonic phagocytosis in alveolar macrophages (Perry et al, 1999).

4.1.1.2. Cargo recruitment into coated pits

4.1.1.2.1. Signals for internalization in CCVs

Membrane proteins that are internalized in CCVs contain one or more signal sequences in their cytoplasmic domain or domains that direct the protein into clathrin-coated pits. Four types of endocytic signals have been identified. Tyrosine (Y)-based signals, YXX∅ where X represents any amino acid and ∅ represents a bulky hydrophobic amino acid residue, have been identified in

animal cells. These signals are found in constitutively endocytosed receptors such as the TfR (YXRF), the LDLR (NPXY), and some receptors which are only endocytosed following ligand binding, such as the EGFR. Second, various dileucine (LL)-based cytoplasmic sequences also function as signals for coated pit uptake and lysosomal delivery of cell-surface proteins. As examples, the CD4 antigen or the human FcRIIB2 that participate in receptor-mediated endocytosis contain dileucine internalization motifs. A third endocytic signal is a phosphorylated serine-rich domain at the COOH-terminus of many GPCRs. A fourth type is constituted by motifs involving ligand-induced phosphorylation of serine residues and the ubiquitination machinery. Several TKs or kinase-linked receptors undergo ligand-stimulated ubiquitination at the plasma membrane, such as the EGFR. These receptors are then internalized and degraded in response to ligand stimulation (for reviews, see Mukherjee et al, 1997; Clague, 1998; Marsh and McMahon, 1999; D'Hondt et al, 2000).

4.1.1.2.2. Regulation of clathrin-mediated endocytosis

There are three classes of cell surface receptors whose entry into coated vesicles is controlled by sorting signals: (i) receptors (TfR or the LDLR) constitutively concentrated in coated pits; (ii) those (FcRII, and some GPCRs) which require ligand-induced activation for concentration into coated pits; (iii) those (CD4) which are actively retained on the cell surface until their release is triggered by some signalling event. Other receptors, including the insulin receptor and the EGFR, exhibit a combination of these properties (for reviews, see Mukherjee et al, 1997; Schmid, 1997; D'Hondt et al, 2000).

Most constitutively internalized receptors carry tyrosine-containing sorting motifs which remain the best characterized signals for concentration of transmembrane receptors into coated pits, and which are recognized by a common sorting machinery (for a review, see Schmid, 1997). For example, efficient internalization of the TfR depends on the tyrosine-based signal in its cytoplasmic tail, while this motif seems not involved in its recycling (Nordeng and Bakke, 1999).

In contrast, other receptors, such as the FcR that mediate endocytosis of soluble immune complexes, are internalized only after ligand binding. FcRIIB2 is endocytosed through coated pits, while FcRIIB1 is actively excluded from these pits, but is found in membrane domains that are very similar to the domains enriched in cross-linked GPI-anchored proteins (see Mukherjee et al, 1997). So, only FcRIIB2 is able to accumulate in coated pits and thereby to mediate the efficient

internalization of bound ligands. The cytoplasmic domain of FcRIIB2 contains a YSLL motif needed for coated pit localization, and this receptor does not accumulate efficiently in coated pits until after ligand binding (Ukkonen et al, 1986; Matter et al, 1994). Another class of receptors which are internalized in response to ligand binding are GPCRs. GPCRs transduce information provided by extracellular stimuli into intracellular second messengers via their coupling to heterotrimeric G proteins. Agonist activation of GPCRs also initiates the feedback desensitization of GPCR responsiveness, i.e. the internalization of GPCRs and the coupling of GPCRs to heterotrimeric G protein-independent signal transduction pathways. GPCR desensitization occurs as a consequence of G protein uncoupling in response to phosphorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). The latter promotes the binding of â-arrestins, which not only uncouple receptor from heterotrimeric G proteins but also target many GPCRs for internalization in clathrin-coated vesicles (for a review, see Ferguson, 2001).

Other receptors exhibit a combination of ligand-induced activation for concentration into coated pits and signalling events. Indeed, although vesicle-mediated membrane traffic has long been considered to be a constitutive process, it is now suggested that signal-transduction pathways also play critical roles in the regulation of protein and membrane trafficking. For example, ligand binding and signal transduction allow the EGFR to initiate coat assembly and promote its own regulation. It has been proposed that cytoplasmic internalization motifs in signalling receptors might become exposed only upon activation of the intrinsic TK domain (for a review, see Mukherjee et al, 1997). Binding of EGF to its tyrosine kinase receptor (TKR) on the plasma membrane results in a striking redistribution of receptor-ligand complexes: activated receptors first dimerize, concentrate into clathrin-coated pits, and are then internalized via clathrin-coated vesicles for delivery to lysosomes where they are degraded, resulting in the depletion of EGFRs from the cell surface and the attenuation of mitogenic effects. EGFR-TK is required for the down-regulation of activated EGFR. Indeed, EGF bound to wild-type receptor is efficiently sequestred into coated pits, while sequestration of kinase-deficient receptors occurs inefficiently and at the same basal level of endocytosis of unoccupied receptors (Lamaze and Schmid, 1995). Following ligand binding, stimulated EGFR endocytosis occurs, and a kinase substrate is required for endocytosis of the EGFR. It has been shown that Eps15, an endocytic protein that is tyrosine phosphorylated by EGFR, is necessary for such function. Thus, tyrosine phosphorylation of Eps15 represents a molecular determinant, in addition to those contained in the receptors themselves,

which is involved in the differential regulation of constitutive *vs* regulated endocytosis (Confalonieri et al, 2000).

4.1.1.2.3. Cargo recruitment: interaction of cargo with AP-2 or arrestins

The first class of proteins that allow cargo recruitment are AP-2. Both tyrosine-based and dileucine-based signals interact with AP-2 complexes. Another class of proteins that may operate in a manner similar to AP complexes are the β-arrestins (for a review, see D'Hondt et al, 2000). They interact with endocytic cargo, clathrin, and phosphoinositides (PI) and play a clear role in endocytosis (D'Hondt et al, 2000). β-arrestins are believed to function as specialized adaptor proteins and are involved in targeting of many GPCRs for internalization in clathrin-coated vesicles (for reviews, see Clague, 1998; Ferguson, 2001).

4.1.1.2.4. Regulation of interaction of AP complexes with membrane

One of the best-characterized systems for recruitment into vesicles comes from studies of clathrin and adaptors. Adaptors interact with trafficking signals located in the cytoplasmic tails of membrane proteins, but, this is not sufficient to explain their interaction with membranes (Kirchhausen et al, 1997). AP receptors, rather than trafficking signals, have indeed been postulated to recruit APs to specific membranes, and one candidate for such function is synaptotagmin, an ubiquitous transmembrane protein. AP-2 recruitment to synaptotagmin is stimulated synergistically by tyrosine-based endocytic motifs and by PLD (for a review, see D'Hondt et al, 2000; see section 7.2.4.2.). In addition, AP complexes are subject to regulation by such factors as pH, inositol-phosphates, GTPases, like ARF and heterotrimeric G proteins, and PLD.

ARFs are small guanine-nucleotide-binding proteins, which regulate membrane traffic and organelle structure in eukaryotic cells. The inactive GDP-bound form of ARF is soluble whereas the active form binds tightly to the membrane. ARFs operate on membrane surfaces where they encounter their effectors and regulators. These include: (i) guanine nucleotide exchange factors (GEFs); (ii) GTPase-activating proteins (GAPs); (iii) lipid-modifying enzymes; and (iv) cytosolic coat complexes, such as COPI. Mammalian ARFs are divided into three categories: (i) class I (ARF1-ARF3), which are involved in trafficking in the ER-Golgi and endosomal systems; (ii)

class II (ARF4, ARF5), about which virtually nothing is known; (iii) class III (ARF6), which operates exclusively in the endosomal-plasma membrane system.

ARF1 does not appear to be required for the recruitment of AP-2 onto the plasma membrane, but supports recruitment of AP-2 to late endosomes (West et al, 1997). It is localized to the Golgi complex and is a common regulator of COPI, AP-1 and AP-3 recruitment (Ooi et al, 1998). ARF1 binding to endosomal membranes is regulated by endosomal pH, which explains the pH dependence of COPI binding to endosomes (Gu and Gruenberg, 2000).

ARF6 is localized to the endosomal/plasma membrane system and has no effect on the Golgi-associated coat proteins (Peters et al, 1995). ARF6 is indeed unique among members of the ARF family in that its membrane association is unaffected by brefeldin A (for brefeldin A, see section 10.4). Roles for ARF6 are numerous: (i) it is involved in endosomal recycling to the plasma membrane; (ii) it is present at the apical surface of MDCK cells, where it plays a role in modulating clathrin endocytosis (for a review, see Donaldson and Jackson, 2000); (iii) ARF6 is implicated in FcγR-mediated phagocytosis in macrophages in which ARF6 is an important regulator of cytoskeletal alterations (for a review, see Donaldson and Jackson, 2000; Zhang et al, 1998); and (iv) it controls actin assembly for pinosome motility, and this notably involves PLD and TK (Schafer et al, 2000).

Heterotrimeric G proteins also play a role in coat recruitment. For example, COP proteins are recruited to phagosomal membranes by a mechanism that involves heterotrimeric GTP-binding proteins and a brefeldin A-sensitive ARF (Beron et al, 2001).

PLD could also play a role in this context. Indeed, PLD helps AP-2 recruitment on plasma membrane and endosomes, but also on lysosomes (see section 7.2.4.2.).

4.1.2. Other coat and associated proteins

4.1.2.1. Other AP complexes: AP-1, AP-3, AP-4 and AP-180/CALM

The AP-1 complex associates with the TGN and directs transport of the lysosomal enzymes to endosomes. Like with AP-2 adaptor, the interaction of AP-1 adaptor with trafficking signals does not appear sufficient to explain their interaction with membranes. One factor that affects

recruitment in some cases is ARF1, which influences recruitment of AP-1 complex to Golgi membranes (for a review, see Riezman et al, 1997).

The AP-3 complex localizes to the TGN and to a more peripheral compartment that partly colocalizes with endosomal markers (Simpson et al, 1996; 1997). The AP-3 complex is required for a non-clathrin-mediated budding event from the TGN, and might be involved in trafficking to lysosomes and related organelles. However, the precise pathway that it mediates is still not clear (Simpson et al, 1996; 1997). AP-3 complex recognizes signals in the tails of certain lysosomal membrane proteins (see Riezman et al, 1997).

AP-4 is localized in the perinuclear region of the cell. It is associated with non CCVs in the region of the TGN. Its association with membranes is regulated by ARF. AP-4 is of relatively low abundance but it is expressed ubiquitously (Hirst et al, 1999; see Robinson and Bonifacino, 2001).

One of the major components of brain CCVs is AP-180, which is able to bind to clathrin triskelions and cages and promote the assembly of clathrin-coat *in vitro* but also to bind phosphoinositides (PI) and inositol phosphates. CALM has been proposed as a nonneuronal homologue of AP-180 because it was detected in many tissues. The bulk of cellular CALM was associated with the membrane fractions of the cell and localized to clathrin-coated areas of the plasma membrane. Clathrin is the major binding partner of CALM and clathrin-CALM interaction seems to be regulated by multiple contact interfaces. It has been suggested that CALM is an important component of coated pit internalization machinery, possibly involved in the regulation of clathrin recruitment to the membrane and/or the formation of the coated pit (Tebar et al, 1999).

4.1.2.2. COPs

COPI and COPII participate in anterograde and retrograde transport between the ER and the Golgi apparatus. Moreover, COPI also plays roles in pinocytic and phagocytic pathways.

COPI is a multimeric complex known to be involved in budding from the Golgi and endosomes. It is composed of seven subunits (α, β, β', γ, δ, ε and ζ) that are recruited to the membrane. Endosomal COPI proteins are involved in the biogenesis of multivesicular intermediates which achieve transport from early to late endosomes (Gu and Gruenberg, 1999).

ARF1 regulates pH-dependent COP functions in the early endocytic pathway (Gu and Gruenberg, 2000).

ε-COP, one of the seven subunits, acts early in the endocytic pathway, most likely achieving the normal sorting and recycling functions of early endosomes. It has been shown that, in the absence of ε-COP, many consequences are observed on endocytic trafficking. Tf appeared to be internalized as efficiently (Gu et al, 1997) or not (Daro et al, 1997), whereas Tf recycling was markedly inhibited. Sorting of receptor-bound EGF to lysosomes was reduced. Internalization into endosomes and recycling back to the plasma membrane of a fluorescent derivative of sphingomyelin (SM) was not changed. Uptake and accumulation of HRP were reduced, its recycling back to the cell surface was increased, and the tracer was not transported from early to late endosomes. No acidification defect of endosomes was observed, the structure of early endosomes was dramatically changed into clusters of thin 50-60 nm tubules lacking multivesicular domains, and MVB formation was also inhibited (Daro et al, 1997; Gu et al, 1997). Interestingly, β-, β'- and ζ-COP were still recruited onto endosomal membranes when ε-COP was missing. So, COP function in endosome transport requires the presence of α- and ε-COP and COP binding involves β-, β'- and ζ-COP. Together, all these results suggest that the formation of the COP coat onto endosomal membranes may occur in at least three steps: (i) membrane recruitment which may be mediated by the β-, β'- and ζ-COP subunits through the sequential involvement of a pH sensor and, (ii) a GTP-binding protein ARF; (iii) MVBs biogenesis which may be dependent on the presence of α- and/or ε-COP (Gu et al, 1997; Gu and Gruenberg, 1999; 2000).

Whereas COPI is not essential for particle internalization of IgG-opsonized red blood cells (RBC) and for phagosome fusion with endosomes and lysosomes (Botelho et al, 2000a), COPI is recruited to phagosomal membranes by a mechanism that involves heterotrimeric GTP-binding proteins and ARF, and is involved in the recycling of components from phagosomes to the cell surface. Indeed, in cells devoid of ε-COP, retrieval of TfR from phagosomes became inefficient (partial inhibition of TfR recycling) (Beron et al, 2001).

4.2. Coat invagination and vesicle scission

The mechanism by which the neck of the coated pit membranes pinches off to generate coated vesicles remains unclear. Besides structural elements of the coat (the clathrin triskelions

and the clathrin AP complexes), a growing number of proteins associated with clathrin-coated pits and vesicles are being identified. The formation of clathrin/AP-2 adaptor-coated vesicles at the plasma membrane (clathrin-mediated endocytosis) has been the most extensively studied budding event, with around 25 proteins identified as involved in clathrin-mediated endocytosis. Most are large multidomain proteins and are thought to coordinately constitute an extensive network of protein-protein and protein-lipid interactions. The AP-2 adaptor complex plays pivotal role: it interacts directly with cargo proteins and the clathrin coat and is responsible for recruiting, directly or indirectly, many of the accessory proteins. These include dynamin and related proteins (amphiphisins, synaptojanin) as well as Eps15/epsin (Simpson et al, 1999; see Owen and Luzio, 2000).

These proteins interact using a variety of defined structural modules involved in protein-lipid (pleckstrin homology [PH] domain) or protein-protein recognition modules (Src homology domain [SH3 domain], coiled-coil domain, proline-rich domain [PRD], and Eps15 homology [EH] domain) and are likely regulated by phosphorylation and dephosphorylation reactions. A variety of protein-protein interactions are made possible by the combination of interaction modules: SH3 domains interact with PRD in their binding partners; EH domains interact with NPF (asparagine-proline-phenylalanine); etc (for reviews, see Marsh and McMahon, 1999; Owen and Luzio, 2000).

4.2.1. Dynamin and related proteins (amphiphisin, synaptojanin)

Dynamin, a 100 kDa cytoplasmic GTPase, is an essential component of vesicle formation in different endocytic pathways. Three differentially expressed isoforms of mammalian dynamin have been identified: dynamin-1 and -3, exclusively expressed in neuronal tissues and testes respectively, and the ubiquitous dynamin-2. Each leads to four different alternatively spliced isoforms. The various dynamin proteins are localized to distinct membrane compartments (Cao et al, 1998).

Dynamin possesses several domains: (i) the GTPase domain containing three GTP-binding motifs and a self-assembly region; (ii) a middle domain with potential self-assembly properties; (iii) a PH domain implicated in membrane binding; (iv) a GED domain, or coiled-coil domain, essential for self-assembly and stimulated GTPase activity; (v) a C-terminal PRD which contains several SH3-binding sites (Danino and Hinshaw, 2001).

How dynamin is recruited to clathrin-coated pits has received particular attention. Dynamin-2 is stimulated by SH3-containing proteins such as amphiphisin, acidic phospholipids and the cytosolic protein synaptojanin: (i) the SH3 domain of amphiphisin binds to a PRD in dynamin; (ii) the interaction of dynamin with acidic phospholipids is pivotal to its ability to form collars on invaginating vesicles and is mediated through its PH domain, and a preference for phosphatidylinositol 4,5-bisphophate (PI 4,5-P_2) may help to specify the region of the membrane in which dynamin oligomerization takes place; (iii) the cytosolic protein synaptojanin is a phosphatidylinositol (PtdIns) phosphatase, whose activity may be important in adjusting the inositol lipid composition of membranes and thereby regulating dynamin recruitment. Since clathrin and dynamin compete for binding to amphiphisin, it explains how amphiphisin can recruit dynamin specifically to the neck of the buds (for a review, see Marsh and McMahon, 1999).

Purified dynamin readily self-assembles into rings or spirals. It was proposed that dynamin wraps around the necks of budding vesicles where it plays a key role in membrane fission (Hinshaw, 2000). Dynamin is a force-generating molecule capable of constricting an underlying membrane. Self-assembly and oligomerization into ordered structures (rings and spirals) is essential for its function, and three domains of dynamin are involved in self-assembly. Moreover, dynamin has a relatively low affinity for GTP and a high rate of stimulated GTP hydrolysis (Danino and Hinshaw, 2001). A new model of membrane fission has recently been proposed that involves conformational changes of the dynamin collar on the one hand, and on the other hand local modifications of the lipid composition of the neck membrane. In this model, dynamin may serve to isolate a piece of membrane between two rings of dynamin to allow activity to be focused upon only the piece of membrane where fission will be achieved. It is supposed that changes in either the intrinsic curvature of the membrane, membrane tension or osmotic pressure can cause fission of the isolated membrane segment (Kozlov, 2001).

A specific role for dynamin and for GTP in the initial stages of receptor-mediated endocytosis was evidenced. Indeed, mutant forms of dynamin block Tf uptake and clathrin-coated vesicle formation, and alter distribution of clathrin heavy chain and α- but not γ-adaptin. This is in agreement with the demonstration that the appendage domain of α-adaptin is a high-affinity binding site for dynamin (Herskovits et al, 1993; Damke et al, 1994; Wang et al, 1995). Likewise, expression of a mutant dynamin disrupts high-affinity binding of EGF, but not ligand-induced recruitment of EGFR to clathrin-coated pits (Ringerike et al, 1998).

In addition to its key role in clathrin-coated vesicle formation, dynamin is involved at different levels of the pinocytosis and phagocytosis pathways. Dynamin-2 participates in membrane traffic at the TGN (Jones et al, 1998) and in late endosome dynamics and trafficking of the CI-MPR (Nicoziani et al, 2000). Dynamin-2 also localizes to forming phagosomes and a mutant form of dynamin-2 inhibits phagocytosis at the stage of membrane extension around the particle (Gold et al, 1999), a role which has been shown for all of the phagocytic receptor systems examined (zymosan, complement-opsonized RBCs or IgG-opsonized RBCs). Moreover, inhibition of PI 3-kinase prevents the recruitment of dynamin-2 to the site of particle binding; so, the activation of PI 3-kinase is upstream of dynamin in mediating phagocytosis. It has been speculated that dynamin's role in phagocytosis is related to its capacity to recruit membrane to nascent phagosome. However, it is also possible that dynamin is involved in the scission of the neck behind the phagosome, similar to its role in endocytosis, but no enrichment of dynamin at the scission site of the phagosome is found (Gold et al, 1999).

4.2.2. Eps15 and EH-domains

Eps15 was initially identified as a major cytosolic substrate for ligand-activated EGFR-TK (see section 4.1.1.2.2.), but it also seems to play a role in vesicle scission. It is composed of a central domain, which is likely to form a coiled-coil, and a NH_2-terminal domain, which has three repeats termed the EH domains. EH domains are protein recognition motifs that mostly bind sequences containing NPF motifs in epsin, CALM, and synaptojanin that can bind to Eps15 but also to amphiphisin, which itself binds to dynamin. Moreover, Eps15 interacts with α-adaptin (for reviews, see Marsh and McMahon, 1999; D'Hondt et al, 2000; see Figure 5).

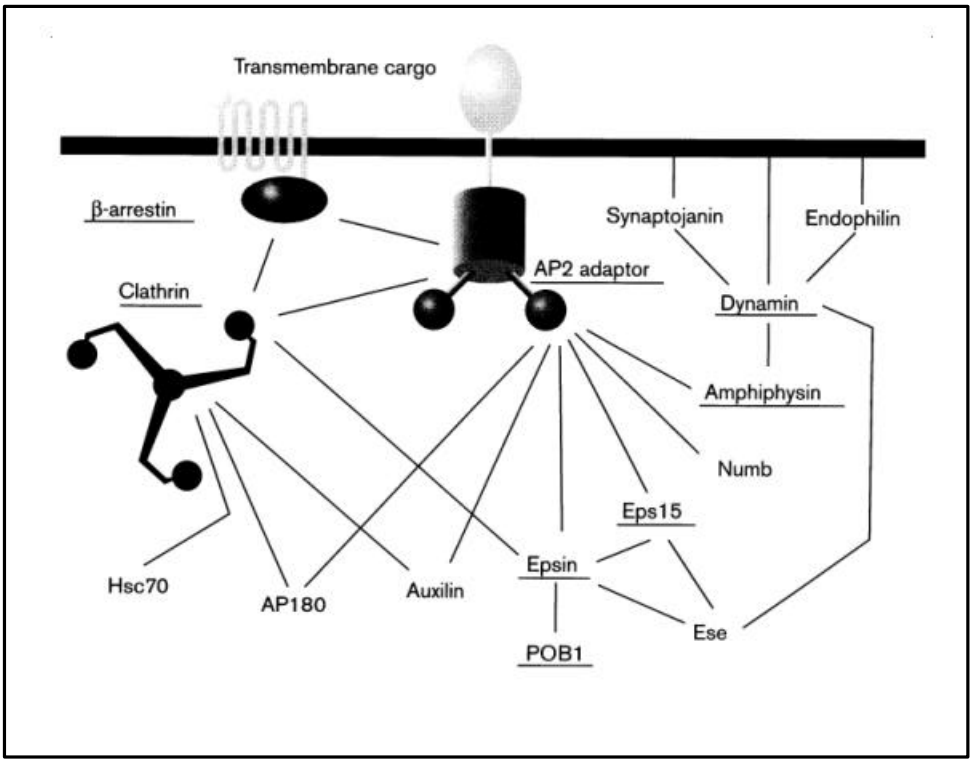


Figure 5. Schematic representations of protein-protein interactions that occur during clathrin-coated vesicle formation at the plasma membrane. (From Owen and Luzio, 2000). Most of the represented proteins are described in the text.

5. Transport-vesicle targeting on the endocytic pathway

Targeting of transport vesicles to the correct membrane destination involves a large set of proteins, to efficiently carry out all steps involved in delivering a newly formed transport vesicle to its target. It requires: (i) the actin- and tubulin-based cytoskeleton to transport a vesicle from one part of the cell to another; (ii) proteins to collect and restrain vesicles at or near their cognate target membranes (tethering); (iii) a core layer of proteins which interact to bring vesicle membranes in close apposition to by pass “hydration force” (docking); and (iv) thereby driving membrane fusion.

Each of these processes is regulated by accessory proteins, to enhance the spatial and temporal control of membrane-trafficking events. Although soluble NSF attachment protein (SNAP) receptors (SNAREs) are clearly important for membrane fusion, the last step of targeting, an increasing body of information indicates that they are not sufficient to ensure that the steps preceeding membrane fusion (tethering and docking of vesicles) are carried out. For tethering and

docking, Rab GTPases are described to recruit tethering and docking proteins such as rabaptin-5 and EEA1.

In this section, we will focus on tethering, docking and fusion in general, and will successively discuss: (i) the factors involved in tethering and docking; (ii) those involved in fusion; (iii) the membrane mixing process; and (iv) the "kiss and run hypothesis" as an alternative process for exchanging material.

5.1. Tethering and docking

Membranes are tethered together prior to interaction of v-SNAREs and t-SNAREs (target SNAREs), that lead to fusion. SNAREs are divided into two subfamilies: one which was originally found enriched on vesicles (v-SNAREs), and the other which was originally associated with target membranes (t-SNAREs). However, this terminology may be misleading and the alternative names of R- and Q-SNAREs have been proposed. Tethering is defined as the formation of links that extend over distances > 25 nm from a given membrane surface. Docking is the holding of membranes within a bilayer's width (< 5-10 nm) of one another. A common feature of many proteins that operate in vesicle tethering and docking is their propensity to form highly extended coiled-coil structures. Among tethering and docking proteins are Rabs and EEA1 (for a review, see Pfeffer, 1999).

5.1.1. Rabs

Because Rab GTPases act prior to SNAREs in membrane docking and fusion, it is unlikely that SNAREs by themselves would play the leading role in vesicle targeting, a role attributed to Rab GTPases and their effectors. However, it is also still possible that not only SNAREs, but also Rab effectors, may participate in the fusion reaction (for a review, see Stenmark and Zerial, 2001).

Rabs belong to a family of about 40 members. They are small GTPases that regulate membrane transport in endocytosis and phagocytosis. Different Rab are localized to distinct compartments and regulate distinct trafficking steps. Rabs are important in vesicle budding, facilitate transport along the cytoskeleton and participate in docking and fusion (for a review, see Somsel Rodman and Wandinger-Ness, 2000).

5.1.1.1. Rabs in endocytosis

Twelve Rabs have been localized to the endocytic pathway of mammalian cells; eight have been functionally characterized and four are epithelial-specific (for a review, see Somsel Rodman and Wandinger-Ness, 2000). Table 3 shows the intracellular location and the role of these Rabs in the endocytic pathway.

Rab	Main intracellular localization	Function
Rab4	Early and recycling endosomes	Endocytic recycling to plasma membrane
Rab5	Clathrin-coated vesicles and Early endosomes	Endocytosis and early endosome Fusion
Rab7	Late endosomes	Transport from early to late endosomes
Rab9	Late endosomes	Transport from late endosomes to the trans-Golgi
Rab11	Golgi and recycling endosomes	Export from the Golgi via endosomes, apical basolateral endocytic recycling
Rab15	Early and recycling endosomes	Inhibitor of endocytic internalization
Rab17	Epithelial specific; apical Recycling endosome	Transport through apical recycling endosomes
Rab18	Epithelial specific; kidney dense Apical tubules and basolateral Domain of intestine	Uncharacterized
Rab20	Epithelial specific; kidney dense Apical tubules	Uncharacterized
Rab22	Endosomes and plasma membrane	Uncharacterized
Rab24	Endoplasmic reticulum, Golgi and endosomes	Uncharacterized
Rab25	Epithelial specific; apical Recycling endosome	Transport through apical recycling endosomes

Table 3. Intracellular location and roles of Rabs in the endocytic pathway. (Adapted from Somsel Rodman and Wandinger-Ness, 2000).

Although it is well known that different Rabs are localized to different structures, a recent study has shown that distinct Rabs could be seen in the same organelle. Indeed, both sorting and recycling endosomes are structured into morphologically distinct domains enriched in Rab4, Rab5 and Rab11. Early endosomes in the cell periphery contain mostly Rab5 and Rab4 and little Rab11. Conversely, recycling endosomes in the pericentriolar area mainly contain Rab4 and Rab11. So, a variety of endosomal populations may arise from combinatorial arrangements of individual

domains. Interestingly, fast recycling seems to require a rapid sorting of Tf from a Rab5 to a Rab4 domain on the same endosome (Sönnichsen et al, 2000).

5.1.1.2. Rabs in phagocytosis

Rab5 is also involved in phagocytosis. Rab5(Q79L), a GTPase deficient mutant, acting as a dominant-positive mutant, stimulates non-specific phagocytosis of "naked" latex beads internalized through undefined receptors, but not of Fc- (latex beads opsonized with IgG) or C3- (latex beads opsonized with complement) receptor-mediated phagocytosis (Duclos et al, 2000). J774 macrophages transfected with Rab5 show transient activation and recruitment of Rab5 to newly formed phagosomes that contain *E. coli* (removal of Rab5 from the phagosomes after 1-2 min)(Roberts et al, 2000). In addition to its role in uptake by phagocytosis, Rab5 also regulates endosome-phagosome fusion and live microorganisms can trigger this process by recruiting Rab5 to the phagosomal membrane (Alvarez-Dominguez et al, 1996). Rab5 is in fact involved in regulating the duration and/or frequency of fusion between phagosomes and endosomes in macrophages, allowing these organelles to engage in "kiss and run" interactions (see section 5.4.). This enables the transfer of the solute content of endocytic organelles to phagosomes without significant increase of phagosome size (Duclos et al, 2000). Likewise, Rab5 can also modulate fusion between phagosomes (Duclos et al, 2000).

Rab11 is present in several intracellular organelles including endosomes and nascent phagosomes. Expression of Rab11(25N), a GTP binding-deficient mutant, leads not only to a decreased rate of Tf efflux but also impairs FcγR-mediated phagocytosis. In contrast, expression of Rab11(70L), a GTPase-deficient mutant, leads to an accelerated Tf efflux and enhances phagocytosis. This shows that macrophages may use a rapidly mobilizable endocytic compartment to support phagocytosis and that Rab11 participates in the recruitment of this compartment to the macrophage cell-surface (Cox et al, 2000).

5.1.1.3. The cycle of Rabs

Rab activity is affected by multiple factors and, through multiple effectors, influences endocytic pathways at the level of budding, cytoskeletal transport and docking/fusion. The combination of specific Rab regulators and specific effectors provides a platform for coordinate regulation of endocytic pathways.

Like ARF and other Ras-like proteins, Rabs cycle between a GDP-bound (off) and a GTP-bound (on) conformation. Rab GTPases are reversibly attached to membranes by insertion of their geranylgeranyl groups with the lipid bilayer. Geranylgeranylated Rab GTPases are presented to, and removed from membranes as complexed by Rab GDP dissociation inhibitor (GDI), then GDI dissociation occurs. Upon Rab recruitment to the membrane, nucleotide exchange is regulated by specific GEFs, so that conformation switch from GDP- or GTP-bound states allows for fusion. GAPs interact with Rabs to stimulate GTP hydrolysis and inactivate Rab. This allows for recognition by GDI, Rab release from membranes and recycling (see Figure 6).

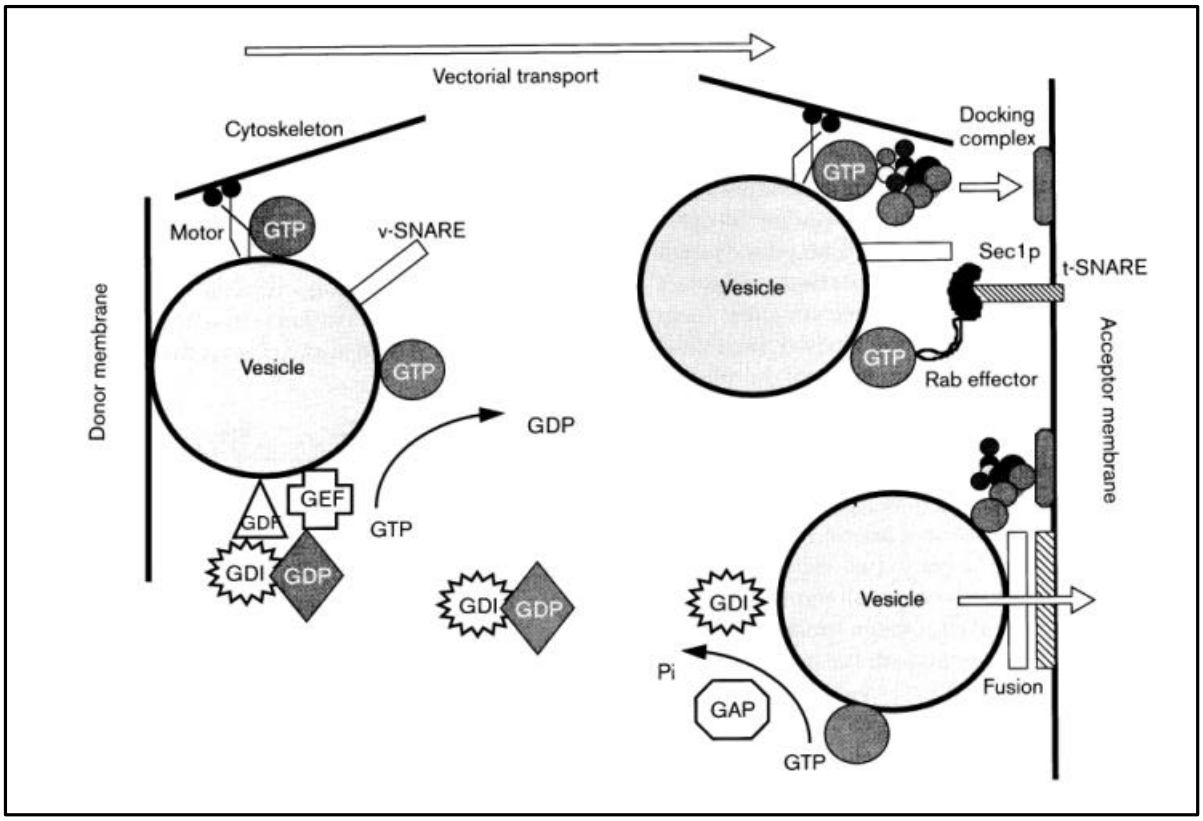


Figure 6. Model of the functional cycle of Rab proteins. Rab proteins are incorporated into a transport vesicle, either during or after its formation. The attachment process of Rab GDP-GDI complexes involves several proteins and is coupled to GDP/GTP exchange catalyzed by GEF proteins. Rab GTP interacts with a select set of effectors to target the vesicle to appropriate sites on the acceptor membrane. The effectors include motor proteins and docking complexes. During or after docking of the vesicle with the acceptor membrane, other sets of Rab effectors activate SNAREs through interactions with SNARE regulatory proteins, such as Sec1p. After fusion of the vesicle, GTP hydrolysis is mediated by GAP. GDI releases Rab GDP from membranes and Rab/GDI complexes are re-used for another round of transport. (From Chavrier and Goud, 1999).

Some GEFs have now been identified: rabex-5, which catalizes GDP/GTP exchange upon delivery of Rab5 to membranes. Rab3AGAP and tuberin are GAPs for the Rab3 and Rab5 subfamilies respectively (for reviews, see Chavrier and Goud, 1999; Somsel Rodman and Wandinger-Ness, 2000).

GTP hydrolysis is not coupled to fusion but rather Rab conformation determines its ability to recruit docking factors from the cytosol onto membranes. One GTPase may have numerous effectors, some of which may mediate vesicle tethering prior to SNARE complex formation (see Stenmark and Zerial, 2001). So, the primary function of Rabs is to recruit cytosolic docking factors that are needed for vesicle translocation, tethering/docking and fusion.

5.1.1.4. Regulation of endocytosis by Rabs

The role of Rab5 in docking and fusion processes has been the most extensively studied. Rab5 catalyzes both heterotypic and homotypic fusion processes, but Rab5 GTP on CCVs is perhaps longer lived than on endosomes, ensuring efficient heterotypic vesicle fusion and restraining homotypic endosome fusion (for a review, see Pfeffer, 1999). In heterotypic fusion, vesicle formation should be rate-limiting, and Rabs would favor transport of vesicles to their targets, rather than support fusion between organelles. By contrast, in homotypic fusion between two organelles, the overall level of Rab-GTP could modulate fusion rates. For example, in endosome-endosome fusion, cells would rely on Rab5 turn-over to maintain the size of the endosome compartment, in particular to prevent the formation of large vacuoles (for a review, see Pfeffer, 1999).

In addition to their roles in membrane docking and fusion, it is currently recognized that they also play roles in membrane budding and vesicle transport. Membrane budding to form vesicles requires complex interactions among multiple proteins including ARF, coatomers, adaptins and clathrin. Accumulating evidence shows that active Rabs are also required to vesicle budding, although their precise roles remain to be determined. For example, it has been suggested that Rabs could facilitate cargo selection but would not be involved in coated pit formation *per se* (for a review, see Somsel Rodman and Wandinger-Ness, 2000).

It has recently been recognized that Rabs are intimately associated with cytoskeletal proteins for the regulation of transport along microtubules. For example, movement of Rab5-

labelled early endosomes on microtubules depends on active Rab5 and a plus-end directed kinesin (see also section 9.5.1.). Moreover, Rab5-stimulated microtubule motility is separable from Rab5-dependent endosome docking and fusion, suggesting that activated Rabs serve as a scaffold for multiple effectors. In this view, through the temporal control of effector protein binding, individual Rabs may control multiple events necessary for the transport of cargo between compartments. Moreover, Rab5 and its effectors bind to actin and Rab5 is sometimes actively required for the reorganization of actin stress fibers. Likewise, an intimate interaction between Rab11 and the actin cytoskeleton has been shown to be critical for TfR recycling (for a review, see Somsel Rodman and Wandinger-Ness, 2000; see also section 9.5.1.).

5.1.2. Rab5 effectors: rabaptin-5 and EEA1

Rab5 is found on the cytosolic side of early endosomes, endocytic vesicles and the plasma membrane. Expression of a GTPase-deficient mutant (Rab5Q79L), acting as dominant-positive mutant, accelerates endocytosis and leads to the formation of giant early endosomes. Conversely, the expression of a mutant with preferential affinity for GDP (Rab5S34N) inhibits endocytosis and causes the formation of very small early-endocytic profiles (for a review, see Stenmark and Zerial, 2001). Rab5 does not act alone, but rather has many effectors. The first one to be identified was rabaptin-5, a dimeric coiled-coil protein found in a complex with rabex-5, and which is recruited to early endosomes in a Rab5- and GTP-dependent manner. Rabenosyn-5 is another Rab5 effector, which contains a FYVE finger domain and is recruited in a PI 3-kinase-dependent fashion to early endosomes (for a review, see Stenmark and Zerial, 2001).

The rabaptin-5/rabex5 complex is essential but not sufficient for both the heterotypic fusion between endocytic vesicles and early endosomes and the homotypic early-endosome fusion. Endocytic membrane fusion requires several other Rab5 effectors. Among these is EEA1, a large, predominantly coiled-coiled, endosomal protein with a zinc-binding FYVE finger, which appears to be a core component of the membrane docking and fusion machinery, whereas the main role of rabaptin-5/rabex-5 might be to maintain a high local level of endosomal Rab5:GTP. By this way, EEA1 may be efficiently recruited to endosomes, tethers endosomes together and acts upstream of SNAREs to trigger endosome fusion (for a review, see Stenmark and Zerial, 2001). Interestingly, whereas Rab5 and rabaptin-5 are symmetrically localized between CCVs and early endosomes, EEA1 is recruited selectively on membranes of early endosomes, suggesting that EEA1 is a tethering molecule that provides directionality to vesicular transport from the plasma membrane to

the early endosomes (Rubino et al, 2000). In addition, although the function of EEA1 is enhanced by Rab5, high levels of EEA1 can tether endosomes in the absence of Rab5 function (for a review, see Waters and Pfeffer, 1999). Other molecules are likely to co-operate with EEA1 and the rabaptin-5 complex in endocytic membrane docking and fusion, such as PI 3-kinase (for a review, see Stenmark and Zerial, 2001; see also section 7.2.1.2.4.).

5.2. Fusion

5.2.1. NSF/SNAP/SNAREs

5.2.1.1. Regulation of fusion and the cycle of SNAREs

The hexameric ATPase, NSF, is required for membrane traffic in mammalian cells. The ATPase activity of NSF is regulated by SNAP and the NSF/SNAP complex binds to SNAP receptors, the SNAREs. The cytoplasmic portions of SNAREs contain several α-helical regions, heptad (seven-amino-acid) repeats in their sequences. The first and fourth residues of each repeat are hydrophobic amino acids, so that one face of the α-helix is hydrophobic. The assembled v-SNARE/t-SNARE complex consists of a bundle of four helices, one of which is supplied by the v-SNAREs and the other three by t-SNAREs. In the plasma membrane, the t-SNARE consists of the protein syntaxin which supplies one helix, and a SNAP-25 protein which contributes the other two. In intracellular membranes, SNAP-25 is replaced by a build-up of a “heavy chain” homologous to syntaxin and two separate non-syntaxin “light chains” (Fukuda et al, 2000). When the four helices of the core fusion complex interact, these faces pack against one another to produce the hydrophobic core of a coiled coil (Weis and Scheller, 1998). However, close proximity of two membranes is not sufficient for fusion and implies an active mechanism. v-SNAREs require anchors capable of spanning both leaflets, whereas t-SNAREs do not, so long as the anchor is sufficiently hydrophobic. Fusion is inhibited as the length of the linker connecting the helical bundle-containing rod of the SNARE complex to the anchors is increased. It has been suggested that one activity of the SNARE complex promoting fusion is to exert force on the anchors by pulling on the linkers (McNew et al, 2000).

Whereas the v-SNARE/t-SNARE complexes can form on the same membrane (*cis*) or between SNAREs in different membranes (*trans*), fusion clearly requires the formation of *trans*-

SNARE complexes, i.e. complexes between v-SNAREs on the one membrane and t-SNAREs on the other one. Pre-existing SNARE complexes are first dissociated (primed) through the activity of NSF/SNAP. Primed SNAREs on a vesicle interact with primed SNAREs on a target membrane. The SNAREs form tight complexes through parallel coiled-coil interaction, and the energy released may power membrane fusion. After fusion, the SNARE complexes reside in the target membrane, and are disrupted by the action of NSF. A second fusion event could thereafter occur (for a review, see Stenmark and Zerial, 2001). It has been shown that *trans*-SNARE complexes are functionnaly resistant to NSF, and they become so at the moment they form and commit to fusion. This explains why NSF does not disrupt these *trans*-SNARE complexes as well as *cis*-SNARE complexes, allowing SNARE-dependent fusion to occur in a cytoplasm containing NSF, SNAP and ATP. The sensitivity of *cis*-SNARE complexes to NSF ensures the avaibility of free SNAREs before fusion and, after fusion, the separation of v-SNAREs from t-SNAREs to permit their recycling to donor membranes (Weber et al, 2000; see Figure 7 in section 5.2.2.).

The SNARE hypothesis is proposed to cover all targeted fusion events during secretion and endocytosis, except: (i) the apical vesicle transport in MDCK cells, which was shown to be independent of four different components essential to the SNARE hypothesis, NSF, α-SNAP, Rab and a v-SNARE (Wilson, 1995); (ii) in addition to NSF, another N-ethylmaleimide (NEM) sensitive factor might also be involved in late steps of the endocytic pathway and this factor may be membrane-associated (Robinson et al, 1997); (iii) NSF may interact with other membrane receptors that are not part of the docking complex NSF/SNAP/SNARE, since, depending on its nucleotide-bound state, NSF may interact with membranes by binding to endosomes diffusely and not only at sites where vesicle docks (Colombo et al, 1996).

5.2.1.2. Roles of NSF/SNAP/SNARE in endocytosis

The endocytic apparatus consists of an heterogeneous complex of tubules and vesicles, that undergo continuous rounds of fusion and fission with both newly internalized and preexisting vesicles. Maintenance of the steady-state number and size of endocytic vesicles indicates tight regulation and coupling of the rates of vesicle fusion and fission. Moreover, membranes retain their biochemical identity, indicating a selectivity of fusion events, which is determined by the ability of the organelles to recognize each other through NSF and SNARE molecules.

Although each endosomal age cohort is able of homotypic fusion, they are restricted in their ability to undergo heterotypic fusion with either older or younger endosomes. Indeed, in an *in vitro* fusion assay of endocytic vesicles isolated at defined stages of maturation of alveolar macrophages, a 4-min endosome (i.e. isolated 4 min after internalization) can fuse with a 4- or 8-min endosome (i.e. isolated 4 or 8 min after internalization respectively) but shows a diminished ability to fuse with either 12-min endosome or lysosome. Conversely, a 12-min endosome is able to fuse with lysosomes, but has a markedly reduced ability to fuse with endosomes earlier than 8 min. This demonstrates a change in endosome fusion properties that occurs between 8 and 12 min after internalization, implying biochemical changes in the molecules that regulate fusion or specify vesicle interactions.

Tethering and fusion of endocytic vesicles with early endosomes and of early endosomes with each other, is mediated by a process involving Rab5, the t-SNARE syntaxin 13 and phosphatidylinositol 3-phosphate (PI 3-P) (Luzio et al, 2000). Two SNAREs, syntaxin 7 and VAMP-7 (vesicle associated membrane protein-7), are involved in fusion of vesicles in the late endocytic pathway (Mc Vey Ward et al, 2000). Lysosomes are able to fuse directly with late endosomes to form hybrid organelles in which degradation of endocytosed material takes place and from which lysosomes can be reformed. Three alternative hypotheses are available to explain content mixing between late endosomes and lysosomes: (i) vesicular transport between the two organelles; (ii) the “kiss and run” process (see section 5.4.); and (iii) direct and complete fusion between late endosomes and lysosomes with subsequent recovery of lysosomes for re-use. Although no evidence supporting vesicular transport between late endosomes and lysosomes has emerged, there is clear evidence for direct fusion of these organelles. In a cell-free content-mixing assay, it has been shown that fusion between late endosomes and lysosomes is ATP-, cytosol- and temperature-dependent, requires the presence of NSF and SNAPs, and is inhibited by Rab-GDI. Although no specific tethering factors have yet been identified for late endosome-lysosome fusion, the evidence of their existence is strong. Moreover, there is no convincing evidence as to which specific SNAREs are required for fusion of late endosomes and lysosomes. Together, these characteristics are consistent with the action of the fusion machinery used at many other sites on the endocytic pathway (Luzio et al, 2000).

5.2.1.3. Roles of NSF/SNAP/SNARE in phagocytosis

Phagocytosis is associated with a net increase in cell surface area, suggesting the concommitant occurrence of exocytosis. It has indeed been demonstrated that v-SNARE-dependent secretion of endomembranes is essential for optimal completion of particle internalization during phagocytosis. This effect is relevant to both IgG- and non-IgG-opsonized particles (Hackam et al, 1998).

Syntaxins 2, 3 and 4, three SNAREs, are present on phagosomal membranes of murine peritoneal macrophages and of the murine macrophage cell line J774, suggesting that they may participate in phagosomal maturation. However, the density of syntaxins on the phagosomal membrane was found to be comparable with that on the surface membrane, suggesting that preferential fusion of vesicles with the phagosomal membrane is not solely the result of segregation of the syntaxins to this organelle (Hackam et al, 1996).

The role played by NSF/SNAP/SNARE is also illustrated in phagosome-endosome and phagosome-lysosome fusions. First, live *Salmonella* helps Rab5 binding on the phagosomes, and possibly activates the SNARE which leads to recruitment of α-SNAP for subsequent binding with NSF. These recruitments promote fusion of the phagosome containing live *Salmonella* with early endosomes and inhibition of their transport to lysosomes. So, the live *Salmonella*-driven fusion of phagosomes with the early endocytic compartment may extend the period of bacterial residence in the less acidic early endosomal compartment, thereby inhibiting the transport of the live *Salmonella* to lysosomes (Mukherjee et al, 2000). This fusion requires cytosol, ATP, NSF and Rab5, and is sensitive to GTPγS (Funato et al, 1997). Second, fusion of lysosome with phagosome also depends on energy and cytosol, and occurs via microtubule-dependent transport, and requires Rabs and NSF (Funato et al, 1997).

5.2.2. Calcium/annexins/calmodulin

Although it is clear that SNAREs play an indispensable role in membrane fusion, it is also obvious that *trans*-SNARE complexes are not the terminal catalysts of fusion.

Moreover, endosomes can fuse by two mechanisms, one that has an absolute requirement for calcium, and one that does not require calcium. Externally added calcium has a minor effect on

endosome fusion, but, in the presence of calcium, endosomes fuse even under conditions that normally not support fusion. Calcium-dependent fusion has several distinctive characteristics, since neither ATP nor cytosol are required. But how can two mechanisms of endosome fusion operate in the cell? Intracellular calcium is normally low and endosome fusion might proceed by a cytosol-dependent mechanism. However, under certain conditions, a local or global increase of calcium could activate the other fusion mechanism (Mayorga et al, 1994).

Calcium exerts its effect through one or more calcium-binding proteins that undergo a conformational change upon interaction with the ion, such as annexins and calmodulin. Annexins are calcium-binding proteins with a wide distribution in most polarized and nonpolarized cells. Several annexins including annexin I, II, IV, VI, VII and XIIIb have been directly implicated in different steps of the intracellular trafficking pathways and they are all associated with the endocytic compartment (Mayorga et al, 1994; for a review, see Gerke and Moss, 1997). In J774 mouse macrophages, whereas annexins I, II, III, IV and V are present on both the plasma membrane and phagosomes, the localization on other organelles differs, with annexins I, II, III and V on early endosomes and only annexin V on late endosomes. Annexin I and II distribute non-uniformly along the plasma membrane and co-localize with F-actin at the sites of membrane protusions (Diakonova et al, 1997). Annexin I is a major substrate for the EGFR kinase and its phosphorylation by the receptor kinase occurs in MVBs, whereas the bulk of membrane-bound annexin I was identified on the plasma membrane and on the membrane of early endosomes. Annexin II is specifically localized to certain areas of the plasma membrane and to cisternal and tubular regions of early endosomes in different cell types. It is involved in some endosome functions and has been suggested to play a role in a docking and/or fusion process between early endosomes from *in vitro* and *in vivo* studies. Annexin VI co-fractionates with early endosomes, and is also found in the late endocytic compartment in a number of cell-types. It stimulates endocytosis and is involved in the trafficking of LDL to the prelysosomal compartment. It was suggested that, after stimulating endocytosis at the cell surface, annexin VI remains bound to endocytic vesicles to regulate entry of LDL into the prelysosomal compartment (Grewal et al, 2000).

Calmodulin is another calcium binding protein which has also been implicated in various intracellular transport events, such as phagocytosis, transcytosis, receptor recycling and lysosomal transport. The demonstration that calmodulin activity regulates endosome fusion both *in vivo* and *in vitro*, leads to the suggestion that it plays a general role in vesicular traffic. Calcium-induced

fusion between endosomes is stimulated by calmodulin. Calmodulin might act directly in membrane trafficking or indirectly by modulating an essential component of the vesicular transport machinery. The putative downstream effectors for calmodulin in endosome fusion are EEA1 (this early endosome-associated protein has a calmodulin-binding domain), the calmodulin-dependent protein kinase II, and synaptotagmin (both a calcium-binding protein and a calmodulin-binding protein) (Colombo et al, 1997).

To integrate SNARE and calcium requirements, it has been proposed that a *trans*-complex could signal the completion of docking to later acting components, possibly by release of calcium from the lumen. This could trigger a machinery to catalyze bilayer mixing via the calcium/calmodulin complex (for a review, see Mayer, 1999; see Figures 7 A and B).

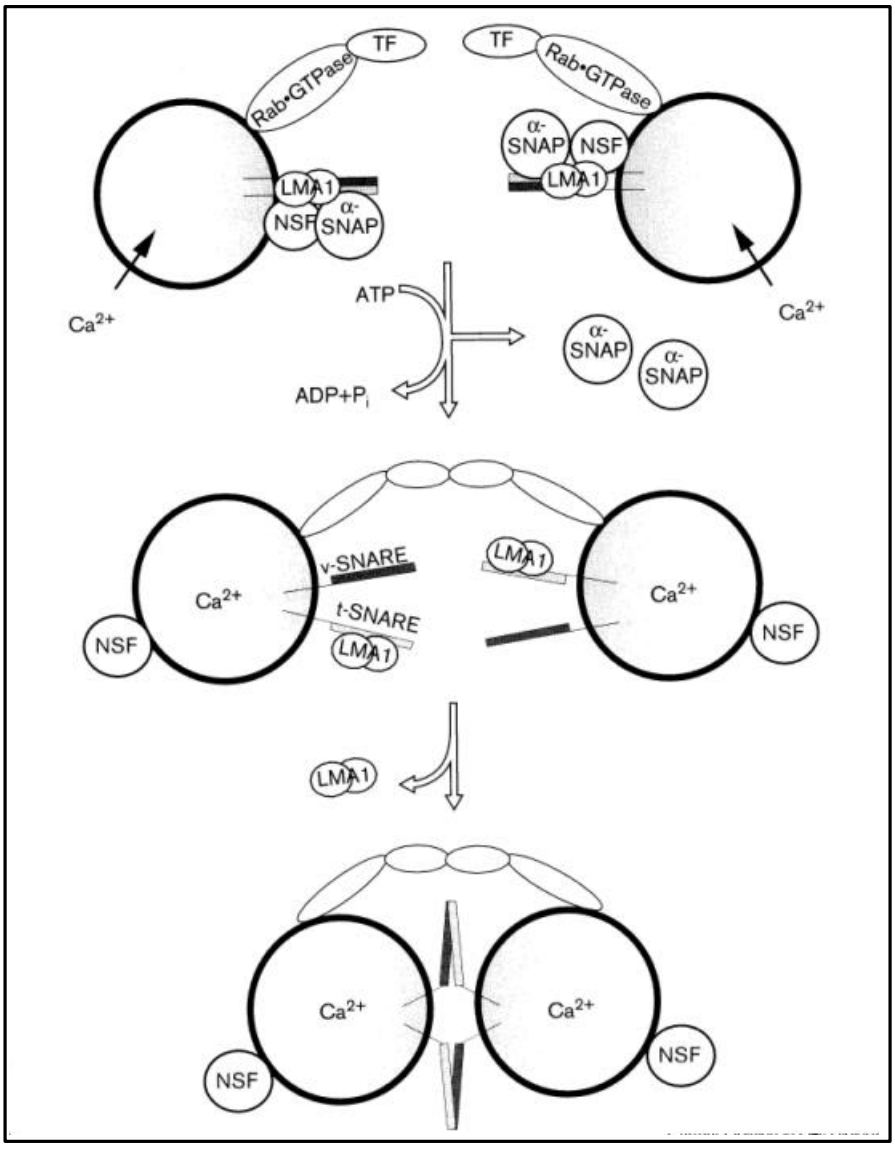


Figure 7 A. Working model of early events in intracellular membrane fusion. Membranes initially bind to each other via a system of tethering factors and GTPases. NSF, together with its cofactor á-SNAP, splits the *cis* of v-t SNARE complexes. This reaction is termed priming. The resulting activated state is preserved by interaction of the LMA1 complex (low molecular weight activity 1) until SNAREs pair again in *trans.* Throughout, a high luminal Ca^{2+} concentration is established and maintained by active uptake. (From Mayer, 1999).

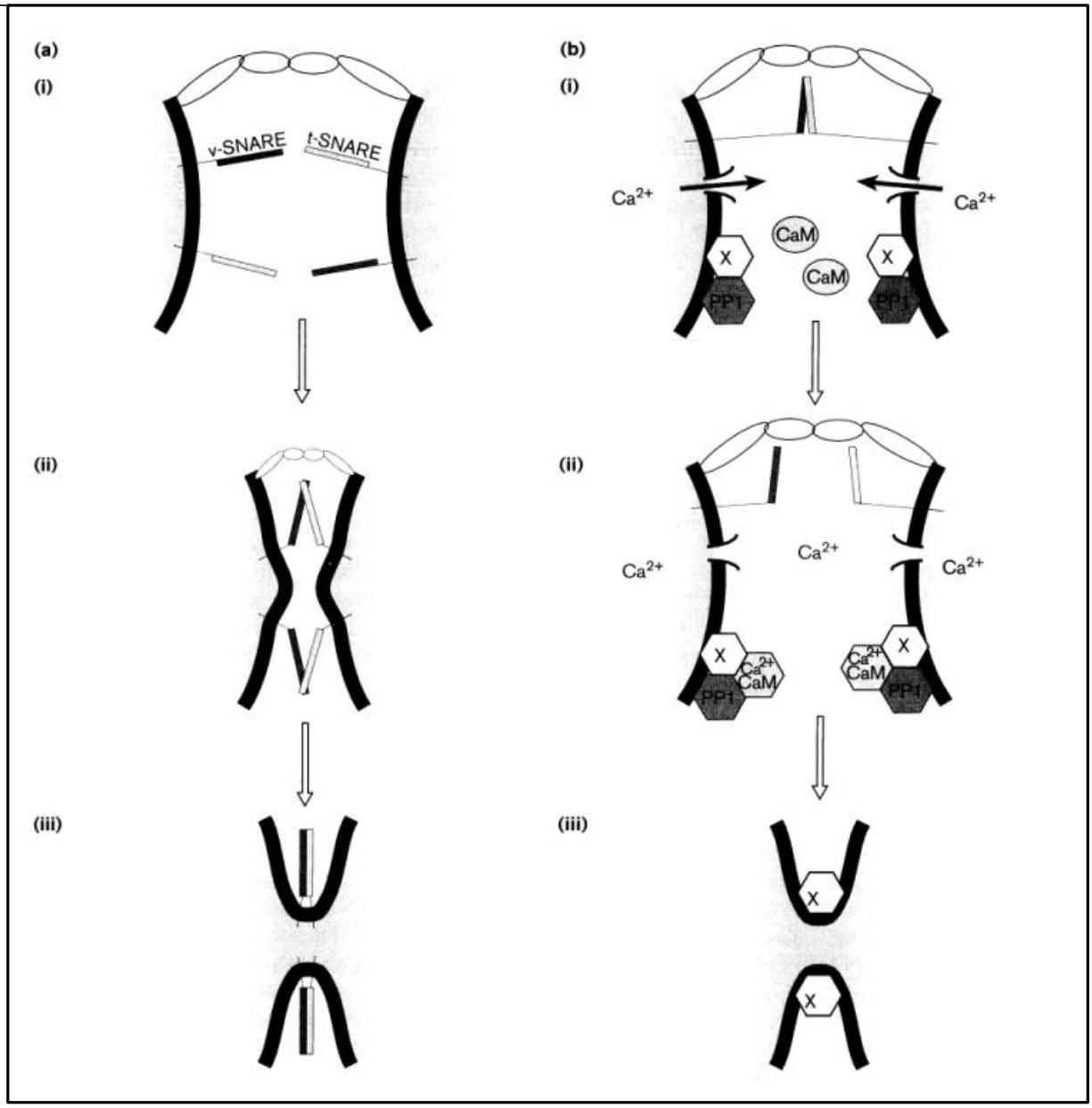


Figure 7 B. Alternative views on late events in membrane fusion. **(a)** SNARE driven fusion. (i) Upon the initial interaction (ii) *trans*-SNARE pairs form. The energy released by complex formation is used to force membranes together and perhaps to introduce some conformational strain, which (iii) promotes fusion. **(b)** SNAREs function at an intermediate stage. (i) *Trans*-SNARE complexes (v-t) form before fusion. This, or some other process involved in docking, produces a signal opening Ca^{2+} channels in the membrane. SNARE complexes may be disassembled after this stage. (ii) Ca^{2+} is released and binds to calmodulin (CaM), which then binds more tightly to the membranes. It enters a complex with protein phosphatase 1 (PP1) and other as yet unidentified factors (X). (iii) These factors could finally promote bilayer/contents mixing. (From Mayer, 1999).

5.3. Membrane fusion *per se*

The fusion *per se* of two lipidic membranes can be summarized in a three-steps process: (i) the close approach of the two membranes; (ii) removal of water from the area of contact; and (iii) localized disruption of the bilayers necessary to rearrange them into a single continuous structure. For the last step, most of the models assume the involvement of inverted lipid structures such as inverted micelles or hexagonal phase which serve as intermediates in the membrane fusion event.

The net force acting between two lipid bilayers at a distance shorter than 30 Å is dominated by a repulsive hydration force, and closer approach of the membrane requires removal of bound water from the headgroup of the lipid molecules. This explains why lipids with a low headgroup hydration, like phosphatidylethanolamine (PE), generally enhance membrane fusion in model membrane systems. Indeed, the PE headgroup binds fewer water molecules than the headgroup of phosphatidylcholine (PC), and it has been speculated that this lower hydration favors the formation of the nonlamellar hexagonal phase by unsaturated PE (Burger and Verkleij, 1990).

A large number of fusogenic lipids have been proposed to be involved in membrane fusion. Among these are, lysolecithin, which can form micelles, as well as monoacylglycerides and cis-unsaturated fatty acids, which increase membrane fluidity (Burger and Verkleij, 1990). These fusogens can induce a change in the membrane lipid organization from the bilayer to the hexagonal phase (Hope and Cullis, 1981). Moreover, acidic phospholipids, especially phosphatidic acid (PA) and phosphatidylserine (PS), have been proposed to play a crucial role in the induction of biomembrane fusion in conjunction with calcium (Burger and Verkleij, 1990).

5.4. “Kiss and run” hypothesis

Despite the dynamic fusion activity, phagosomes and endosomes maintain a relatively stable size over time. Based on this, it has been proposed that phagosomes repeatedly fuse with endocytic organelles without fully exchanging their membranes, a process referred to as the “kiss and run” hypothesis. This process would have the advantage to allow organelle to exchange contents without the complete mixing of their membranes, limiting the extent of recycling.

This mechanism has been postulated to account for rates of exchange between plasma membrane and lysosomes (Draye et al, 1988). Evidence for “kiss and run” exchanges has been

obtained from various biological systems including: (i) exocytosis-endocytosis coupling (Fesce and Meldolesi, 1999); (ii) phagosome-endosome interaction (Desjardins et al, 1994; 1997; Duclos et al, 2000); (iii) endosome-endosome interaction (Berthiaume et al, 1995); and (iv) lysosome biogenesis (Storrie and Desjardins, 1996). The first, or conventional, model of coupled exocytosis-endocytosis predominates at low calcium concentration. After fusion of the vesicle with the plasma membrane, an aqueous pore is formed, followed by flattening of the vesicle in the plasma membrane, the formation of a clathrin-coat and detaching from the plasma membrane. By contrast, "kiss and run" fusion is favoured at high calcium levels. In this process, the vesicle fuses briefly with the plasma membrane and widening of the pore ensues. Quick resealing and recycling of the vesicle then follow (Fesce and Meldolesi, 1999). Protein transfer between phagosomes and the endocytic compartments may also occur by "kiss and run". In this model, phagosomes and endocytic vacuoles do not coalesce into one organelle, but rather, membranes and luminal content are exchanged between the two compartments by a momentary fusion followed by fission. Rab5 seems to be involved in the regulation of this "kiss and run" fusion (Duclos et al, 2000). Third, Berthiaume et al have proposed that endocytic compartment communication could involve transient and incomplete fusions between organelles. By this model, they explained a possible mechanism for size-selective movement through endocytic compartments, based on the idea that organelle communication transfers small molecules from one compartment to another more readily than large molecules (Berthiaume et al, 1995). Finally, it has been proposed that lysosomes are maintained within cells by a series of "kiss and run" fusion events with the late endosome/prelysosome compartment (Storrie and Desjardins, 1996). Although the benefit of avoiding full exchange of membranes is not clear, this model would prevent escape of a lysosomal matrix of supramolecular assemblies of soluble enzymes complexed with lipids (Jadot et al, 1997).

6. Sorting mechanisms during endocytosis

Sorting is a key requirement of the endocytic apparatus since not all of the membrane constituents are directed at the same rate or into the same endocytic vesicle and since the constituants internalized by the same route end up into different destinations. The traffic of macromolecules within and through vesicular organelles is directed both by physical sorting as well as by signal sorting (Mukherjee et al, 1997).

6.1. Bulk sorting

Molecular sorting that occurs without signals is called “bulk sorting”. Physical sorting is based on physical properties (pH and shape) and dynamic (regulation of fusion and vesicle formation) of organelles, the size of solutes and lipids, and cargo partitioning between membranes or the luminal fluid (see Mukherjee et al, 1997).

6.1.1. Fluid content

Soluble tracers, such as fluorescein isothiocyanate-dextran (FITC-dextran), accumulate in sorting endosomes and are delivered to lysosomes. This is based on the tubulovesicular morphology of sorting endosomes, soluble tracers being in the vesicular portion, giving rise to a physical sorting (for a review, see Mukherjee et al, 1997).

Another sorting factor which occurs without signal is the size of molecules. Small solutes recycle from macrophages to the extracellular medium more efficiently, and move more easily between endocytic organelles than do large solutes. Moreover, uniform mixtures of different-sized fluorescent dextrans can segregate by size into distinct lysosomes (Berthiaume et al, 1995). Three mechanisms are proposed for size-selectivity through endocytic compartments, to explain how small molecules are transferred better than large ones: (i) packing of small molecules is more dense than large ones; (ii) organelle communication could involve transient and incomplete exchange (“kiss and run” model); (iii) tubular extension create long narrow lumina along which macromolecules would diffuse at different rates (Berthiaume et al, 1995).

6.1.2. Lipids

The majority of lipids are in tubules of sorting endosomes and recycle to the cell surface. The tubulovesicular geometry of the sorting endosomes largely accounts for the efficient sorting of membrane components: up to 80 % of the sorting endosome membrane area is in its tubules, indicating that the majority of the lipid in the sorting endosome membrane should be in tubules. Moreover, recycling receptors traffick through the same early endosomal compartments and at the same rate as membrane lipids such as C_6-NBD-SM, implying that exit from sorting endosomes,

and the subsequent delivery back to the plasma membrane, is generally a “default pathway” for membrane components (see Mukherjee et al, 1997).

However, consistent with a possible role for microdomains, certain lipids can be sorted within the endocytic system so that they have a higher likehood to enter late endosomes. As examples, we have to mention: (i) the non-random internalization of lipids, using C_6-NBD-SM; (ii) during phagocytosis of large latex beads, cells specifically exclude from phagosomes lipids with long and saturated tails but not lipids with unsaturated tails that preferentially partition into fluid regions; and (iii) in sorting endosomes, fluid domain-preferring lipids, which have short and/or unsaturated tails, exit the sorting endosomes more rapidly and efficiently, whereas lipids with long and saturated tails are better retained and then trafficked to the late endosomes/lysosomes. Preferential retention of certain lipids in organelles has also been evidenced: (i) late endosomes contain an extensive system of internal vesicles in which a negatively charged lipid, LBPA, is concentrated; and (ii) the ERC is an important cellular cholesterol pool (for a review, see Mukherjee and Maxfield, 2000). Two possible mechanisms by which preferential retention of one lipid class in a given organelle might be achieved: (i) the vesicular region of sorting endosomes might be composed of more rigid membrane domains, as compared to the tubules, therefore favoring partition of long saturated chain lipid analogs into this region; (ii) the inclusion into inward budding vesicles in late endosomes, where curvature most likely plays a role, and in which retention of LBPA may be due to the shape and the charge of this molecule (for a review, see Mukherjee and Maxfield, 2000).

In polarized cells, lipids can also be sorted. For example, in HepG2 cells, it has been shown that: (1) trafficking of GlcCer and SM from their site of synthesis, the Golgi apparatus, to the apical plasma membrane occurs by a direct transport process; (2) trafficking of both lipids from the basolateral plasma membrane to the apical plasma membrane (transcytosis) occurs via bulk flow; (3) sorting of sphingolipids, which is taking place in a SAC, occurs in the reverse transcytotic pathway (from the apical to the basolateral plasma membrane) and allows GlcCer to remain located in the apical region and SM to redistribute to the basolateral domain (van Ijzendoorn et al, 1997; Hoekstra et al, 1999).

6.2. Signal sorting

Signal sorting implies information contained in the structure of the macromolecule. Sorting signals within membrane proteins are required for: (i) receptor-mediated endocytosis from the plasma membrane, (ii) trafficking between endosomes and the TGN, (iii) recycling from endosomes to the plasma membrane, and (iv) movement within the endosomal system.

6.2.1. Sorting of membrane proteins during receptor-mediated endocytosis

Recruitment into coated pits is an example of signal-mediated sorting. The most critical information for internalization is encoded by a short stretch of amino acids located within the cytoplasmic domain of membrane proteins, such as a tyrosine-based motif (TfR or LDLR), or a leucine-based sorting motif (FcRIIB2) (for a review, see Höning, 2001; see section 4).

The rate at which proteins are internalized via CCV-mediated endocytosis is quite variable, from less than 1 % / min (in case of preferential exclusion) to more than 40 % / min (in case of active recruitment). Differences in the affinity of the cytoplasmic sorting signals for the adaptor complexes may contribute to these different rates (for a review, see Höning, 2001).

6.2.2. Entry into the endocytic pathway via the TGN

Delivery of proteins from the TGN to lysosomes is a route taken up by MPR46 and MPR300, which mediate the delivery of soluble lysosomal enzymes harbouring a mannose 6-phosphate (MP) recognition signal. The receptors, together with their bound enzymes, are thought to be concentrated in AP-1 containing clathrin-coated pits at the TGN. Delivery from the TGN to endosomes is also a route followed by most lysosomal membrane proteins, which are delivered to lysosomes via the endosomal system without appearance at the plasma membrane. This delivery is thought to be signal mediated, and for some proteins, signals originally described as internalization signals are also utilized for sorting at the TGN (e.g. the tyrosine-based motif of Lamp-1) (for a review, see Höning, 2001).

The question of which endosomal subcompartment receives the different membrane proteins is still not well understood. AP-1, AP-3 and AP-4 are localized to some extent to the TGN. The localization of different adaptors to the TGN has led to the hypothesis that each may

play a role in sorting at different exit sites from the TGN, with one adaptor mediating sorting to an early endosomal intermediate while another may be involved in the sorting to late endosomes (for a review, see Höning, 2001).

6.2.3. Sorting from endosomes to the plasma membrane

Many receptors that mediate the uptake of macromolecules release their ligands in endosomes and then recycled to the cell surface. This pathway is generally considered as a "default pathway". Despite the high-efficiency default recycling, an additional contribution of signal-mediated sorting to enhance the recycling efficiency of some proteins cannot be ruled out, and the presence of coat on the tubules that come out of sorting endosomes has been proposed to play such a role (Stoorvogel et al, 1996). The glucose transporter Glut-4 in adipocytes or the MHCII in antigen presenting cells recycle to the plasma membrane from specialized endosomal subcompartments (for a review, see Höning, 2001).

6.2.4. Sorting en route to late endosomes/lysosomes

A number of studies suggest that lysosomal targeting is signal-mediated, and sequences on the retained proteins have been proposed to play a role in trafficking to late endosomes. Perhaps the clearest example of this is the EGFR that requires an active cytoplasmic kinase domain for entry into internal vesicles of the MVB. Other late endosomal/lysosomal targeting signals have been identified in the cytoplasmic tail of the MPR, the MHCII, lysosomal membrane proteins (Lamps and lysosomal integral membrane proteins [Limps]) and the lysosomal acid phosphatase (LAP), suggesting that entry into MVBs does not occur by default (see Mukherjee et al, 1997; Zaliauskiene et al, 2000). Lamp-1, Lamp-2, Limp-I and Limp-II are members of the family of proteins thought to protect the limiting membrane from degradation by lysosomal enzymes. LAP is a soluble lysosomal enzyme, which is first transported to endosomes as an integral membrane protein, then cleaved to meet with the soluble cargo directed to lysosomes. Common to all these lysosomal proteins is a short cytoplasmic tail of 11-20 amino acid residues, which has been shown to contain the important intracellular sorting signals. While Lamp-1, Lamp-2, Limp-I and LAP all harbour tyrosine-based sorting motifs, the tail of Limp-II is carrying a leucine-based signal. The itinerary of the membrane proteins after biosynthesis involves delivery from the TGN to endosomes and further transport within the endosomal system to lysosomes. Sorting of LAP is different since it is first delivered to the plasma membrane after biosynthesis, and finally

transported to lysosomes (for a review, see Höning, 2001). Sorting along the endocytic pathway to lysosomes is signal mediated, requiring tyrosine- or leucine-based targeting signals. Recycling receptors have similar signals, yet these proteins seldom enter the later stages of the endocytic pathway, and the tyrosine motif of LAP would bind to the relevant sorting factor with only low affinity, thus causing low-fidelity endosomal sorting and prominent recycling to the cell surface. Indeed, the same motif operates as efficient internalization signal as well as lysosomal targeting signal, but the cytoplasmic tail positional requirements for lysosomal signals appear to be much more restricted. This suggests that the overall structure of the cytoplasmic tail differently affect motif recognition at each site (White et al, 1998; Höning, 2001).

In addition to the structural features in the cytoplasmic tails of membrane proteins that are important for internalization and/or lysosomal targeting, the role of the transmembrane domain was recently evidenced. Polar residues contained in the transmembrane domain of down-regulated cell-surface receptors, mediate receptor self-association, leading to down-regulation and lysosomal targeting. It has even been suggested that recycling and down-regulated receptors can be segregated without the need of a specific cytoplasmic tail sorting determinant (Zaliauskiene et al, 2000).

Sorting proteins that bind lysosomal membrane proteins like Lamp-1 or LAP include AP-1, AP-2 and AP-3. Another class of cytoplasmic sorting factors that has been localized to endosomes are the COP proteins, and especially COP-I which was thought to play a role in trafficking from early to late endosomes (for a review, see Höning, 2001).

6.3. Interplay of physical- and signal-sorting

Several examples of interplay between physical and signal sorting are given in this section, and concerne sorting of receptor/ligand complexes, for which physical sorting plays also a role in addition to signal sorting.

Accumulation of LDL in the sorting endosomes is an example of physical sorting based on molecule size. Once they enter the acidified endosomes, LDL molecules are released from their receptors. The LDL accumulation in sorting endosomes can be accounted for by the retention of most of the fluid volume in the vacuolar portion of sorting endosomes. The diameter of LDL molecules (approx. 22 nm) is nearly half the internal diameter of the tubules associated with

sorting endosomes, suggesting that these large particles could be excluded from these tubules. Similarly, association of EGFR in the inward invaginations of MVBs is another example of endosome retention given by organelle characteristics (for a review, see Mukherjee et al, 1997).

Receptor recycling is considered as a default pathway based on the tubulovesicular geometry of sorting endosomes. Indeed, many mutant receptors with very short cytoplasmic domains have been shown to be internalized very slowly but recycled efficiently by cells. For example, TfR mutant lacking the entire cytoplasmic tail was found to recycle rapidly (Höning, 2001). This suggests that recycling occurs by "default" for the bulk of the internalized plasma membrane, and that superimposed active sorting is necessary to target membrane components to alternate locations, such as late endosomes/lysosomes or the TGN (for a review, see Mukherjee et al, 1997).

The situation is more complex for FcγRII, the recycling of which depending on the type of ligand bound: internalization of monovalent ligands bound to this receptor is followed by rapid recycling of the receptor and ligand back to the cell surface; by contrast, if immunocomplexes are bound to FcγRII, both the ligand and the receptor are delivered to lysosomes and undergo degradation (for a review, see Höning, 2001). Likewise, aggregation and oligomerization of proteins can play a role in sorting endosome retention. An aggregated complex in a membrane is a specialized membrane subdomain that is excluded from the default recycling pathway, perhaps because this domain is too large and/or rigid to be included in the sorting endosome's tubules (for a review, see Mukherjee et al, 1997).

Specialized lipid microdomains themselves can mediate membrane protein sorting, and, shortly after endocytosis, recycling receptors can be detected in tubular extensions of early endosomes. It has recently been shown that a variety of endosomal populations may arise from combinatorial arrangements of individual domains and cargo molecules move through distinct domains on endosomes. Three major populations of endosomes are involved in TfR movement: one that contains only Rab5, a second with Rab4 and Rab5, and a third with Rab4 and Rab11. So, TfR enters the cell via Rab5 containing structures, fast recycling is achieved by rapid sorting from Rab5 into Rab4-positive domains on the same endosome, whereas at steady state TfR accumulates in Rab4/Rab11 containing membranes. Based on these characteristics, the TfR route is best described assuming two recycling stations with different kinetic properties; the first is filled maximally within 5 min, subsequently more than two-thirds of the remaining TfR will accumulate

in the second station after 25 min. Fast recycling seems to require a rapid sorting of TfR from a Rab5 to a Rab4 domain on the same endosome (Sönnichsen et al, 2000).

7. Membrane properties, lipid metabolism and regulation of membrane trafficking

Membranes play a central role in the structure but also in function of cells. Among the latter is endocytosis. Indeed, several membrane properties and lipid metabolism are involved at distinct stages of endocytosis. This section shows the role played by some membrane properties, such as fluidity and asymmetry, and by lipid metabolism in the regulation of membrane trafficking.

7.1. Membrane properties and regulation of membrane trafficking

We will here focus on heterogeneity of lipid distribution (distribution among membranes, membrane asymmetry, lipid rafts, and distribution in polarized cells), and on membrane tension and fluidity.

7.1.1. Heterogeneity of lipid distribution in membranes of the endocytic apparatus

7.1.1.1. Determinants for membrane lipid distribution

The major components of membranes are proteins and lipids (see Figure 8). The most striking feature of membrane lipids is their enormous diversity. The major lipid classes are pictured in Figure 9. Glycerophospholipids are the most commonly found membrane lipids. One of the glycerol hydroxyls is linked to a polar phosphate-containing group and the other two hydroxyls are linked to hydrophobic groups. Most glycerophospholipids have the phosphate at the *sn*-3 position. The polar head groups of glycerophospholipids are varied, giving rise to PC, PS, PE, PG, PA and PI. Sphingophospholipids contain the same kinds of polar substituents as do the glycerophospholipids, but the hydrophobic group is a ceramide. Sphingomyelin (ceramide 1-phosphorylcholine) is widely found in animal cell plasma membranes. Glyceroglycolipids are polar lipids in which the *sn*-3 position of glycerol forms a glycosidic link to a carbohydrate such as

galactose. Sphingoglycolipids have a glycosidic linkage to the terminal hydroxyl of ceramide. The carbohydrate moeity can range from a single sugar to very complex polymers. In addition, cholesterol is a compact, rigid hydrophobic entity with a polar hydroxyl group.

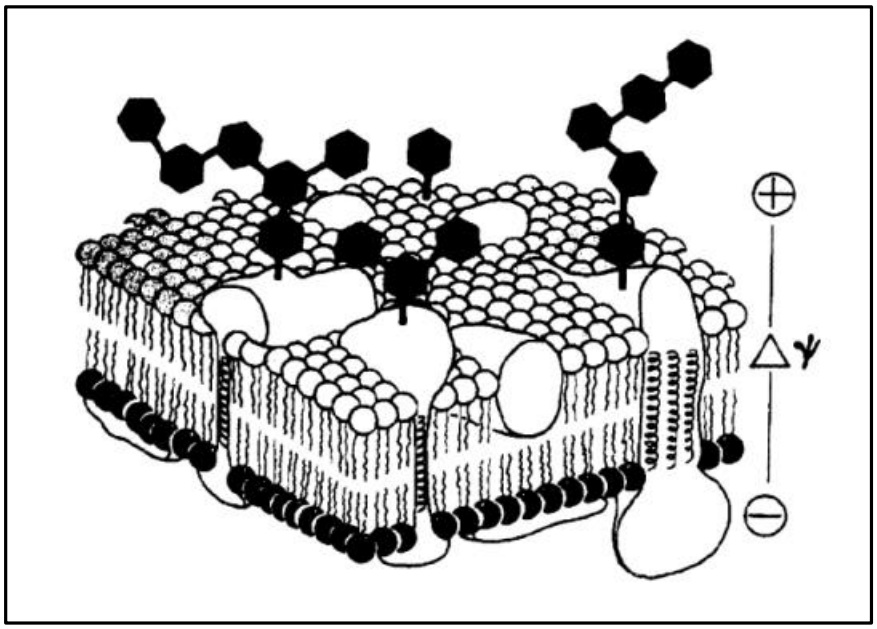
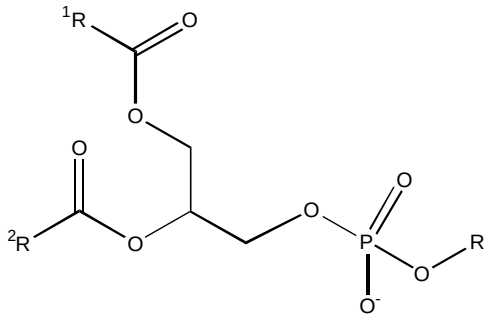
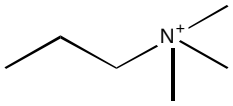


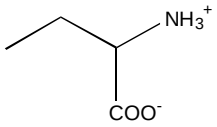
Figure 8. The topography of membrane protein, lipid and carbohydrate in the fluid mosaic model of a typical eukaryotic plasma membrane. Phospholipid asymmetry results in the preferential location of PE and PS in the cytosolic monolayer. Carbohydrate moieties on lipids and proteins face the extracellular space. Äø represents the transmembrane potential, negative inside the cell.



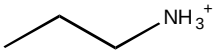
phosphatidylcholine (PC)
zwitterion

R = 

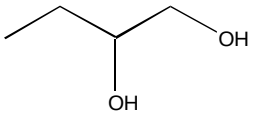
phosphatidylserine (PS)
anion

R = 

phosphatidylethanolamine (PE)
zwitterion

R = 

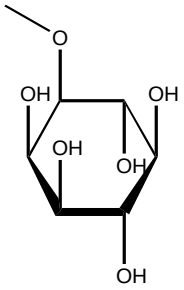
phosphatidylglycerol (PG)
anion

R = 

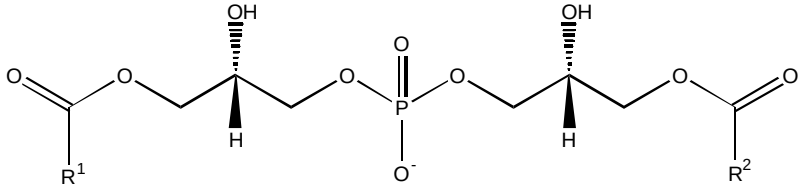
phosphatidic acid (PA)
anion

R = - H

phosphatidylinositol (PtdIns)
anion

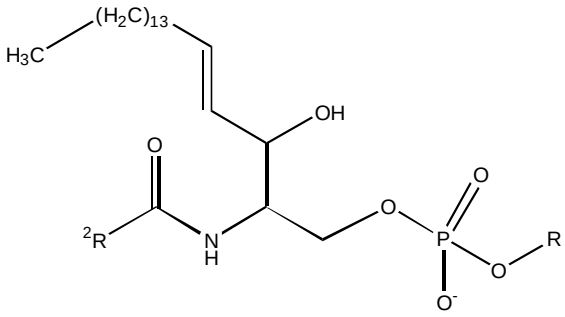
R = 

LYSOBISPHOSPHATIDIC ACID (LBPA)

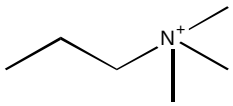


SPHINGOPHOSPHOLIPIDS

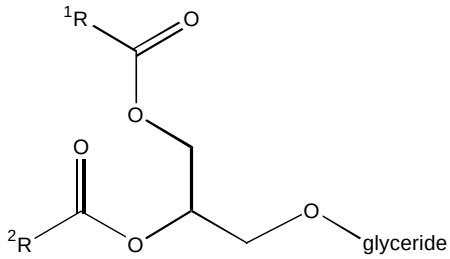
sphingomyelin (SM)
zwitterion



R =

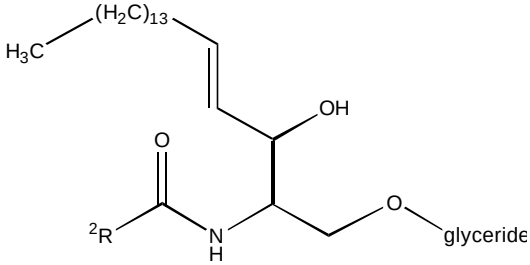


GLYCEROGLYCOLIPIDS



SPHINGOGLYCOLIPIDS

glucosylceramide (GlcCer)



glyceride =

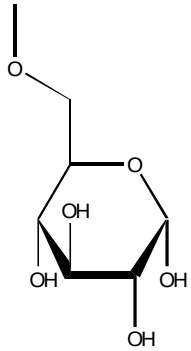


Figure 9. Structures of membrane lipids, illustrating the variety of polar head groups. R^1 and R^2 are saturated or unsaturated fatty acids linked to the glycerol of phospholipids or to the sphingosine of sphingolipids. R is the polar head group of phospholipids. Glycerides in the glycolipids are numerous, such as glucose in glucosylceramide.

Chemical and biophysical properties of lipids are important determinants for their membrane distribution. Among these determinants, we have first to mention shape of lipids, and therefore the size of head group (see Figures 9 and 10), which may promote positive or negative curvature and thereby modulate invagination, vesiculation and tubulation. As examples, PE and ceramide are lipids with a small, low hydrated headgroup which have tendency to form inverted non lamellar phases. LBPA is a highly hydrophobic acidic phospholipid which is predicted to be also cone-shaped (Kobayashi et al, 1999).

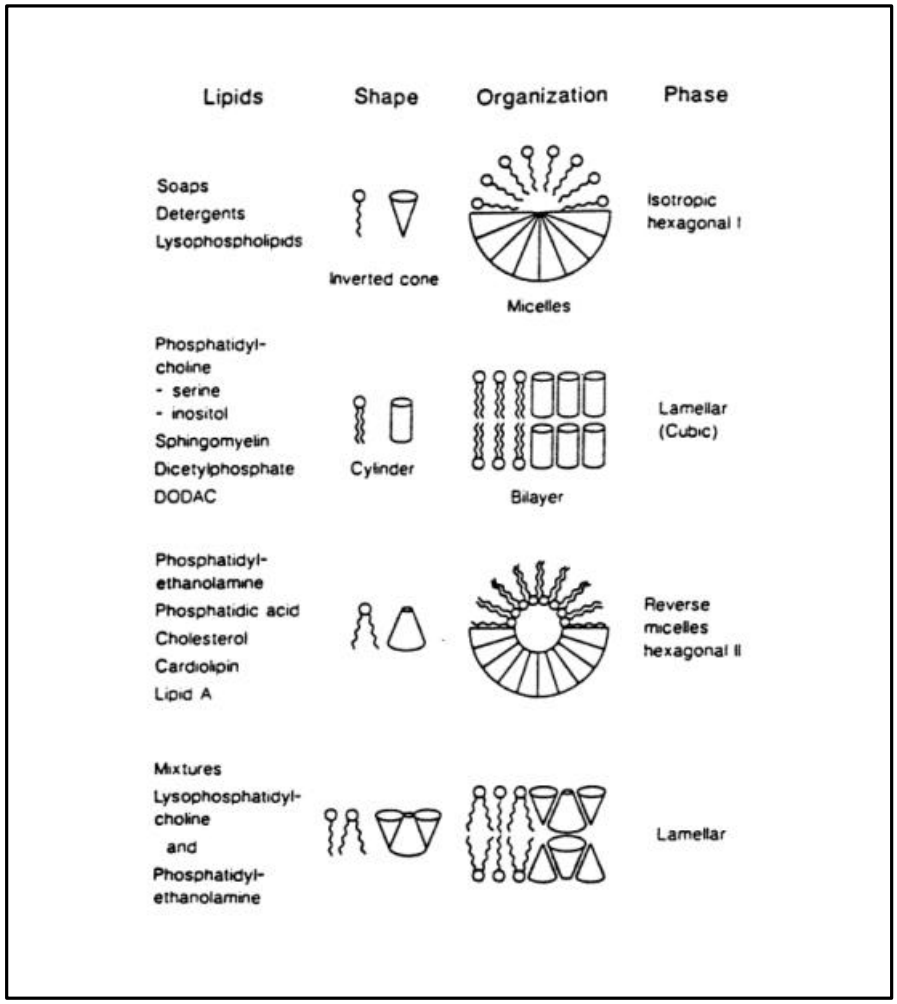


Figure 10. Organization of lipids in function of their shapes. (From Lasic, 1993)

A second determinant is lipid head-group-specificity, which notably influences the trafficking of sphingolipids. Indeed, in HepG2 cells, sorting of sphingolipids in the reverse transcytotic pathway (from the apical to the basolateral plasma membrane) is taking place in the SAC and allows GlcCer to remain located in the apical region and SM to redistribute to the basolateral domain (van Ijzendoorn et al, 1997; van Ijzendoorn and Hoekstra, 1998; Hoekstra et al, 1999; see also Figure 9).

Third, length and saturation of the alkyl/acyl chains affect their partitioning into domains of different fluidity (see Mukherjee and Maxfield, 2000). For example, SM possesses a high degree of saturation, leading to higher transition temperature and higher microviscosity of the membranes compared to PC.

Fourth, the presence of cholesterol has pronounced effects on the phase behavior of a lipid bilayer (Silvius et al, 1996), interacts specifically with some lipid head groups (Brown, 1998) and has tendency to self-associate (see Mukherjee and Maxfield, 2000).

Fifth, ability to associate with adjacent proteins or lipids including self-association into microdomains and transbilayer interactions (for a review, see Mukherjee and Maxfield, 2000).

7.1.1.2. Evidence and roles of heterogeneity of lipid membrane distribution

The heterogeneity of lipid membrane distribution is first illustrated by the presence of specific lipids at various stages of the endocytic process: endocytic and plasma membranes contain more PS, SM, glycolipids and cholesterol than other organelles, with less PC; likewise, LBPA, a rare negatively charged lipid, concentrates in the inner involutions of late endosomes (Kobayashi et al, 1998; 1999). Secondly, lipids are distributed asymmetrically across the plasma membrane bilayer: whereas PC, SM and glycolipids are enriched in the exoplasmic leaflet, the aminophospholipids are concentrated on the cytosolic face. Thirdly, lateral lipid distribution in biological membranes is heterogenous: sphingolipids and cholesterol form microdomains in the exoplasmic leaflet of the plasma membrane, and the existence of rafts of PI 4,5-P_2 was also evidenced. Heterogeneity of lipid membrane distribution is also illustrated by large (micron scale) domains with differences in lipid composition, which can be detected in specialized cells such as polarized cells.

7.1.1.2.1. Distribution among membranes: the case of cholesterol and LBPA

Among the several lipids that are heterogeneously distributed among endocytic membranes, we here focus on cholesterol and LBPA. Cholesterol, predominantly found in the plasma membrane, the ERC and the TGN, is concentrated with sphingolipids in domains (the so called “rafts”) that undergo lateral phase-separation from cholesterol-poor domains. LBPA is in late endosomal fractions, which contain high amounts of triglycerides and cholesterol esters, and a

limited subset of phospholipids, among which is LBPA. This lipid is not easily degraded by phospholipases, and which accounts for approx. 15 % of the total phospholipids in the fractions. It is present in the internal late endosomal membranes (forming specialized microdomains), the composition of which is therefore different from that of the limiting endosomal membrane (Kobayashi et al, 1999).

Cholesterol is necessary for different internalization mechanisms. First, depletion of cholesterol (filipin, nystatin or methyl-β-cyclodextrin; see section 10.3.) blocks internalization through caveolae and leads to flattening of caveolae (for a review, see D'Hondt et al, 2000). It also functions in internalization through clathrin-coated pits, and removal of cholesterol leads to flattening of clathrin-coated pits, indicating that cholesterol affects the morphology and curvature of the plasma membrane (for a review, see D'Hondt et al, 2000). Thirdly, the role of cholesterol in non-clathrin-mediated endocytosis was evidenced by using cholesterol derivatives, such as poly (ethylene glycol) cholesteryl ethers (PEG-chols). These derivatives of cholesterol would not move across the hydrophobic membrane core by flip-flop, due to the bulky and hydrophilic PEG moeity, therefore resulting in the expansion of the outer monolayer and thereby counteracting the membrane invagination process. It has been demonstrated that the incorporation of PEG-chols in the outer leaflet of the plasma membrane drastically reduces the internalization of liposomes and fluid-phase endocytic tracers, and preferentially inhibits clathrin-independent as compared with clathrin-dependent endocytosis. It was suggested that the distribution of PEG-Chols reduced the membrane dynamics essential for clathrin-independent invagination of membrane, based on the model in which the expansion of the outer monolayer by insertion of PEG-Chols could counteract the membrane invagination process. Since clathrin-dependent endocytosis was less sensitive than clathrin-independent endocytosis, the results further suggested that invagination due to the generation of membrane curvature by spherical assembly of clathrin triskelions can overcome such energetic constrains (Vertut-Doï et al, 1996; Ishiwata et al, 1997; Baba et al, 2001).

Cholesterol also acts at the level of endosomal sorting. GPI-anchored proteins are recycled at a 3-fold slower rate than C_6-NBD-SM or TfRs. The resulting intracellular retention of GPI-anchored proteins presumably depends on the levels of cholesterol in the endosomal membrane since GPI-anchored protein trafficking becomes similar to that of other recycling components upon cholesterol cell depletion. Cholesterol-dependent endocytic sorting of GPI-anchored proteins is consistent with the involvement of specialized lipid domains, or rafts, in endocytic sorting (Mayor et al, 1998).

At the level of late endosomes, in fibroblasts of patients with Niemann-Pick syndrome type C (NPC), lipoprotein-derived cholesterol mainly accumulate in the LBPA-rich late endosomes. Retention of excess cholesterol in the late endosomes of NPC cells might cause an overall change in membrane elasticity, compromising budding of transport vesicles (Mukherjee and Maxfield, 1999).

The question remains, however, how cholesterol participates in endocytosis. Cholesterol may define spatial specificity by recruiting the endocytic machinery to a membrane site, and this function may occur either as part of lipid rafts, or independently of its association with sphingolipids. Cholesterol may also be necessary for the formation of membrane curvature at the plasma membrane (for a review, see D'Hondt et al, 2000).

A second heterogeneously distributed lipid is LBPA, a highly hydrophobic acidic phospholipid. LBPA is predicted to be cone-shaped and this characteristic may contribute to the highly curved membrane regions found inside these compartments. Conversion of LBPA to PA by endophilin I, a downstream effector of dynamin, mediates vesicle formation and membrane fission at the nerve terminal plasma membrane, presumably by inducing negative curvature (for a review, see D'Hondt et al, 2000). Within the endosomal system, LBPA may provide a platform for lipid-lipid or lipid-protein interactions necessary for the protein sorting function and/or membrane trafficking. Among these is the sorting of the multifunctional receptor MPR/IGF2 (for MP-bearing ligands and insulin-like growth factor 2). This receptor delivers newly synthesized lysosomal enzymes, which carry the MP signal, from the TGN to late endosomes, and must recycle back to the TGN for reutilization. In late endosomes, most of the receptor was found to colocalize with LBPA (Kobayashi et al, 1998).

7.1.1.2.2. Membrane asymmetry

The outer leaflet of animal plasma membrane is mainly composed of choline-phospholipids (PC, SM), while amino-phospholipids reside in the inner leaflet. Choline-lipids undergo a slow and passive transmembrane diffusion whereas amino-phospholipids are transported inwardly by an ATP-dependent carrier, the amino-phospholipid translocase or "flippase" (Devaux and Zachowski, 1994). The amino-phospholipid translocase could play an important role in the control of membrane curvature, particularly in the initiation of vesicle formation (Devaux and

Zachowski, 1994). Plasma membrane asymmetry drives vesicle formation during endocytosis. Indeed, when phospholipid density at the inner leaflet is increased by adding to living cells exogenous aminophospholipids (PS or PE), which can be translocated from the outer to the inner leaflet by the ubiquitous flippase, both bulk-flow and clathrin-dependent internalization of plasma membrane proteins are accelerated. At the opposite, lyso-α-PS, a closely related aminophospholipid that is not recognized by the flippase, or a cholesterol derivative, PEG-Chol, that also remains in the outer leaflet, inhibited endocytosis (Farge, 1995; Farge et al, 1999).

Another example where asymmetry plays a role is in phagocytosis. The appearance of PS into the outer leaflet of blood cells is responsible for their recognition and phagocytosis by macrophages (Devaux and Zachowski, 1994).

7.1.1.2.3. Lateral heterogeneity and lipid rafts

The dynamic formation of liquid-ordered phase domains within a liquid-disordered matrix is an important regulator of molecular interactions in membrane trafficking. Although cholesterol-sphingolipid rafts are probably not the only such domain (see also domains of PI 4,5-P_2; domain being defined as localized regions with non-random lipid compositions), at present they provide the best defined system to study lipid-mediated protein trafficking.

Rafts are viewed as dynamic assemblies of cholesterol and sphingolipids that form in the exoplasmic leaflet of cellular bilayer membranes. The mean radius of the raft assemblies is approx. 25 nm (Pralle et al, 2000). In rafts, sphingolipids would associate laterally with one another in the exoplasmic leaflet, probably through weak interactions between the carbohydrate heads of the glycosphingolipids. Cholesterol is present on both leaflets and fills the space under the head group of sphingolipids or extends the interdigitating fatty acyl chain in the apposing leaflet (see Simons and Ikonen, 1997). However, the role of cholesterol is likely to be more than a spacer between nearby sphingolipids (Brown, 1998). Since these domains could selectively incorporate or exclude proteins, thereby might modulate protein-protein and protein-lipid interactions. Functionally specialized membrane regions with differences in lipid distribution were first analyzed in cells with anatomically distinct plasma membrane domains, such as polarized cells. Cholesterol-sphingolipid domains also form in cells that do not have a stably polarized architecture.

A prerequisite for raft assembly is tight control of metabolism and flow of the lipids involved. Both vesicular and non-vesicular transport mechanisms apply, although lipids with large polar head groups essentially follow vesicular transport pathways. SM and complex glycosphingolipids depend on vesicular trafficking to reach the plasma membrane and to cycle via the endocytic compartments. In comparison to sphingolipids, cholesterol moves more freely, partially utilizing membrane trafficking routes and partially relying on other systems (for a review, see Ikonen, 2001).

The first indication for a role of rafts is provided by the cholesterol and sphingolipid dependence for vesicle formation. Depletion of cholesterol blocks internalization via both caveolae and clathrin-coated pits, suggesting that rafts are incorporated in both types of endocytic structures (for a review, see Ikonen, 2001). Hypotheses were proposed for the involvement of cholesterol-based membrane microdomains in budding and fission, with consideration of physical parameters such as viscosity and raft topology: (i) the microdomain would tend towards a higher viscosity than in non-microdomain membrane, if there is a high degree of attractive intermolecular interactions between the lipids and raft-preferring proteins; (ii) if microdomains are sitting on either side of a neck, they are in a ring topology; (iii) cholesterol-based microdomains in the external leaflet might influence the lipid composition and phase in the cytoplasmic leaflet, resulting, for example, in an enrichment of PI required for signal transduction; and (iv) since more ordered structures are higher in density, the area per lipid head group in microdomains will be smaller, so that if one leaflet can be raft and the other not, the difference in the leaflet's area would curve the membrane into a bud facing the cytoplasm (for a review, see Huttner and Zimmerberg, 2001). However, this part is speculative and is only based on consideration of physical parameters of rafts. The role of the other raft components (sphingolipids and especially ceramide) in regulating endocytosis could also be related to their capacity to form domains. However, this role is even less clear. Exogenous sphingomyelinase induces the formation of endocytic vesicles in an ATP-independent manner (Zha et al, 1998), and Holopainen et al (2000) demonstrated that generation of ceramide by addition of sphingomyelinase is able to induce vesicle formation *in vitro*. This was explained by: (i) the lateral phase separation of ceramide-enriched domains; (ii) the area difference between adjacent monolayers; (iii) the negative spontaneous curvature; and (iv) the increase bending rigidity of the ceramide-containing domain. However, the relevance of this phenomenon remains to be explored (for a review, see Ikonen, 2001).

Rafts are also believed to be present in early endocytic organelles and to play a role in sorting. Late endosomes and lysosomes have a different lipid composition, being enriched in neutral lipids (triglycerides and cholesterol esters) and selected phospholipids, and thus normally depleted of rafts (for a review, see Ikonen, 2001). However, some lysosomal storage diseases result from the accumulation of lipids in degradative compartments of the endocytic pathway. Examples are the Niemann-Pick syndrome type C (NPC) which is characterized by the accumulation of cholesterol, and Niemann-Pick syndromes type A and B (NPA and NPB) which show accumulation of SM. It has been hypothesized that lysosomal storage diseases can be caused by the accumulation in late endosomes and/or lysosomes of both lipids, which are normal constituents of lipid rafts (see Simons and Gruenberg, 2000). A raft-dependent transport between endosomes and the Golgi apparatus has also been suggested. Indeed, there is a redistribution of membrane proteins and impairment of late endosome to Golgi transport of the MPR in NPC. Moreover, both decreasing and increasing the cellular cholesterol content reduce ricin transport from endosomes to the Golgi apparatus. This indicates that a certain level of cholesterol is required to retain the normal structure and function of the Golgi apparatus, as both an increase and a decrease of cholesterol seem to induce fragmentation, and that the right level of cholesterol is essential for efficient endosome to Golgi transport (for a review, see Ikonen, 2001).

The cortical actin cytoskeleton, thought to provide constraints for the lateral mobility of rafts, would increase raft stability and segregate raft components from more mobile ones. Annexins may be important for these processes, as they promote the formation of membrane-cytoskeleton complexes and could thus control raft assembly. Recent evidence suggests that actin may be directly involved in the intracellular movement of transport vesicles containing rafts (for a review, see Ikonen, 2001).

Raft-dependent signalling and endocytic uptake may also be coupled (see also section 2.2.3.). EGF induced activation of the mitogen-activated protein kinase (MAPK) pathway involves caveolin internalization. The transcytotic delivery of albumin is coupled to signalling and vesicle formation induced by a caveolar albumin docking protein. Signal transduction and uptake mechanisms through FcR, in lymphocytes which lack caveolae, suggest that rafts may serve as platforms for triggering signalling cascades (see Ikonen, 2001).

7.1.1.2.4. Heterogeneity of lipid distribution in polarized cells

Another example of heterogeneity of lipid membrane distribution is illustrated by large (micron scale) domains with differences in lipid composition, which can be detected in polarized cells. Indeed, these latter exhibit cell-surface polarity with a basolateral plasma membrane domain, which faces the blood circulation and adjacent cells, and an apical domain, which is in contact with the external environment, such as the bile canaliculus in hepatocytes or the lumen in epithelial cells. These membrane domains differ both in lipid and protein composition. To generate and maintain this polarized membrane composition, specific vectorial flow of membrane carriers must be ensured. This implies that apically and basolaterally destined lipids and proteins must be segregated and sorted along the biosynthetic pathway. In addition, there must be appropriate targeting of endocytozed material that is returned to the plasma membrane and this emphasizes the role of endocytosis in maintenance of cell surface polarity (Mukherjee et al, 1997). For example, the trafficking and sorting of sphingolipids along both pathways- the biosynthetic and the endocytic- is demonstrated in HepG2 cells. In these cells, it has been shown that: (1) trafficking of GlcCer and SM from the Golgi apparatus to the apical plasma membrane occurs by a direct transport process; (2) trafficking of both lipids from the basolateral plasma membrane to the apical plasma membrane (transcytosis) occurs via bulk flow; (3) sorting of sphingolipids, which is taking place in a SAC, occurs in the reverse transcytotic pathway and allows GlcCer to remain located in the apical region and SM to redistribute to the basolateral domain (van Ijzendoorn et al, 1997; Hoekstra et al, 1999).

7.1.2. Membrane tension and fluidity

7.1.2.1. Determinants of membrane tension and fluidity

Membrane tension is a complex parameter that plays an important role in many cellular functions, including membrane trafficking and cell motility. Its role in endocytosis comes from the observation that a decrease in tension in the membrane correlates with acceleration of endocytosis. It is possible to measure membrane tension of biological membranes from the force that is exerted on membrane tethers. Tethers can be formed by pulling on membrane-attached beads with lazer tweezers and the displacement of the beads in the lazer tweezers gives a rapid readout of the force on the beads. This can be viewed by a video enhanced differential interference contrast microscope equiped with lazer optical tweezers (Dai and Sheetz, 1999).

The tether force involves a viscous or dynamic component and a static force. Several components are involved in the viscous component of tether formation, including: (i) the membrane viscosity; (ii) the drag of the cytoskeleton; and (iii) the shear between the two halves of the bilayer. The static component of the tether force involves: (i) the in-plane tension in the membrane bilayer, which is dependent upon balance of endocytosis and exocytosis, and hence the ratio of plasma membrane area to the cell volume for a given cell shape, as well as upon osmotic pressure across the membrane, which in turn is related to the concentration of protein at the cytoplasmic face of the plasma membrane; and (ii) the membrane-cytoskeleton adhesion (Dai and Sheetz, 1995; 1999).

Since both membrane tension and membrane cytoskeleton adhesion contribute to the tether force, it could be interesting to separate plasma membrane from the cytoskeleton, which occurs in membrane blebs. Membrane blebs are spherical membrane extensions that can be seen at the periphery of eukaryotic cells during mitosis for example or on cells injured by physical and chemical stress. When the membrane tension term is substracted from the apparent membrane tension over the cytoskeleton, the membrane cytoskeleton adhesion term can be estimated (Dai and Sheetz, 1999).

Membrane viscosity, or fluidity, is only a small part of membrane tension, and refers to the degree of ordering and motion in the hydrocarbon core of the lipid bilayer. More precisely, a decrease in membrane fluidity corresponds to an increase of the membrane lipid order, to the hindrance of rotational diffusion and to the reduction of translational movements. Membrane fluidity, which could be determined from fluorescence measurements using DPH probes, is influenced by fatty acyl modifications (see section 7.1.2.2.) and by an increasing cholesterol content, but not by phospholipid head group modification (see Spector and Yorek, 1985). Membrane fluidity is also influenced by calcium, since addition of this ion to suspensions of isolated rat hepatocyte plasma membranes decreases the membrane lipid fluidity (Storch and Schachter, 1985), and by the presence of the clathrin-coat on endocytic vesicles (Liaubet et al, 1994).

7.1.2.2. Roles of membrane tension and fluidity in endocytosis and phagocytosis

Membrane fluidity is important in determining endocytic and phagocytic activities (Calder et al, 1990). Indeed, decrease of membrane fluidity by increase of saturated/unsaturated fatty acid ratio in the phospholipids of macrophages decreases both fluid-phase endocytosis of HRP and receptor-mediated phagocytosis of particles attached to the FcR (Mahoney et al, 1977). Likewise, macrophages enriched with saturated fatty acids showed a decrease in their ability to phagocytose unopsonized zymozan particles and those enriched with polyunsaturated fatty acids showed enhancement of phagocytic capacity.

Clathrin-dependent and clathrin-independent endocytosis can also be differentiated by means of membrane fluidity measurements. The clathrin coat has a strong influence on the membrane fluidity of endocytic vesicles, since the presence of coat increases the membrane lipid order, hinders rotational diffusion and reduces translational movements (Liaubet et al, 1994). By following membrane fluidity on the very early events of endocytosis, it is possible to correlate these measurements with the relative contribution of endocytosis via coated or non-coated vesicles, and it has been shown that the highest possible contribution of initial non-coated vesicles would be 50 % in mouse fibroblasts of the L929 cell line and 65 % in bone marrow-derived macrophages (Coupin and Kuhry, 1999).

Membrane tension seems to play a key role in membrane budding. The driving force for membrane budding can arise from at least three other biological mechanisms than clathrin polymerization (see section 4). One of these mechanisms, related to the physical elastic properties of the plasma membrane, is membrane tension asymmetry. Indeed, at decreasing the external osmotic pressure, a sudden inhibition of endocytosis was observed, but the endocytosis could be recovered upon increasing the phospholipid number asymmetry (Rauch and Farge, 2000).

7.2. Lipid metabolism and regulation of membrane trafficking

PI kinases produce a variety of phosphoinositides (PI) which play essential roles in membrane trafficking event involving regulation of membrane budding, motility and fusion. Sphingolipid metabolism via the SM cycle, which involves the formation of ceramide by the action of a sphingomyelinase, also plays an important regulatory role in intracellular vesicle transport. In

addition, phospholipases, such as phospholipase A_2 (PLA_2), PLC and PLD (see Figure 11), play an integral role in endocytosis and phagocytosis.

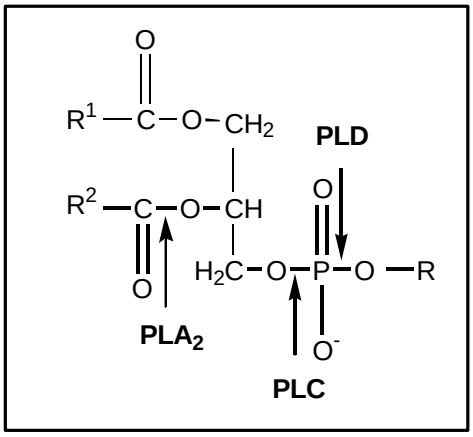


Figure 11. Schematic representation of the cleavage of a typical phospholipid by different phospholipases.

7.2.1. Roles of PI kinases and PI in membrane trafficking

Unlike the head group of other phospholipids, the inositol ring is a highly versatile substrate that can be modified at several positions (see Figure 12). PI are synthesized at the cytosolic face of cellular membranes where they recruit and/or activate effectors proteins. Highly localized changes in the levels of PI are rapidly mediated through the action of kinases and phosphatases at specific regions of the membrane, providing a means for the temporal and spatial regulation of membrane budding, motility and fusion. Specific PI are implicated in endocytic and phagocytic trafficking pathways. Among products of PI-kinases are PI 4,5-P_2 and 3-phosphoinositides (3-PI), the latter functionning more at post-internalization steps, by contrast to the former.

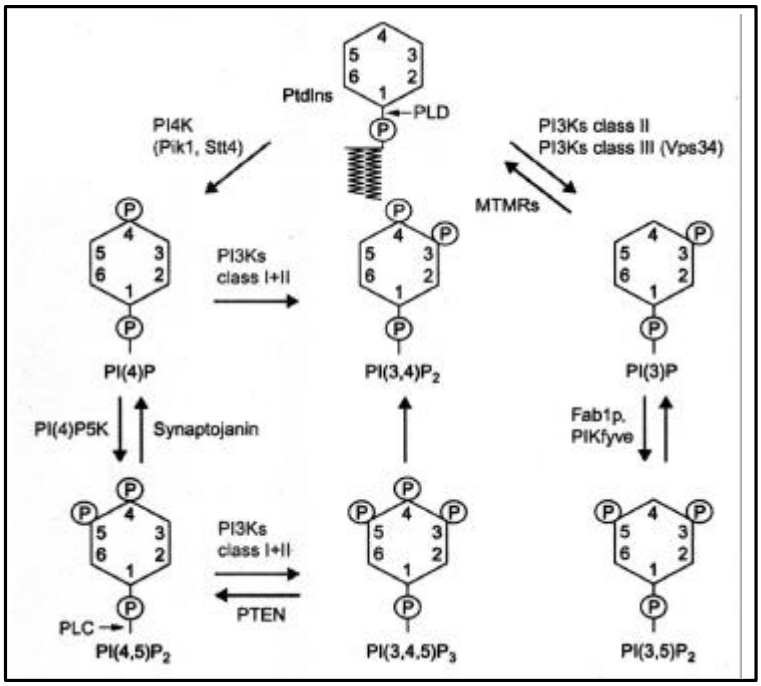


Figure 12. Phosphoinositides (PI). The inositol ring of phosphatidylinositol (PtdIns) can be phosphorylated in the 3-, 4- and 5-positions, giving rise to seven different PI. The most important phosphorylation and dephosphorylation pathways are indicated. (From Simonsen et al, 2001).

7.2.1.1. PI 4,5-P_2

The fact that PI 4,5-P_2 regulates spatially localized membrane events led to the suggestion that PI 4,5-P_2 synthesis must be localized to specific membrane domains. PI 4,5-P_2 is distributed non-uniformly on the plasma membrane as well as on trafficking vesicles, and may be located in domains. In addition, it has been shown that the PI 4,5-P_2-binding PH domain of PLCδ1 transiently accumulates at active sites of phagocytosis such as actin-rich membrane projections at ruffling zones. Immunocytochemistry with PI 4,5-P_2 antibodies also detects discontinuous, raft-like clusters of PI 4,5-P_2. These may result from the coalescence of PI 4,5-P_2-rich microdomains, which are similar to sphingolipid/cholesterol-rich rafts. Local production of PI 4,5-P_2 requires recruitment of PI kinases: phosphatidylinositol 4-phosphate (PI 4-P) 5-kinases at active sites of cytoskeleton assembly (membrane ruffles, sites of phagosome formation) and on Golgi membranes. Members of the ARF family mediate their recruitment and activation (for a review, see Martin, 2001).

PI 4,5-P_2 is involved in clathrin-mediated endocytosis in which it seems to be a key regulator of the formation, scission and uncoating of CCVs. Indeed, several proteins involved in the formation, scission and uncoating of CCVs bind to PI 4,5-P_2. First, proteins implicated in the formation of CCVs, including epsin, CALM and AP180, bind specifically to PI 4,5-P_2. Second, dynamin which is involved in scission of CCVs from the plasma membrane, have also been found to bind to PI 4,5-P_2. Dynamin can penetrate into the acyl chain region of membrane lipids, a process that is stimulated by PA, PI 4-P and PI 4,5-P_2. Interaction of dynamin with endophilin, a lipid-modifying enzyme that converts lyso-PA into PA, seems to be important for the invagination of CCVs, suggesting that endophilin may stimulate the mechanochemical activity of dynamin (for a review, see Simonsen et al, 2001). Third, synaptojanin, a protein involved in scission and uncoating of CCVs, is also able to bind to PI 4,5-P_2. It dephosphorylates PI 4,5-P_2 into PI 4-P, interacts with the SH3 domain of endophilin, and this interaction seems to be important both for the late stages of invagination and scission and for the uncoating of CCVs. So, synaptojanin seems to function both in the uncoating of CCVs and as a negative regulator of the clathrin coat interaction with the plasma membrane, probably by dephosphorylation of PI 4,5-P_2 (for a review, see Simonsen et al, 2001).

Roles for negatively charged lipids, notably PI 4,5-P_2, in endocytosis involve the activity of a PI 4,5-P_2-dependent PLD stimulated by ARF (Jones and Wessling-Resnick, 1998).

PI 4,5-P_2 is also involved in phagocytosis and macropinocytosis. The PI 4,5-P_2-binding PH domain of PLCδ1 transiently accumulates at active sites of phagocytosis and concentrates at dynamic actin-rich membrane projections and ruffles (for a review, see Martin, 2001). Constitutive macropinocytosis depends on sequential activation of the class I PI 3-kinase and the PtdIns-specific PLC (Amyere et al, 2000), which cleaves PI 4,5-P_2 into diacylglycerol and inositol-1,4,5-trisphosphate.

7.2.1.2. PI 3-kinases and 3-PI

PI 3-kinases play essential roles in endocytosis, phagocytosis, macropinocytosis and vesicle fusion.

7.2.1.2.1. PI 3-kinases are divided into three classes

There are three classes of PI 3-kinases: (i) class I, which includes the p85-p110 PI 3-kinase complex, and uses PI 4,5-P_2 as its main *in vivo* substrate, giving rise to phosphatidylinositol-3,4,5-trisphosphate (PI 3,4,5-P_3); (ii) class II, which is represented by PI 3-kinase C2α and PI 3-kinase C2β, the major substrate of PI 3-kinase C2α being also PI 4,5-P_2; and (iii) class III, the major substrate of which being PtdIns, giving rise to PI 3-P. The lipid-kinase activity of most PI 3-kinase isoforms is blocked by LY294002 and by wortmannin (Shepherd et al, 1996).

7.2.1.2.2. Roles of PI 3-kinases and 3-PI in endocytosis

Endocytosis of the fluid-phase tracer HRP into Baby Hamster Kidney cells (BHK cells) was originally reported to be inhibited by wortmannin: a > 50 % decrease in the initial rate of tracer internalization (1 min), a concommitant decrease in the volume of early endosomes and an increased efficiency of recycling were observed. It has been suggested that effects of wortmannin reflect an inhibition of a CCV-mediated uptake pathway (Clague et al, 1995). Moreover, the drug blocked the stimulation of HRP uptake by Rab5, and it was suggested that PI 3-kinase plays an important role in regulation of early endosome fusion, probably via activation of Rab5 (Li et al, 1995). However, in several other types (e.g. Rat-1 fibroblasts), wortmannin and LY294002 have no effect on fluid-phase endocytosis (Amyere et al, 2000).

PI 3-kinases seem also play roles in receptor-mediated endocytosis, since internalization, intracellular accumulation and release of Tf in BHK-21 and Trvb-1 cells were slowed by wortmannin. The drug also blocked the constitutive endocytosis mediated by the MR in J774 macrophages (Li et al, 1995; Shepherd et al, 1996). Again, there was no appreciable effect of wortmannin and LY294002 on receptor-mediated endocytosis of Tf in Rat-1 fibroblasts (Amyere et al, 2000).

A more direct evidence for the implication of PI 3-kinase in endocytosis is that PI 3-kinase C2α directly binds to clathrin and that clathrin stimulates the catalytic activity of this enzyme, especially towards PI 4,5-P_2. Endogenous PI 3-kinase C2α is found in CCVs at the plasma membrane and in the TGN, and overexpression of PI 3-kinase C2α inhibits clathrin-mediated

endocytosis and sorting in the TGN. Moreover, PI 3-kinase C2α and PI 3-kinase C2β are recruited to activated growth factor receptors (for a review, see Simonsen et al, 2001).

PI 3-kinase also plays a key role in signal transduction during endocytosis. For example, endocytosis and trafficking of immune complexes and activation of PLD by the FcγRI require distinct PI 3-kinase activities: activation of a p85-dependent PI 3-kinase (class I) is involved in the initial endocytosis of immune complexes, and a distinct p85-independent PI 3-kinase isoform (class III) is necessary for the subsequent vesicular trafficking of internalized immune complexes for degradation (Gillooly et al, 1999).

7.2.1.2.3. Roles of PI 3-kinases and 3-PI in phagocytosis and macropinocytosis

FcγR-mediated phagocytosis is accompanied by a rapid increase in the accumulation of PI 3,4,5-P_3 *in vivo*, and addition of wortmannin inhibits phagocytosis but not FcγR-directed actin polymerization. The block occurs during pseudopod extension, not during the very early phases (F-actin accumulation) or late phases (phagosomal closure) of ingestion and could be bypassed when requirements for pseudopod extension were minimized. Moreover, decreasing bead size from 6 to 1 µm, and hence the magnitude of pseudopod extension required for particle engulfment, relieved the inhibition of phagocytosis by wortmannin by up to 80 %. So, pseudopod extension requires participation of TKs, actin polymerization and PI 3-kinases. All are necessary for the coordinated extension of pseudopod that culminate in the formation and eventual closure of the phagosome (Cox et al, 1999; see also section 2.3.5.). The accumulation of 3-PI during phagocytosis is transient, terminating shortly after sealing of the phagosomal vacuole, due to the dissociation of the type I PI 3-kinase from the phagosomal membrane combined with the persistent accumulation of PI phosphatases (Marshall et al, 2001).

Constitutive macropinocytosis also involves PI 3-kinase, since it depends on sequential activation of the class I PI 3-kinase and the PtdIns-specific PLC (Amyere et al, 2000).

7.2.1.2.4. Roles of PI 3-kinases and 3-PI in endosome fusion

PI 3-kinase is required for early endosome fusion, since it has been shown that the homotypic fusion between early endosomes from BHK cells is blocked by addition of wortmannin

(Jones and Clague, 1995). The role of PI 3-kinase on endosome fusion is best understood. PI 3-P, which is highly enriched on early endosomes (and in the internal vesicles of multivesicular endosomes), plays a major role in endocytic trafficking, by recruiting Rab effectors. Among these are EEA1, rabaptin-5 and rabenosyn-5, which are essential for homo- and heterotypic early endosome fusion and are recruited to early endosomes by Rab5-GTP and PI 3-P (see also section 5.1.2.). But what directs the localized production of PI 3-P on early endosomal membranes? It has been shown that PI 3-kinase is itself an effector of Rab5, and that the association of EEA1 with the endosomal membrane requires Rab5-GTP and PI 3-kinase activity. The identification of EEA1 as a direct Rab5 effector provides a molecular link between PI 3-kinase and Rab5. Rab5 thus plays a dual role in the recruitment of EEA1 to membranes, first through a direct binding to EEA1, and secondly, through the stimulation of PI 3-P production on endosome membranes (Simonsen et al, 1998; for reviews, see Simonsen et al, 2001, and Stenmark and Zerial, 2001).

7.2.2. Roles of sphingomyelinase, sphingolipids and ceramide in membrane trafficking

Sphingolipids are predominantly present in the external leaflet of the plasma membrane. The major mammalian sphingolipid is SM, but various sphingoglycolipids with sugars or sulfates bound to ceramide are also present in mammalian cells (see structures in section 7.1.1.). The SM cycle involves the formation of ceramide, which is obtained, in cellular membranes, upon the hydrolytic removal of the phosphocholine moeity of SM by sphingomyelinase (see Figure 13). Sphingolipid metabolism via the SM cycle plays an important regulatory role in intracellular vesicle transport, including endocytosis, phagocytosis, and vesicle fusion.

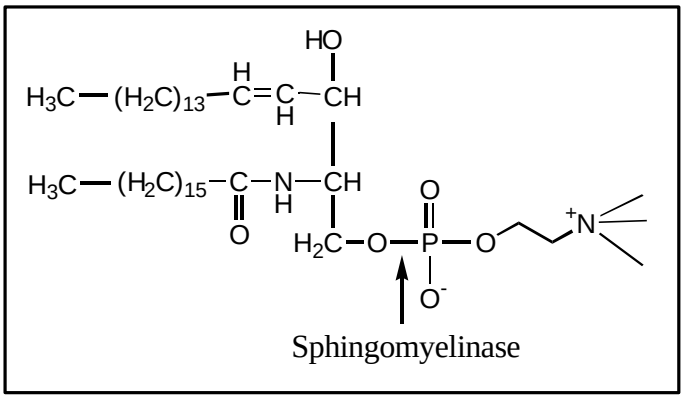


Figure 13. Cleavage of a typical sphingophospholipid, sphingomyelin, by sphingomyelinase.

7.2.2.1. Sphingomyelinases are divided into four classes

There are various forms of sphingomyelinases in the extracellular medium and in the secretory and endocytic compartments of many cells (Hinkovska-Galcheva et al, 1998b; Schissel et al, 1998; Tabas, 1999): (i) a lysosomal acid sphingomyelinase; (ii) a cytosolic Mg^{2+}-independent neutral sphingomyelinase; (iii) a plasma membrane-associated Mg^{2+}-dependent neutral sphingomyelinase; and (iv) a secreted Zn^{2+}-dependent sphingomyelinase.

7.2.2.2. Roles of sphingomyelinase and ceramide in endocytosis

Sphingomyelinase could play a role in vesicle formation during endocytosis by hydrolysis of SM on the plasma membrane. Treatment of ATP-depleted macrophages and fibroblasts with exogenous sphingomyelinase rapidly induces formation of numerous vesicles that pinch off from the plasma membrane. These vesicles are approx. 400 nm in diameter and lack discernible coats since there is no detectable enrichment of either clathrin or caveolin. When ATP is restored to the cells, the sphingomyelinase induced vesicles are able to deliver fluid-phase tracers to late endosomes/lysosomes and return recycling receptors, such as TfR, back to the plasma membrane (Zha et al, 1998). The possibility that ceramide induces vesicle formation was also demonstrated by treatment of cells with C_6-ceramide, which induces the formation of endocytic vesicles and causes the enlargement of late endosomes and lysosomes in fibroblasts. This induction is time- and dose-dependent, rapid and reversible (Li et al, 1999). Using giant liposomes, the role of ceramide in vesicle budding has been related to asymmetry and microdomain formation. Vectorial formation of vesicles, i.e. endocytosis and shedding, was induced by the asymmetrical formation of ceramide by sphingomyelinase in either the outer or inner leaflet, respectively. A model was proposed in which: (i) the lipids in the monolayer are randomly distributed; (ii) hydrolytic cleavage of the phosphocholine headgroup from SM by sphingomyelinase generates ceramide, a lipid with a small, less hydrated headgroup; (iii) the formed ceramide segregates into a domain; and (iv) because of the tendency of ceramide to form inverted non lamellar phases, an invagination is formed by the ceramide-enriched domain (Holopainen et al, 2000). However, the relevance of these roles of ceramide is not established and remains to be determined.

7.2.2.3. Roles of sphingomyelinase and ceramide in phagocytosis

Sphingomyelinase may also be involved in phagocytosis. For example, endogenous acidic sphingomyelinase activity triggers phagocytosis of *Neisseria gonorrhoeae* bound to syndecan in human cells that are normally inactive for phagocytosis. Moreover, complementation of acidic sphingomyelinase-deficient fibroblasts from Niemann-Pick syndrome patients restored *Neisseria gonorrhoeae* induced signalling and entry processes. The bacteria can activate a signalling cascade through PC-specific PLC that activates the target cell's sphingomyelinase, resulting in conversion of SM in ceramide (Grassme et al, 1997). Also, in human polymorphonuclear neutrophils (PMNs) engaged in IgG-dependent phagocytosis, the SM cycle is restricted to the plasma membrane where intracellular targets of ceramide action, such as PLD, are localized. During the phagocytosis of antibody-coated erythrocytes, ceramide is generated, and ceramide-1-phosphate is formed by a calcium-dependent ceramide kinase, found predominately in the neutrophil plasma membrane and having an optimal activity at pH 6.8 (Hinkovska-Galcheva et al, 1998a; 1998b).

7.2.2.4. Roles of sphingomyelinase and ceramide in fusion

Vesicle (large unilamellar vesicles) membrane fusion is induced by the concerted activities of sphingomyelinase and PLC. When both enzymes are added together, their joint hydrolytic activities give rise to leakage-free vesicle aggregation, lipid mixing and aqueous contents mixing, i.e. vesicle fusion. The enzymes appear to be coupled through their reaction products (Ruiz-Argüello et al, 1998).

7.2.3. Roles of PLA_2 and arachidonic acid in membrane trafficking

PLA_2 catalyzes the production of arachidonic acid and is involved in endocytosis, phagocytosis and membrane fusion.

7.2.3.1. PLA2 are divided into several classes

The classification of PLA_2 is based on Ca^{2+}-dependence (Ca^{2+}-dependent PLA_2 and Ca^{2+}-independent PLA_2) and other biochemical characteristics (for reviews, see Mayer and Marshall, 1993; Ackermann and Dennis, 1995; Roberts, 1996). Ca^{2+}-dependent PLA_2 are divided into groups I, II, III and IV and are all dependent on Ca^{2+} either for catalytic activity or for association

with membrane phospholipid substrates: (i) groups I, II and III (secretory PLA_2 [$sPLA_2$]) which require mM Ca^{2+}, a pH of 7-10 for optimal activity, and are extracellularly localized or cell-associated; and (ii) group IV (cytosolic PLA_2 [$cPLA_2$]) which requires µM Ca^{2+}, a pH of 7-10 for optimal activity, and is localized intracellularly. Ca^{2+}-independent PLA_2 ($iPLA_2$) include three categories: (i) the lysosomal (or acidic) $iPLA_2$, with an optimum pH range of 4.5-7.5; (ii) the brush-border membrane $iPLA_2$; and (iii) the intracellular (cytosolic/membrane) $iPLA_2$.

7.2.3.2. Roles of PLA_2 and arachidonic acid in endocytosis

Addition of arachidonic acid or stimulation of arachidonic acid production by secretory PLA_2 selectively accelerated apical endocytosis of ricin in MDCK cells without affecting basolateral endocytosis (Llorente et al, 2000).

A role for PLA_2 in the trafficking of some receptors, such as the asialoglycoprotein receptor, was also evidenced. Indeed, inhibition of endosome fusion by an inhibitor of PLA_2 affected the trafficking of asialoglycoprotein receptor but not those of Tf or iron (Young, 2000).

7.2.3.3. Role of PLA_2 and arachidonic acid in phagocytosis

Arachidonic acid is required for IgG-mediated phagocytosis in monocytes and arachidonic acid release is mediated by a Ca^{2+}-independent phospholipase. During the early stages of phagocytosis, plasma membrane is sequestred in intracellular vesicles that provide membrane for the forming phagosome via fusion events that require arachidonic acid (Lennartz et al, 1997).

7.2.3.4. Role of PLA_2 and arachidonic acid in fusion

It is well known that Rab5 and NSF promote fusion among early endosomes. Mayorga et al showed that endosome fusion along the endocytic pathway also requires a PLA_2 activity. Indeed, several PLA_2 inhibitors blocked endosome fusion in a broken-cell preparation and inhibition was reversed by addition of arachidonic acid (Mayorga et al, 1993). A model was proposed, in which NSF and PLA_2 activities are required downstream of Rab5 (Barbieri et al, 1996).

7.2.4. Roles of PLD and PA in membrane trafficking

PLD catalyze the hydrolysis of PC to PA and choline and are involved in endocytosis, phagocytosis, endosome fusion and cytoskeletal dynamics.

7.2.4.1. PLD are divided into two classes

PLD are divided into PLD_1 and PLD_2. The PLD_1 exhibits PC-specific PLD activity and is activated by ARF and PI 4,5-P_2. PLD_1 has been renamed PLD_{1a} after another splice variant has been identified, PLD_{1b}. PLD_1 is highly expressed in kidney and lung, but detectable levels were also observed in other tissues. ARF-dependent PLD_1 activities in multiple cellular membranes include the nuclear enveloppe, ER, Golgi apparatus, transport/secretory vesicles and plasma membrane. PLD_1 appears to be localized mainly in organelles and vesicles of the endosomal/lysosomal compartment. PLD_2 seems to be expressed to various extents in almost every tissue and cell type studied. PLD_2 may be positively regulated by ARF. Moreover, it has recently been showed that PLD_2 interacts with the EGFR, that RTKs regulate PLD_2 activity and that PLD_2 localization and/or activity are regulated by tyrosine phosphates. As opposed to PLD_1, whose localization in most cells seems to be confined to intracellular membranes, PLD_2 was found localized in the plasma membrane (for a review, see Liscovitch et al, 2000).

7.2.4.2. Roles of PLD and PA in endocytosis

A first role for PLD is the recruitment of AP-2 on plasma membrane, endosomes and lysosomes. First, PLD helps AP-2 recruitment on plasma membrane and endosomes: (i) exogenous PLD stimulates AP-2 recruitment onto endosomal compartment, while neomycin, which inhibits endogenous ARF-dependent PLD by binding to its cofactor PI 4,5-P_2, inhibits this event; (ii) neomycin also has a strong effect on plasma membrane AP-2 binding, indicating that the recruitment of AP-2 onto both membranes may require PLD. Interestingly, neither exogenous PLD nor neomycin has any effect on AP-1 recruitment. So, different mechanisms are used for the recuitment of AP-1 and AP-2 (West et al, 1997). In addition to recruitment on plasma membrane and endosomes, PLD may be involved in AP-2 recruitment onto lysosomes, by the production of PA and by its involvement in the production of PI 4,5-P_2. PI 4,5-P_2, generated by the sequential action of a lysosome-associated type II PI 4-kinase, a soluble type I PI 4-P 5-kinase and a PLD_1-like enzyme located on the lysosomal surface, are involved in clathrin-coat assembly on lysosomal

membranes. The PLD_1-like enzyme produces PA which potently stimulates PI 4-P 5-kinase activity. A positive feed-back loop between PI 4-P 5-kinase and PLD was evidenced. Both PI 4,5-P_2 and PA are important regulators of AP-2 recruitment and clathrin-lattice assembly onto lysosomes: (i) PA activates a putative AP-2 selective docking molecule in a manner analogous to the way ARF activates an AP-1 specific docking site at the TGN; and (ii) in addition to serving as a cofactor for PLD_1, PI 4,5-P_2 might contribute to the nucleation of coated structures directly. So, the mechanisms that underly coat assembly on lysosomes are likely to be similar to those occuring at the cell surface (Arneson et al, 1999).

A second role for PLD is signal transduction during receptor-mediated endocytosis. In mammalian cells, cellular PLD activity is normally low and increases on agonist stimulation of a variety of cell surface receptors. For example, a role for PLD in signalling that facilitates EGFR endocytosis has been evidenced: in response to EGF, the EGFR is internalized and then degraded, the internalization of the EGFR involving endocytosis which is dependent upon PLD, and the degradation of EGFR being also dependent upon PLD (Shen et al, 2001).

7.2.4.3. Roles of PLD in phagocytosis

PLD also functions in the regulation of phagocytosis. FcγR-mediated phagocytosis is accompanied by TK-dependent activation of PLD which functions to regulate the ingestion of IgG-opsonized particles, each class of macrophage FcγRs (FcγRI, RII and RIII) being able to stimulate PLD activity. Likewise, PLD is activated via TKs during phagocytosis by macrophages of *Mycobacterium tuberculosis* or opsonized zymozan (Kusner et al, 1996). PLD activity is also a target of ceramide action which is produced by the SM cycle (Hinkovska-Galcheva et al, 1998b; Kusner et al, 1999).

7.2.4.4. Roles of PLD and PA in fusion and in cytoskeletal dynamics

The conversion by PLD of membrane PC to PA results in the replacement of a non fusogenic phospholipid with a fusogenic one, and in the increase of the potential for interaction between membranes and the annexins (Blackwood et al, 1997). Particle motility induced by ARF6 notably involved PLD (Schafer et al, 2000).

7.2.5. Roles of PLC in membrane trafficking

PLC, which hydrolyses PI 4,5-P_2 (one of its major substrate) to produce diacylglycerol and IP_3, plays roles in endocytosis, phagocytosis and macropinocytosis.

7.2.5.1. PLC are divided into several classes

Based on substrate specificity, PI-PLC and PC-PLC are distinguished (Dennis et al, 1991; Roberts, 1996). PI-PLC, which catalyzes the hydrolysis of PI, is present in most mammalian cells. Mammalian PI-PLC can be divided into four types: PLC-α, PLC-β, PLC-γ and PLC-δ. All four enzymes hydrolyze PtdIns, PI 4-P and PI 4,5-P_2, but not PI 3-P, phosphatidylinositol 3,4-bisphosphate (PI 3,4-P_2) or PI 3,4,5-P_3. Moreover, PI-PLC can be divided into cytosolic PI-PLC (cPI-PLC) and secreted PI-PLC (sPI-PLC). PC-PLC is represented by non-specific PLC enzymes, often characterized by their action toward PC molecules.

7.2.5.2. Roles of PLC in endocytosis

PLC participates in endocytosis via signal transduction and active maintenance of PI 4,5-P_2 levels is required for meaningful PLC signalling. As a first example, endocytosis of FcγRI is regulated by two distinct signalling pathways: one of them involves the activation of PLC-β and PKC but is PI 3-kinase-independent, and the other requires the activation of PI 3-kinase and is PKC-independent (Norman and Allen, 2000; see also section 7.2.1.2.2.). As a second example, EGFR signalling implies the PLC pathway, and this is spatially restricted at a point between PLC-γ1 phosphorylation and PI 4,5-P_2 hydrolysis, perhaps because of limited access of EGFR-bound PLC-γ1 to its substrate in endocytic trafficking organelles. Only cell surface receptors effectively participate in PLC function and active EGFR in internal compartments stimulate little if any hydrolysis of PI 4,5-P_2 (Haugh et al, 1999).

7.2.5.3. Roles of PLC in phagocytosis and macropinocytosis

The presence of PI 4,5-P_2 and active PLCγ at the phagosome is essential for effective particle ingestion. During FcγR-mediated phagocytosis of IgG opsonized particles, a sequence of coordinated steps in phospholipid metabolism has been described: (i) a focal, rapid accumulation

of PI 4,5-P_2 accompanied by recruitment of type Iα PI phosphate kinase to the phagosomal cup, (ii) followed by disappearance of PI 4,5-P_2 as the phagosome seals; (iii) loss of PI 4,5-P_2 correlates with mobilization of PLCγ and with the localized formation of diacylglycerol; (iv) neither PI 4,5-P_2 nor diacylglycerol are detectable in late phagosomes; and (v) phagosomal actin decreases in parallel with or shortly after PI 4,5-P_2. So, the presence of PI 4,5-P_2 and active PLCγ at the phagosome was shown to be essential for effective particle ingestion. It has been suggested that PLC activity is required to induce actin disassembly and remodeling during cup formation and phagosomal closure (Botelho et al, 2000b; see section 2.3.5.).

As already mentioned, constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of PI 3-kinase and PI-specific PLC (Amyere et al, 2000).

8. Acidification of endosomal and phagosomal compartments and acidotropic accumulation

8.1. The vacuolar H^+-ATPase

The vacuolar H^+-ATPase is an universal component of eukaryotic organisms. It is present on membranes of many organelles, such as endosomes, lysosomes, phagosomes, the Golgi apparatus and secretory granules, where its proton-pumping action creates the low intravacuolar pH found (for a review, see Finbow and Harrison, 1997).

The vacuolar H^+-ATPase is a member of a family of multi-subunit proton pumps. It is composed of a membrane sector and a cytosolic catalytic sector. It pumps protons from the cytoplasm to the lumen of the vacuole using the energy released by ATP hydrolysis. So, it is part of the cellular machinery that regulates the cytosolic pH. This pump is electrogenic, i.e. the movement of a proton results in the translocation of net charge that is not directly coupled to the transport of another ion to maintain electroneutrality, thereby creating an electrical potential difference across the membrane (for a review, see Finbow and Harrison, 1997).

8.2. Acidification of endosomal compartments: control and roles in endocytosis

Acidification of endosomal compartments, its regulation and its roles in endocytosis have been extensively described in Mukherjee et al (1997) and Clague (1998).

Extensive characterization of endosomal compartment pH using endocytosed fluorophores such as fluorescein has been carried out in Chinese Hamster Ovary cells (CHO cells) and in other fibroblasts. In CHO cells, sorting endosomes have a pH of 5.9-6.0, the recycling compartment of 6.4-6.5, whereas late endosomes have a pH below 6.0 and lysosomes are even more acidic, with a pH between 5.0 and 5.5.

Endosomal acidity is maintained by an ATP-dependent proton pump. Various mechanisms may account for different acidities in different endosomal compartments. In some cells, acidification of early endosomes is counteracted by the electrogenic Na^+/K^+ pump. This pump generates an inside positive membrane potential that makes it more difficult to pump protons inside the endosome. This pump, which recycles back to the plasma membrane from early endosomal compartments, is present on early endosomes but not on late endosomes, thus allowing the late endosomal compartment to acidify to a greater extent. Chloride conductance also regulates the pH of endosomes and lysosomes. It has been suggested that changes in membrane potential could affect the steady-state pH of an endosome by implying changes in the coupling between proton translocation and ATP hydrolysis by the vacuolar proton pump as the proton concentration and/or positive charge in the compartment builds up.

Acidification is an essential property for endocytic sorting and activity of lysosomal enzymes. The acidic environment is used to dissociate many ligands, including insulin, EGF, LDL, from their receptors within sorting endosomes. This could result from a pH-dependent conformational change in the receptors. Another example of pH-dependent conformational change is the release of iron from Tf in the sorting endosomes, while apo-Tf remains bound to its receptor in the acidic endosomes. Some ligands require a lower pH than that found in the early endosomes to be released from their receptors; it is the case for MP ligands that are released from the CI-MPR at about pH 5.8, corresponding to the pH found in the late endosomes. The low pH of late endosomes and lysosomes also ensure the optimal environment for the activity of lysosomal enzymes that have acidic pH optima.

In addition, the control of the internal pH of endosomal compartments plays a role in membrane traffic. First, agents that neutralize endosomal pH such as ammonium chloride or monensin (see section 10.1.), inhibit the transport of the fluid-phase endocytic tracer HRP between early and late endosomes (Sullivan et al, 1987; Cupers et al, 1997). Second, upon inactivation of the vacuolar H^+-ATPase by bafilomycin A1 (see section 10.1.), trafficking between early and late endosomes continues, but that to lysosomes is blocked, indicating that transport from late endosomes to lysosomes depends on the vacuolar H^+-ATPase (van Weert et al, 1995). ECV formation from endosomes is inhibited by bafilomycin treatment, whilst initial internalization and recycling are essentially unchanged. The mechanism by which internal pH alters these properties remain unclear. It seems that the requirement for an acidic endosomal lumen reflects the existence of an allosteric transmembrane pH-sensor governing the assembly of the budding apparatus at the cytosolic surface of the early endosomes. Consistent with this, is the finding that β-COP associates with endosomes in a pH-sensitive manner and is required for an *in vitro* assay of ECV formation.

8.3. Acidification of phagosomes: control and roles in phagocytosis

Characterization of phagosomal pH using ingestion of FITC-labelled *Staphylococcus aureus* has been carried out in thioglycolate-elicited murine macrophages. After ingestion, the pH of the phagosomal compartment decreased rapidly, reaching a steady state value of 5.8-6.1 (Lukacs et al, 1990).

The substrate requirement and inhibitory profile of phagosomal acidification suggested that proton translocation across the phagosomal membrane was mediated by a vacuolar H^+-ATPase (Lukacs et al, 1990). Concerning the origin of the phagosomal proton pump, it seems that acidification is initiated prior to lysosomal fusion (Lukacs et al, 1990). This is consistent with the finding that phagosomal acidification is detectable before phagolysosomal fusion occurs and prior to the appearance of lysosomal enzyme activity in the phagosomes (McNeil et al, 1983). It seems, however, that the vacuolar H^+-ATPase accumulates in the phagosomal membrane as it matures within the cell (for a review, see Caron and Hall, 2001).

Whereas the vacuolar H^+-ATPase appears to be the main determinant of phagosomal acidification, fractionation studies have also revealed the incorporation of Na^+/H^+ exchanger 1

(NHE1) into the phagosomal membranes. NHE1 is the predominant isoform of the NHE in macrophages, which are ubiquitous membrane transport proteins that play a major role in the regulation of intracellular pH and of cellular volume. NHE catalyzes the electroneutral exchange of extracellular Na^+ for intracellular H^+, a process which is inhibited by amiloride. Measurements of pH indicate that NHE1 is functional in the phagosomal membrane. NHE could therefore contribute importantly to phagosomal acidification. However, it has been shown that it is not the case, and this has been supported by the finding that the phagosomes are virtually devoid of Na^+/K^+ ATPases. Considering the latter observation, it is conceivable that mature phagosomes attain a much more acidic pH than endosomes due, at least in part, to the effective removal of Na^+/K^+ ATPases (Hackam et al, 1997). In conclusion, NHE, but not Na^+/K^+ ATPases, are present in phagosomes. Although present in the phagosomal membrane in a functional state, NHE1 does not contribute to the acidification of phagosomes, which is made by vacuolar H^+-ATPases (Hackam et al, 1997).

Acidification of the phagosomal compartment is crucial to its microbicidal functions: (i) the elevated proton concentration is directly lethal for certain microorganisms; (ii) it promotes the spontaneous dismutation of superoxide to hydrogen peroxide; (iii) it is a prerequisite for phagosome-lysosome fusion and it provides optimal conditions for the activity of certain hydrolytic enzymes (Lukacs et al, 1990).

8.4. Acidotropic accumulation

The term «lysosomotropic » has been used to designate all substances that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake. Indeed, in addition to endocytosis, another route is taken up for molecules to accumulate into lysosomes, consisting of intralysosomal trapping by protonation. This is the route used by weak bases, which can diffuse readily across biological membranes in unionized form, but not or very slowly in ionized form (for a review, see de Duve et al, 1974). Since these substances are also able to accumulate in other acidic compartments than lysosomes, we prefer using the term « acidotropic » to designate these compounds.

If the membrane separates two regions of different pH, bases accumulate on the acid side. These considerations have led to the model described by de Duve (1974). In this model, the

penetration of a weak base (B) through the plasma membrane into the cytoplasm and then through the lysosomal membrane into the lysosome is presented in Figure 14.

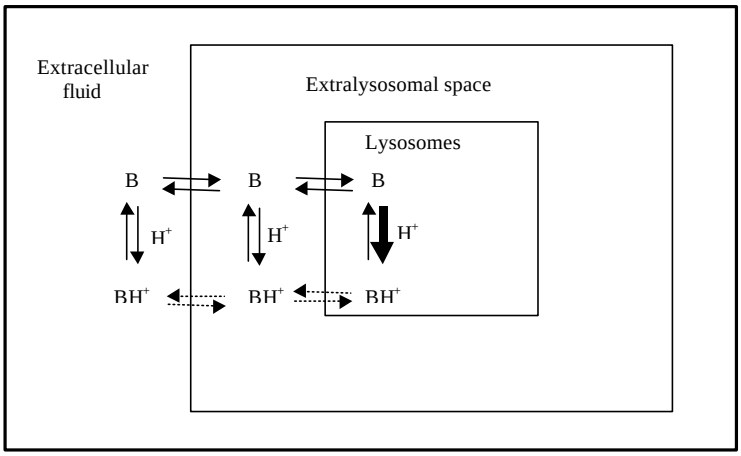


Figure 14. Model of intralysosomal trapping of weak bases. (From de Duve et al, 1974).

From a series of equations, it has been deduced that the maximal concentration ratio (F) of the total concentration of the substance in the lysosomes to that in the extracellular medium is defined as : $F = ([H^+]_{lysosomes} / [H^+]_{extracellular\ fluid})^n$, where n represents the number of ionizable functions present in the substance (for a review, see de Duve et al, 1974). However, two other parameters are important for steady state concentration ratios of bases, the permeability coefficient ratio of the protonated form to the unprotonated form (á), and the pKs of the substance. Several situations can be observed : (i) a high á value results in a decrease of the concentration ratio for all bases, but bases with higher pKs are more sensitive to this effect; (ii) bases with pKs below 8 can never achieve the maximum concentration ratio even when á equals 0; (iii) substances with a pK around 8 are the most likely to show the kind of lysosomotropism; and (iv) bases with high pKs would be expected to penetrate the lysosomes very slowly (for a review, see de Duve et al, 1974).

For example is the uptake of chloroquine. Cells exposed to chloroquine take up this compound avidly, concentrating it many-fold over the surrounding medium in a matter of minutes. The amounts of drug that can be taken up by cells are enormous and is consistent with the model of proton trapping. Chloroquine is a weak base with an appropriate pK of 8.1. Its uptake is accelerated by a rise in extracellular pH. However, the rapid rate of chloroquine uptake is surprising, since the drug has a second basic group of pK 10.1, and therefore occurs in only trace amounts of unprotonated form at physiological pH. This apparent contradiction could be resolved by the observation that the permeability coefficient of the membranes to free chloroquine base must be much higher than for any hydrophilic compound.

9. Role of the cytoskeleton

Two types of motility allow movement of organelles in mammalian cells, microtubules and actin cytoskeleton. The role of microtubules in endocytosis has been extensively described in Lane and Allan (1998), Allan and Schroer (1999) and Kamal and Goldstein (2000), and that of actin cytoskeleton in May and Machesky (2001). In animal cells, movement of many types of organelle seems involve contribution from both microtubule- and actin-associated motor proteins, microtubule-based motility providing long-range organelle transport and microfilaments local movement. The different motility systems may therefore act synergistically within cells to determine organelle distribution and shape, and to promote movement between compartments. The precise balance between the two types of motility may well vary between cells and according to physiological conditions.

In this section, we will describe microtubules and actin filaments and their associated motor proteins. We will also evolve their roles in location and motility of components of the endocytic and phagocytic pathways, as well as the regulation and coordination of motor-dependent organelle motility.

9.1. Microtubules and their associated motor protein complexes

Microtubules are polar, unbranched polymers. Microtubules of the eukaryotic cytoskeleton are composed of an heterodimer of á- and â-tubulin. In non-polarized cells, the minus ends of microtubules are located at the cell center near the centrosomes, whereas the plus ends extend radially to the cell periphery. In polarized cells, the plus ends are located at the basolateral surface, and the minus ends at the apical surface.

Coordinated organelle movements in cells depend upon the actions of motor proteins which use energy derived from the hydrolysis of nucleotides to provide directional motion along the underlying cytoskeleton. Microtubule motors can be classified into two groups: plus end-directed motors, which include kinesin; and minus end-directed motors, which include cytoplasmic dynein. Kinesin is found in many species of animals, although not in budding yeast. This motor translocates towards the plus ends of microtubules. Transport in the opposite direction along microtubules is driven by the microtubule motor dynein. An additional factor, the dynactin complex, is needed for cytoplasmic dynein to move membranes both *in vivo* and *in vitro*.

9.2. Actin filaments and their associated motor protein

Actin microfilaments are localized as cortical actin patches at the cell surface and as actin cables within the cell. It has been suggested that short-range transport takes place on actin filaments by myosin motors. Currently, 15 different members of the myosin superfamily exist and several are membrane associated.

9.3. Microtubules and actin filaments and the endocytic pathway

9.3.1. Role in distribution of early and late endocytic compartments

Organelles and vesicular transport intermediates are localized in association with the polarized microtubule arrays. For example, in fibroblasts, endosomes are dispersed along microtubules toward the plus ends pointed at the cell periphery, whereas the Golgi apparatus, late endosomes, and lysosomes are often clustered near minus ends at the cell center. Lysosomes are highly motile organelles that move mainly along microtubules, tend to be located near the nucleus, and are able to move in the minus end- as well as in the plus end-directions. This microtubule-dependent distribution of organelles is thought to promote efficient transport within the endocytic pathway by utilization of cytoskeletal motors, kinesin and dynein motors. In addition to the role of microtubules, actin-based movements may also be of vital importance throughout the endocytic pathway, and depolymerizing actin filaments can alter distribution of endosomes.

9.3.2. Roles in endocytosis

Depolymerizing actin filaments inhibits endocytosis from the apical but not from the basolateral domain of polarized epithelial cells. Non-polarized cells may also use actin-based motors in this early step. Actin filaments indeed increase the uptake of ligands, a role for the small GTPases Rho and Rac (both of which regulate the actin cytoskeleton) being demonstrated in receptor sequestration. However, the requirement for actin in receptor-mediated endocytosis of mammalian cells has been controversed.

Once material has been endocytozed by receptor-mediated endocytosis, it is delivered to the endosome by a mechanism independent of microtubules. Then, vesicular structures containing ligand tend to move inwards along microtubules, and, indeed, endosomes have been shown to possess cytoplasmic dynein. Microtubule-based movement is also required for the delivery of

material from early endosomes to late endosomes in cell types that have the more vesicular type of endosomes (such as BHKs and MDCKs).

The final step of endocytosis is the transfer of material from late endosomes to lysosomes. Evidence suggests that this transfer may not require intact microtubules in certain cell types *in vivo* and *in vitro*. Myosin has previously been implicated in endocytosis and it has been localized on endocytic organelles, such as endosomes and lysosomes, by biochemical fractionation and immunoelectron microscopy. Although the loss of microfilaments had no obvious effect on the movement of late endosomes and lysosomes in Normal Rat Kidney cells (NRK cells), a role for myosin has been suggested in the late steps of endocytic trafficking from endosomes to lysosomes in other cell types. So, in addition to their roles in internalization by endocytosis, actin filaments increase the delivery of ligands to the degradative compartments downstream of the region where microtubules are required.

In addition, transport between Golgi and endocytic compartments also requires microtubules. It seems that kinesin-I is needed for some aspect of trafficking from Golgi membranes, and that dynein stimulates late endosome to TGN transport of the MPR *in vitro*.

9.4. Microtubules and actin filaments and the phagocytic pathway

9.4.1. Role in distribution of early and late phagosomes

Intracellular phagosome movement requires microtubules. Interestingly, early phagosomes have a strong preference for microtubule plus-ends, whereas late phagosomes do not (Blocker et al, 1998).

9.4.2. Roles in phagocytosis

Despite the great variety of targets and phagocytic receptors, one feature is always conserved in phagocytosis, the dependence of actin cytoskeleton. Rapid polymerization of actin is widely thought to be responsible for the generation of driving forces allowing the plasma membrane to be somehow pushed in structures such as microvilli, lamellipod or pseudopod extensions. Numerous cytoskeletal molecules are recruited to the sites of particle binding. Among these proteins is á-actinin, which can bundle actin filaments and link them to integrins, and talin, vinculin, paxillin and focal adhesion kinase, which are focal adhesion proteins. The focal adhesion is a complex assembly of cytoskeletal and signalling proteins. Both Fcã- and CR3-mediated

phagocytosis are accompanied by the local recruitment of á-actinin, and most of the focal adhesion molecules are conserved in phagocytosis mediated by several distinct receptors. However, there are some differences: paxillin and vinculin are recruited to IgG- as well as complement-dependent phagosomes, but are absent from phagosomes forming around unopsonized zymozan.

The role of microtubule network in phagocytosis also appears to be different among phagocytic receptors. Indeed, CR-mediated internalization requires intact microtubules and is accompanied by the accumulation of vesicles beneath the forming phagosome, suggesting that membrane trafficking plays a key role in CR-mediated phagocytosis. FcγR-mediated phagocytosis and the uptake of latex beads are not sensitive to colchicine, which disrupts the microtubule network.

After the early stages of phagocytosis, phagosomes fuse with endocytic structures, eventually maturing into phagolysosomes using a mechanism facilitated by microtubules or microfilaments: microtubules are needed for the movement of phagosomes containing 0.9 µm polystyrene beads towards the cell centre, although, phagosomes which formed around 3.0 µm beads appeared to use actin filaments. Phagosome translocation via microtubules has been shown, *in vitro*, to occur predominantly towards microtubule minus ends in a cytoplasmic dynein-dependent fashion, but with some kinesin-driven movement as well. Filamentous actin can be seen in close proximity to many, but not all phagosomes of J774 macrophages. These organelles can assemble actin *de novo* on their cytoplasmic surface and this capacity to assemble actin oscillates with a low point at 12h. It has been proposed that this actin assembly process facilitates phagosome/endosome aggregation prior to membrane fusion (Defacque et al, 2000; Guerin and de Chastellier, 2000).

9.5. Regulation and coordination of motor-dependent organelle motility

9.5.1. Coordination with membrane traffic

There must be a regulatory link between membrane traffic and motors. For example, if a transport vesicle is to reach its target compartment within a cell, it is essential that the correct motor protein is sorted into the budding vesicle. It has been suggested that the machinery that ensures the sorting of proteins into vesicles and which controls the budding process also selects

and regulates the appropriate motors. A role for Rab5 in regulating the motility of early endosomes on microtubules was evidenced. Rab5 increases minus-end directed motility along microtubules. Moreover, Rab5 and its effectors bind to actin and Rab5 is sometimes actively required for the reorganization of actin stress fibers. Likewise, an intimate interaction between Rab11 and the actin cytoskeleton has been shown to be critical for TfR recycling (for a review, see Somsel Rodman and Wandinger-Ness, 2000).

9.5.2. How do motors bind to membranes?

Motor proteins have been detected in association with several organelles implicated in the endocytic pathway. An important question arises, how is the motor complex assembled on the organelle surface?

Kinesin binds tightly to organelles and a putative membrane receptor for kinesin is kinectin, a membrane-associated kinesin-binding protein, which might underlie some motor assemblies and has been found on phagosomes. Cytoplasmic dynein, on the other hand, associates less strongly with organelles than does kinesin. Indeed, cytoplasmic dynein appears to be able to associate directly with the lipid bilayer. Likewise, some myosins might bind lipids directly.

Dynactin, the cofactor of dynein, was colocalized with the microtubule-binding protein CLIP-170 (for cytoplasmic linker protein-170) at the plus ends of microtubules. CLIP-170 is a cytoplasmic linker that is thought to tether endosomes to microtubules and associates with growing microtubule ends as they extend into the cell periphery. A model has been suggested in which CLIP-170 first captures endosomes to microtubules and then dissociates, allowing the endosomes to be moved by the motor along the microtubules.

9.5.3. Regulation of motor activity

Dynein is unable to move organelles in the absence of additional cytosolic factors and it has been suggested that motor activation is mediated or enhanced in some way by the dynactin complex. One important function of dynactin may be to increase the processivity of the dynein motor by perhaps providing cargo with an extra site of contact to microtubules.

For kinesin-I, it seems that, in the absence of bound cargo, kinesin motors fold into a compact conformation and are inhibited from moving along microtubules by the cargo-binding tail.

9.5.4. Regulation of the direction of organelle movement

One additional element to consider are the tracks themselves. It seems that this is regulated by the presence of microtubule-associated proteins (MAPs) which can be released by other proteins, allowing organelle motility. *In vitro*, MAPs can inhibit motility and overexpression of MAP4 or tau can inhibit organelle movement and membrane trafficking. Under normal conditions, mechanisms probably exist to clear the microtubule path of binding proteins. These might include mapmodulin, a protein that binds MAPs and prevents them from binding microtubules, or kinases that release MAPs from the microtubule surface. A model has been proposed in which mapmodulin captures the microtubule-binding domains of MAPs, thus releasing MAPs from microtubules and allowing organelle motility to take place in the endocytic pathway.

9.5.5. Regulation of the amount of organelle movement

Another important question is to know what mechanisms exist to ensure that organelle motor activities work together and not in opposition? Although phosphorylation is an obvious mechanism, it has been difficult to make a clear correlation between phosphorylation state and motor activity. However, direct interactions between motors and a number of kinases and phosphatases have been observed.

10. Agents perturbing endocytosis

In this section, we will briefly present some inhibitors which have been used to dissect endocytic and phagocytic pathways. These agents have been classified into 5 categories, based on their capacity to interfere with one or more machineries involved in endocytic and phagocytic trafficking. However, it is important to note that this classification is a simplification, since some agents could be inserted into several categories. When it is possible, the main characteristics of each class of agents are summarized after the respective table.

10.1. Agents perturbing endosome/lysosome acidification

Among the agents able to perturb endosomal/lysosomal pH are: (i) the vacuolar H^+-ATPase inhibitors, such as bafilomycins, concanamycins and megalomicins; (ii) the Na^+-H^+ ionophores, such as monensin; and (iii) weak bases, such as chloroquine, methylamine, primaquine and procaine amide.

Bafilomycins are macrolide antibiotics with a 16-membered lactone ring (Werner and Hagenmaier, 1984). Among these is bafilomycin A1 which is a specific inhibitor of vacuolar-type H^+-ATPase and which has been shown to inhibit lysosomal, phagosomal and plasma membrane H^+-ATPase in mouse macrophages (Tapper and Sundler, 1995). Structuraly related agents, concanamycins, are also selective inhibitors of the vacuolar H^+-ATPase and inhibit acidification of endosomes and lysosomes (Woo et al, 1992). Megalomicins are also macrolide antibiotics, and are composed of an aglycone identical to that of erythromycin, and three sugars, two of which being aminated. Megalomicins are able to strongly inhibit the ATP-dependent acidification of lysosomes *in vitro* and to induce a rapid swelling of these organelles (Bonay et al, 1997).

Monensin is a metabolite of *Streptomyces cinnamonensis* that binds Na^+, K^+ and H^+. In membranes, it acts as a diffusional carrier, mediating one-for-one cation exchange, and accomplished partial equilibration of ions with which it interacts. Because of its lipophilic structure, it could insert into all cellular membranes. The best-documented effect of monensin is the perturbation of the Golgi complex. Another effect of this carboxylic ionophore which can catalyzes the exchange of Na^+ with H^+ across membranes is the disruption of the H^+ gradient that maintains the acid pH of lysosomes (Tartakoff, 1983), showing, at 6 µM, an increase of the endocytic pH above 6.0 instead of 5.0 in control cells (Maxfield, 1982).

Weak bases such as chloroquine accumulate in lysosomes. The antimalarial action of this drug is due to the lysosomal accumulation in the parasite. This leads to an increase of lysosomal pH, to an inhibition of the lysosomal hydrolysis of haemoglobin, and to arrested growth of the parasite (Homewood et al, 1972). Chloroquine at 140 µM is indeed able to raise endocytic vesicle pH above 6.0 instead of 5.0 in control cells (Maxfield, 1982). The same effect on endosomal pH is obtained for the weak base methylamine at a concentration of 10mM (Maxfield, 1982). The anti-malaria drug primaquine is also an amphiphilic weak base. Like other weak bases, this drug is able

to accumulate in endosomes (van Weert et al, 2000). Procaine amide, a weak base with two amino functions and with an amido function, induces cytoplasmic vacuolation (Bond et al, 1975).

Drug	**Experimental model:** **Cell** **Drug concentration** **Endocytic tracer (s)**	**Consequences for trafficking** **(references)**
Bafilomycin A1 (baf)	MDCK Baf 0.5 µM	tubulation of the early endosomes (D'Arrigo et al, 1997)
	BHK Baf 1 µM, 30 min HRP	early endosomes are highly tubular; no significant effect on internalization or regurgitation of HRP; endocytosed HRP does not appear in late endosomes (Clague et al, 1994)
	CHO Baf 0.2/0.5 µM Tf C_6-NBD-SM	does not alter the exit of the TfR from early sorting endosomes nor its subsequent accumulation in the ERC; strongly slows down the rate constant for exit of TfR from recycling compartment; slows down to a lesser extent the exit rate constant for recycling of C_6-NBD-SM (Presley et al, 1997)
	HepG2 Baf 1 µM Tf HRP	the rates of internalization and recycling of Tf are reduced to a comparable extent, thus resulting in little change in TfR expression at the cell surface; transport of HRP to late endosomes continues; transport from late endosomes to lysosomes is blocked (van Weert et al, 1995)
	Rat hepatocytes Baf 1 µM, 15 min asialoorosomucoid albumin and HRP	slows down the rate of accumulation of receptor-mediated (asialoorosomucoid) and fluid (HRP, albumin) tracers; the number of cell-surface asialoglycoprotein receptors is reduced; recycling or regurgitation of both probes is increased; transfer of HRP from late endosomes to lysosomes is inhibited (Mousavi et al, 2001)
Concanamy-cin A/B (Conc A/B)	J774 ConcB 10-25 nM LDL	inhibits lysosomal degradation of oxidized LDL; causes intracellular trapping of their receptors (Woo et al, 1992)

	Rat hepatocytes ConcA 100 nM, 15 min asialoorosomucoid albumin and HRP	slows down accumulation of receptor-mediated and fluid tracers; the number of cell-surface asialoglycoprotein receptors is reduced; recycling or regurgitation of both probes is increased; transfer of HRP from late endosomes to lysosomes is inhibited (Mousavi et al, 2001)
Megalomicin (MGM)	NRK MGM 50 µM, 3 h Fluid-phase tracers EGFR	does not affect fluid-phase accumulation; impairs its delivery to lysosomes and prevented its degradation; inhibits the delivery of EGFR to lysosomes (Bonay et al, 1997)
Monensin (Mon)	Mouse peritoneal macrophages Mon 5/10 µM, 30 min HRP	strongly decreases accumulation of HRP; strongly inhibits transfer of solutes from endosomes to lysosomes (Stenseth and Thyberg, 1989)
	Rat embryo fibroblasts Mon 1 µM, 6-24 h HRP	inhibits HRP accumulation (Wilcox et al, 1982)
	Human fibroblasts/rat liver parenchymal cells Mon 25 µM LDL/ asialoglycoprotein	interrupts the recycling of LDLR/asialoglycoprotein receptor by preventing the receptor from returning to the surface, thereby causing receptor accumulation within the cell (Basu et al, 1981; Berg et al, 1983)
	Rat foetal fibroblasts Mon 2 µM HRP C_6-NBD-GlcCer Tf	accelerates HRP regurgitation; does not affect the recycling of C_6-NBD-GlcCer; Tf recycling is slowed down (Cupers et al, 1997)
Chloroquine (CQ)	Mouse peritoneal macrophages CQ 50/100 µM, 30 min HRP	reduces accumulation of HRP; strongly inhibits transfer of solutes from endosomes to lysosomes (Stenseth and Thyberg, 1989)
	Rabbit alveolar macrophages CQ 0.3/1 mM, 60 min αmacroglobulin-trypsin complexes (αM) HRP	strongly decreases binding, and more strongly accumulation of αM; strongly decreases accumulation of HRP (Kaplan and Keogh, 1981)
	Mouse peritoneal macrophages CQ 30 µM, 30 min	increases phagosome/lysosome fusion (D'Arcy Hart and Young, 1978)

	PMNs CQ 15/150 µM, 90 min Latex beads phagocytosis	reduces phagocytosis of latex beads (Jones and Jayson, 1984)
	Rat peritoneal macrophages CQ 100-1000 µM, 60 min C3b-mediated bacterial phagocytosis	completely inhibits phagocytosis (Antoni et al, 1986)
Methylamine (MA)	Rabbit alveolar macrophages MA 50 mM αmacroglobulin-trypsin complexes (αM) HRP	decreases the surface receptor number for αM; delays αM internalization; decreases fluid-phase accumulation of HRP (Kaplan and Keogh, 1981)
	Human skin fibroblasts MA 10-30 mM α2macroglobulin (αM)	strongly inhibits the accumulation of αM; reduces the number of available receptors; interferes with the recycling of receptors (van Leuven et al, 1980)
Primaquine	HepG2 Primaquine 0.3 mM Tf	interferes with membrane recycling from endosomes to the plasma membrane (van Weert et al, 2000)
Procaine amide (PAm)	Mouse peritoneal macrophages PA 3.68 mM, 30 min Yeast invertase Sheep RBC	reduces pinocytosis of yeast invertase; decreases phagocytosis of sheep RBC (Bond et al, 1975)

Table 4. Agents perturbing endosome/lysosome acidification.

In conclusion, the main perturbations of endocytosis induced by agents impairing endosomal/lysosomal pH are: (i) an inhibition of fluid transfer to lysosomes; (ii) an acceleration of fluid regurgitation; (iii) no effect on membrane recycling; (iv) a decrease of receptor recycling; and (v) an inhibition of phagocytosis.

10.2. Agents inducing lysosomal overloading

Among the agents able to induce lysosomal overloading are: (i) those which perturb endocytosis by the lysosomal accumulation of the drug itself, such as sucrose (Montgomery et al, 1991); and (ii) those which perturb endocytosis probably by the lysosomal accumulation of other compounds, i.e. phospholipids. Phospholipidosis, which refers to an excessive accumulation of phospholipids in the tissues, can be induced by agents that may perturb the regulation of lipid synthesis, utilization and turn-over, resulting in phospholipidosis. Among these are cationic amphiphilic drugs, such as the anorexic chlorphentermine, the antimalarial chloroquine and the antibacterial gentamicin (Kodavanti and Mehendale, 1990). Gentamicin is an aminoglycoside antibiotic which accumulates in lysosomes of rat foetal fibroblasts (Tulkens and Trouet, 1978), and which induces a lysosomal phospholipidosis which has been related to its capacity to inhibit lysosomal PLA_1 *in vitro* (Aubert-Tulkens et al, 1979; Laurent et al, 1982).

Drug	**Experimental model:** **Cell** **Drug concentration** **Endocytic tracer (s)**	**Consequences for trafficking** **(references)**
Sucrose	J774 Sucrose 58 mM, 3 h LY, HRP IgG complexes	inhibits delivery of fluid-phase (LY, HRP) and receptor-bound ligands (IgG) in lysosomes (Montgomery et al, 1991)
Gentamicin	Rat kidney proximal tubules HRP	reduces accessibility of incoming vesicles to lysosomes overloaded with phospholipids (Giurgea-Marion et al, 1986)
Chloroquine	rat hepatocytes	lipid-storage lysosomes are reluctant to fuse (Lüllman-Rauch and Watermann, 1987)
Chlorphen-termine	rat hepatocytes	lipid-storage lysosomes are reluctant to fuse (Lüllman-Rauch and Watermann, 1987)

Table 5. Agents inducing lysosomal overloading.

In conclusion, the main perturbation of endocytosis caused by agents inducing lysosomal overloading is a decreased accessibility to overloaded lysosomes.

10.3. Agents interacting with membranes and/or perturbing membrane properties

Among the agents able to interact with membranes and/or perturb membrane properties are: (i) those which sequester or form complexes with cholesterol, such as methyl-β-cyclodextrin, filipin, nystatin and progesterone; (ii) cationic amphiphiles which interact with membranes and which are known to inhibit clathrin-dependent receptor-mediated endocytosis (Rikihisa et al, 1994; Orlandi and Fishman, 1998; Perry et al, 1999), such as chlorpromazine, dansylcadaverine and amantadine; and (iii) local anesthetics which perturb membrane fluidity, such as benzyl alcohol, procaine, dibucaine and tetracaine.

Cyclodextrins are cyclic oligomers of glucose that have the capacity to sequester lipophiles in their hydrophobic core. Methyl-β-cyclodextrin has been used to remove cholesterol from the plasma membrane (Rodal et al, 1999; Subtil et al, 1999). The polyene antibiotics filipin and nystatin were also used to sequester cholesterol. Filipin is a sterol binding agent that disrupts caveolae and caveolae-like structures. Progesterone is able to form complexes with cholesterol in aqueous solution.

Chlorpromazine, a cationic amphiphile belonging to the phenotiazines, is a membrane-stabilizing agent (Miller and Yin, 1979). Dansylcadaverine is also a primary amine, but it caused no effect on vesicle pH at 1mM (Maxfield, 1982). Amantadine is also a primary amine.

Benzyl alcohol is a neutral local anesthetic which increased membrane fluidity (Friedlander et al, 1987). Procaine, tetracaine and dibucaine are local anesthetics having each two amino functions, characterized by partition coefficients of 2.24, 3.65, 4.62 respectively, and which are therefore able to penetrate membranes (Hendrickson, 1976; Mohd Sidek et al, 1984; Hattori et al, 1997). Local anesthetics are able to permeabilize the plasma membrane due to the destabilization of the lipidic structure (Ohki et al, 1981; Shimooka et al, 1992). They induce cytoplasmic vacuolation, increase the intralysosomal pH and influence the membrane systems of the cell by depressing the phase-transition temperature in a model of cell membrane, thereby increasing membrane fluidity (Ueda et al, 1977; Finnin et al, 1969).

Drug	**Experimental model:** **Cell** **Drug concentration** **Endocytic tracer (s)**	**Consequences for trafficking** **(references)**
Methyl-β-cyclodextrin (CD)	CHO CD 10 mM, 30 min Tf	strong inhibition of TfR internalization, no effect on recycling; accumulation of flat-coated membranes and decrease in deep-coated pits; suggestion: clathrin is unable to induce curvature in membranes without cholesterol (Subtil et al, 1999)
	Hep-2 CD 0-15 mM Tf, EGF ricin	strong inhibition of Tf and EGF endocytosis; little effect on ricin endocytosis; strong inhibition of invagination of clathrin-coated pits (Rodal et al, 1999)
	HeLa CD 10 mM, 15 min GPI-linked diphteria toxin (artificial construction)	no effect on uptake of GPI-linked diphteria toxin; suggestion: uptake independent of both caveolae and CCVs (Skretting et al, 1999)
	neutrophils CD 10 mM, 15 min phagocytosis	inhibition of non-opsonic phagocytosis of *Mycobacterium kansasii*; no effect on phagocytosis of zymozan or serum-opsonized *M. kansasii*; suggestion: entry of non-opsonized bacteria at rafts (Peyron et al, 2000)
Filipin	neutrophils filipin 3 µM, 15 min phagocytosis	inhibition of non-opsonic phagocytosis of *Mycobacterium kansasii*; no effect on phagocytosis of zymozan or serum-opsonized *M. kansasii*; suggestion: entry of non-opsonized bacteria at rafts (Peyron et al, 2000)
	endothelial cells insulin, albumin áM	decreases number of invaginated caveolae; reduces transcytotic transport of insulin and albumin; no effect on clathrin pathways (áM) (Schnitzer et al, 1994)
	coated vesicles from rat liver filipin 300 µM	no effect on coated vesicle membranes; after clathrin removing from coated vesicles, a strong filipin response is obtained; suggestion: the stabilizing influence of the clathrin coat could explain the absence of filipin effect on coated vesicles (Steer et al, 1984)
	CaCo-2 filipin 1.5 µM, 1 h cholera toxin	strong inhibition of internalization (Orlandi and Fishman, 1998)

Nystatin	neutrophils nystatin 27 µM, 15 min phagocytosis	inhibition of non-opsonic phagocytosis of *Mycobacterium kansasii*; no effect on phagocytosis of zymozan or serum-opsonized *M. kansasii*; suggestion: entry of non-opsonized bacteria at rafts (Peyron et al, 2000)
	HeLa nystatin 27 µM, 30 min GPI-linked diphteria toxin (artificial construct)	no effect on uptake of GPI-linked toxin; suggestion: uptake independent of both caveolae and CCVs (Skretting et al, 1999)
Progesterone (PG)	Human fibroblasts PG 32 µM, 60 min	blocks cholesterol transport from endoplasmic reticulum (ER) to caveolae and caveolin accumulates in the lumen of the ER; suggestion: cholesterol transport is linked to caveolin movement, and PG inhibits potocytosis by reducing the cholesterol level of caveolae (Smart et al, 1996)
Chlorpromazine (CL)	human skin fibroblasts CL 40 µM, 4 h LDL	inhibits surface binding, internalization and catabolism of LDL (Miller and Yin, 1979)
	CaCo-2 CL 31 µM, 1 h Cholera toxin (CT)	does not affect CT internalization in contrast to its inhibitory effect on diphteria toxin cytotoxicity (Orlandi and Fishman, 1998)
Dansylcadaverine (DNSC)	Human skin fibroblasts DNSC 0.2-0.4 mM α2macroglobulin (αM)	strongly reduces the accumulation of αM; reduces the number of available receptors; interferes with the recycling of receptors (van Leuven et al, 1980)
	BALB 3T3 EGF	inhibits internalization of EGF; suggestion: the drug prevents EGF from clustering in clathrin-coated pits (Haigler et al, 1980)
	Mouse cells DNSC vesicular stomatitis virus (VSV), α2-macroglobulin (αM)	inhibits the uptake of both VSV and αM; suggestion: one cellular target for DNSC appears to be the clustering of membrane-bound ligands or particles in clathrin-coated pits (Schlegel et al, 1982)
	Macrophages DNSC *Ehrlichia risticii* phagocytosis	strongly inhibits *ehrlichia* internalization into macrophages; suggestion: its internalization depends on the functions of clathrin (Rikihisa et al, 1994)

Amantadine (Am)	Mouse cells vesicular stomatitis virus (VSV), α2-macroglobulin (αM)	inhibits the uptake of both VSV and αM; suggestion: one cellular target for Am appears to be the clustering of membrane-bound ligands or particles in clathrin-coated pits (Schlegel et al, 1982)
	Alveolar macrophages liposomes containing Am 660 mM, 1 h zymozan coated liposomes	strongly inhibits phagocytosis; suggestion: clathrin-mediated receptor/membrane recycling is required for phagocytosis (Perry et al, 1999)
Benzyl Alcohol (BA)	MDCK BA 30 mM, 30 min LY	strong inhibition of LY accumulation (Giocondi et al, 1995)
Procaine	rat liver cells procaine 1/5 mM asialoglycoproteins	reduces the surface binding of asialoglycoproteins decreases the rate of internalization of surface-bound ligands (Tolleshaug et al, 1982)
Tetracaine	rat liver cells tetracaine 1 mM asialoglycoproteins	decreases the surface binding of asialoglycoproteins reduces the rate of internalization of surface-bound ligands (Tolleshaug et al, 1982)
Dibucaine	rat liver cells dibucaine 0.5 mM asialoglycoproteins	strongly decreases the rate of uptake of asialoglycoproteins, acting by a decrease of the binding capacity and of the rate of internalization of surface-bound ligands (Tolleshaug et al, 1982)

Table 6. Agents interacting with membranes and/or perturbing membrane properties.

In conclusion, agents sequestring cholesterol perturb membrane curvature of clathrin-coated pits and caveolae. The cationic amphiphiles chlorpromazine, dansylcadaverine, amantadine and local anesthetics inhibit clathrin-mediated coated pit formation and internalization of ligand-receptor complexes. The neutral anesthetic benzyl alcohol reduces fluid internalization.

10.4. Enzyme modulators

Among the enzyme modulators are: (i) the fungal metabolite wortmannin and LY294002, two structurally-distinct, cell-permeable inhibitors of PI 3-kinase; bromoenol lactone, a membrane-permeable PLA_2 inhibitor (Mayorga et al, 1993); primary alcohols, inhibitors of PLD; neomycin which inhibits endogenous PLD by binding to its cofactor PI 4,5-P_2; and okadaic acid, an inhibitor

of protein phosphatases; (ii) AlF_4^- which interacts with GDP in the nucleotide binding site of heterotrimeric G proteins mimicking the γ-phosphate and converting the G protein to its active GTP-bound form; and mastoparan, a cationic amphiphilic α-helical peptide interacting with the α-subunits of heterotrimeric G proteins to activate them by mimicking their interaction with G protein-coupled receptors (Gil et al, 1991; Carter et al, 1993); and (iii) the fungal metabolite brefeldin A, a macrocyclic lactone, which is known to induce the redistribution of at least three coat components from membranes to the cytosol, β-COP, ARF and γ-adaptin. It is an inhibitor of ARF1 guanine nucleotide exchange. Binding of these coat proteins is directly or indirectly regulated by guanine nucleotides, since pretreatment of cells with AlF_4^- inhibits the action of brefeldin A (Hunziker et al, 1992).

Drug	**Experimental model:** **Cell** **Drug concentration** **Endocytic tracer (s)**	**Consequences for trafficking** **(references)**
Wortmannin (WT)/ LY294002	early endosome fusion *in vitro* WT 1-100 nM LY 1-100 µM	homotypic fusion between early endosomes is blocked; suggestion: PI 3-kinase is required for early endosome fusion (Jones and Clague, 1995)
	BHK WT 25 nM HRP	reduces the initial rate of tracer internalization; increases efficiency of recycling (Clague et al, 1995)
	oncogene-transformed fibroblasts WT 100 nM LY 20 µM	abolishes constitutive macropinocytosis in oncogene-transformed fibroblasts (Amyere et al, 2000)
Bromoenol lactone (BEL)	early endosome fusion *in vitro* BEL 100-500 µM	blocks endosome fusion; suggestion: vesicle fusion along the endocytic pathway requires a PLA_2 activity, in part mediated by the release of arachidonic acid (Mayorga et al, 1993)
Primary alcohols	*in vitro* model system AP-2 coat/lysosomes	prevents the synthesis of PI 4,5-P_2 and blocks coat assembly (Arneson et al, 1999)
	early endosome fusion butan-1-ol 100 mM	partially blocked homotypic fusion between early endosomes; suggestion: a role for PLD in endosome fusion (Jones and Clague, 1997)

Neomycin	*in vitro* assay neomycin 0.1/1 mM	prevents recruitment of AP-2 not only onto endosomes but also onto the plasma membrane; suggestion: both events are mediated by PLD (West et al, 1997)
	cell-free assay neomycin 0.1-0.5 mM	inhibits the rate and extent of endosome fusion (Jones and Wessling-Resnick, 1998)
Okadaic acid	cell-free fusion of endocytic vesicles okadaic acid 1 µM	blocks vesicle fusion (Woodman et al, 1992)
AlF_4^-	A431 AlF_4^- 5-50 µM	coated pit invagination is resistant to AlF_4^-; late events involved in coated vesicle budding are inhibited by this antagonist; so, G proteins are involved in receptor-mediated endocytosis via clathrin-coated pits (Carter et al, 1993)
	phagosomes of J774 AlF_4^-	increases β-COP association to phagosomal membranes, suggestion: β-COP binding to phagosomal membrane involves heterotrimeric G protein (Beron et al, 2001)
Mastoparan	A431 mastoparan 5-70 µM	coated pit invagination is resistant to mastoparan; late events involved in coated vesicle budding are inhibited by this antagonist; so, G proteins are involved in receptor-mediated endocytosis via clathrin-coated pits (Carter et al, 1993)
Brefeldin A (BFA)	polarized MDCK dIgA	increases the tubular appearance of the early endosomal compartment; the morphology of late endosomes is not altered; lysosomes acquire a more tubular appearance; do not alter internalization to and recycling from endosomes; nor the delivery of ligands from early endosomes to degradative endocytic compartments; selectively and completely blocks the transcytosis of dIgA mediated by the pIgR (Hunziker et al, 1992)
	polarized MDCK BFA, 0.7-70 µM, 20 min ricin, HRP	increases apical endocytosis of both ricin and HRP; basolateral endocytosis is unaffected; transcytosis in the basolateral to apical direction is increased for both HRP and ricin (Prydz et al, 1992)

	phagosomes of J774 BFA 200 µM	binding of β-COP to phagosomal membranes is inhibited; suggestion: COP proteins are recruited to phagosomal membranes by a mechanism that involves a brefeldin A-sensitive ARF (Beron et al, 2001)

Table 7. Enzyme modulators.

10.5. Agents perturbing microtubules and microfilaments

Nocodazole, colchicine and taxol are agents acting on microtubules. Colchicine depolymerizes microtubules and inhibits tubulin polymerization; nocodazole is a microtubule inhibitor; taxol stabilizes microtubules.

Cytochalasins bind to actin filaments inhibiting both the association and dissociation of subunits. Jasplakinolide is an actin assembly-promoting drug (Shurety et al, 1998).

Phorbol myristate acetate (PMA) stimulates microtubule and microfilament extension.

Drug	**Experimental model:** **Cell** **Drug concentration** **Endocytic tracer (s)**	**Consequences for trafficking** **(references)**
Nocodazole (Noc)	NRK noc 10 µM, 5 h	translocation of endosomes and lysosomes depends on microtubules and is ceased when microtubules are depolymerized by nocodazole (Matteoni and Kreis, 1987)
	BHK-21 noc 10 µM, 2 h 30 HRP	endocytosis proceeds as in controls; internalized HRP remained in the spherical vesicles and does not appear in late endosomes/lysosomes; suggestion: transport between the early and late endosomes requires intact microtubules (Gruenberg et al, 1989)
	CV-1 noc 3.3 µM, 60 min HRP Tf	does not inhibit fluid-phase endocytosis; reduces initial uptake (5 min) of tf (Hamm-Alvarez et al, 1996)

	K562 noc 10 µM, 2 h TfR	increases the level of surface tfR, which is due to a change in receptor dynamics; TfR endocytosis is slowed; no effect on recycling; little effect on tfR transport from the cell surface to the Golgi complex (Jin and Snider, 1993)
	polarized MDCK noc 33 µM, 2 h pIgR	the biosynthetic and transcytotic pathways to the apical surface are dramatically affected; the apical recycling pathway is slowed; the biosynthetic and recycling pathways to the basolateral surface are less affected (Breitfeld et al, 1990)
Colchicine (Colch)	rat peritoneal macrophages HRP	reduces HRP uptake (Piasek and Thyberg, 1980)
	Vero cells, A431 colch 10 mM, 15 min ricin sucrose, LY Tf	selectively inhibits endocytic uptake from non-clathrin-coated areas of the cell membrane (ricin, sucrose and LY) (Sandvig and van Deurs, 1990)
	human skin fibroblasts colch 5 µM, 3 h LDL	reduces surface binding, internalization and catabolism of LDL (Miller and Yin, 1979)
Taxol	CV-1 taxol 1 µM, 60 min HRP Tf	does not inhibit fluid-phase uptake; reduces the initial uptake (5 min) of tf (Hamm-Alvarez et al, 1996)
	CV-1 taxol 4 µM, 2 h 30 EGF	disruption at the level of the early accumulation of protein and endocytic tracers in endosomes and of the later accumulation in a dense compartment (Sonee et al, 1998)
Cytochala-sins (cyt)	NRK cyt 1 µM, 1 h	endosome and lysosome movement remains unaffected when cells are treated with cytochlasin D; suggestion: it is independent of microfilament network (Matteoni and Kreis, 1987)
	Vero, A431 cyt 20 µM, 15 min ricin sucrose, LY Tf	selectively inhibits endocytic uptake from non-clathrin-coated areas of the cell membrane (ricin, LY and sucrose) (Sandvig and van Deurs, 1990)

Jasplakino-lide (JAS)	polarized MDCK JAS 1 µM, 45 min FITC-dextran, HRP	increases uptake of fluid-phase tracers in a polarized manner (at the basolateral pole); suggestion: a role for filamentous actin in the endocytosis of fluid-phase tracers from the basolateral membrane (Shurety et al, 1998)
Phorbol myristate acetate (PMA)	mouse peritoneal macrophages PMA, 16 nM, 24 h HRP	stimulates pinocytosis and membrane spreading (Phaire-Washington et al, 1980b)
	mouse peritoneal macrophages PMA, 16 nM	stimulates lysosome redistribution; suggestion: cytoskeletal elements regulate the movement and distribution of lysosomes in the macrophage cytoplasm (Phaire-Washington et al, 1980a)
	resident peritoneal macrophages, J774 PMA, 1.6 -1600 nM	stimulates both the rate and extent of fusion of phagosomes with preexisting secondary lysosomes (Kielian and Cohn, 1981)

Table 8. Agents perturbing microtubules and microfilaments.

10.6. Conclusion

In conclusion, we have illustrated in this section that dissection of pinocytosis and phagocytosis have greatly benefited from a variety of drugs. However, almost all of them are unspecific and may affect more than one pathway and/or step of endocytosis and phagocytosis. In addition, drugs selectively inhibiting the earliest step of pinocytosis, in particular those supporting clathrin-independent pinocytosis, have long been lacking (see Dautry-Varsat, 2001).

Interestingly, mutations affecting endocytic proteins show pleiotropic effects and none of these has been found so far to exclusively inhibit the formation of primary endocytic pits supporting fluid-phase endocytosis in mammalian cells. For example, dominant-negative dynamin efficiently blocks formation of clathrin-coated vesicles in mammalian cells, but only transiently affects fluid-phase endocytosis (Damke et al, 1994, 1995). In addition, dynamin was shown to have several other effects, including on phagocytosis (Gold et al, 1999), on the recycling route (van Dam and Stoorvogel, 2001) and at distal stages of the degradation pathway (e.g. Nicoziani et al, 2000). Rab5 dominant-negative mutation slows down fluid-phase and receptor-mediated endocytosis to a comparable extent and causes major endosomal alterations,

indicating that its effect on the formation of primary endocytic vesicles could be indirect (Bucci et al, 1992). So, mutations, like pharmacological agents, show pleiotropic effects.

11. Azithromycin

In this section, we first present pharmacological properties of macrolides, azalides and ketolides in general. In the second part, we will focus on azithromycin, a macrolide antibiotic and the first azalide synthesized.

11.1. Macrolides, azalides and ketolides: pharmacology and clinical applications

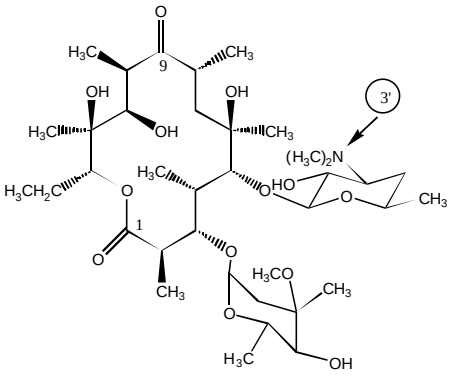
11.1.1. Structure and activity

Macrolides are naturally derived antimicrobial substances that have been available for clinical use for more than fifty years. The word macrolide breaks down into macro (large) and olide (lactone). Macrolides are defined as lipophilic molecules with a characteristic central lactone ring bearing 12 to 16 atoms, few if any double bounds, and no nitrogen atoms. Several amino and/or neutral sugars, namely desosamine and cladinose, are fixed to the lactone ring. Azalides are not included in this definition because they have an endogenous nitrogen in the aglycone ring. Ketolides are erythromycin derivatives characterized by a 3-keto function in place of the cladinose moeity.

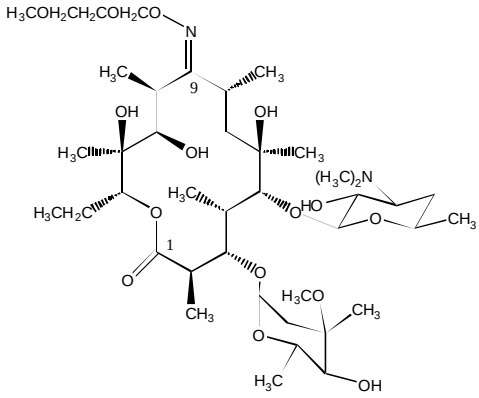
Erythromycin was the leading compound of the macrolide class (see Figure 15). However, this drug is instable at low pH, and the degradation products are inactive against bacteria. Several pharmaceutical and physicochemical modifications (salts and esters) have been proposed to avoid this obstacle, but none was satisfactory.

Therefore, several research groups have synthesized series of new derivatives of erythromycin. The objective was to retain antibacterial activity while improving pharmacokinetics. Various chemical modifications have been proposed, and erythromycylamine (and dirithromycin, its prodrug), roxithromycin, flurithromycin and clarithromycin were produced (see Figure 15).

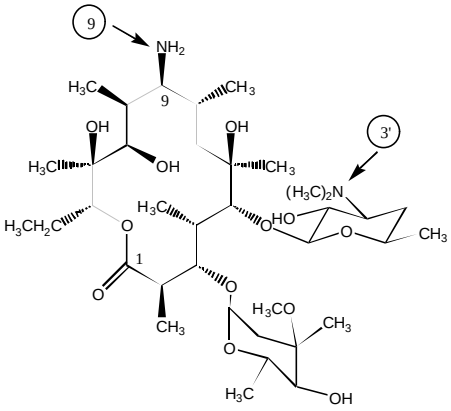
Moreover, a series of 9a-aza-9a-homoerythromycin A derivatives has been synthesized. These compounds contain an unusual expanded aglycone with an endocytic nitrogen, namely azalide (Djokic et al, 1987; Bright et al, 1988). Azithromycin (9-deoxo-9a-aza-9a-methyl-9a-homoerythromycin A), a 15-membered macrolide antibiotic and the first azalide antibiotic, has a methyl-substituted nitrogen in the 9a position in the aglycone ring, markedly improving its acid stability relative to erythromycin A (see Figure 15; see below). 16-membered macrolide antibiotics are an important class of antibacterial agents. They include natural compounds (josamycin, spiramycin, midecamycin) and semi-synthetic compounds (rokitamycin, miokamycin) (for a review, see Bryskier et al, 1993).



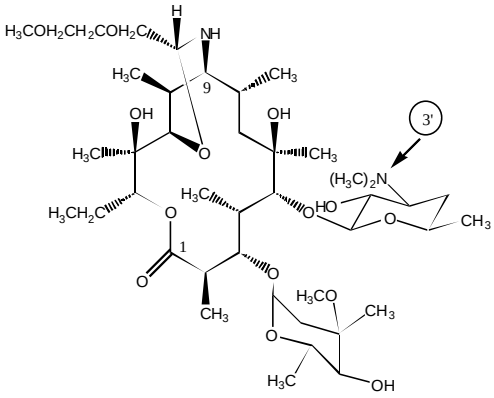
erythromycin



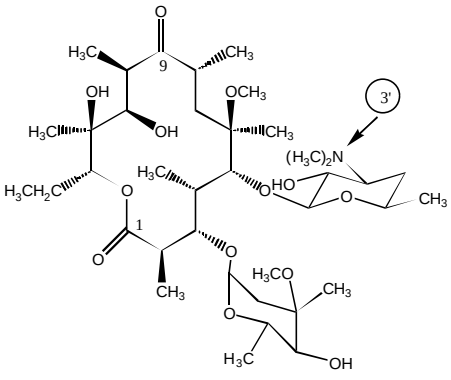
roxithromycin



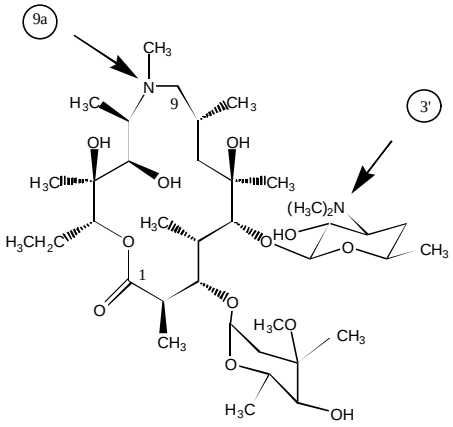
erythromycylamine



dirithromycin



clarithromycin



azithromycin

Figure 15. Chemical structures of some 14-membered macrolide antibiotics and of an azalide, azithromycin.

Emergence of bacterial resistance to macrolide antibiotics, particularly Gram-positive bacteria prompted the search for new macrolide compounds. Recent identified ketolides that remain active against macrolide-resistant strains. Among interesting ketolides reported are HMR 3004 and HMR 3647. HMR 3004 (formerly RU 64004) is more lipophilic than erythromycin A and possesses two substituents with pKa of 5.3 (quinoline ring) and 8.6 (desosamine). HMR 3647 has 3 pKa, 3.0 (pyridinium ring), 5.1 (imidazolium ring), and 8.7 (desosamine). Both ketolides are able to accumulate in human PMNs, showing, after 3 h, cellular to extracellular concentration ratios of 460 and 350 respectively (Vazifeh et al, 1997; 1998).

11.1.2. *In vitro* activity and therapeutic use

Erythromycin demonstrated broad-spectrum antimicrobial activity and was used primarily for respiratory, skin and soft tissue infections. Macrolides exhibit good activity against Gram-positive aerobes and some Gram-negative aerobes. Although the mechanism of action and susceptibility to resistance of azithromycin are similar to those of the macrolide antibiotics, azithromycin's extended spectrum of activity includes Gram-positive and Gram-negative organisms as well as atypical pathogens. Azithromycin possesses better activity than erythromycin against *Haemophilus influenzae, anaerobes, Legionella, Chlamydia* and *Pasteurella multodica* (for a review, see Chu, 1999; Zhanel et al, 2001). Macrolides are indicated mainly for the treatment of upper and lower respiratory tract. In general, they are the preferred treatment for community-acquired pneumonia and alternative treatment for other respiratory infections (Zhanel et al, 2001). They are also used for the treatment of skin infections and sexually transmitted diseases caused by *Chlamydia*. An interesting feature of azithromycin is its use for the treatment of disseminated *Mycobacterium avium* Complex in AIDS patients (Koletar et al, 1999). Ketolides have activity against macrolide-resistant bacteria (for a review, see Chu, 1999).

11.1.3. Mechanism of action and resistance

Macrolides reversibly bind to the 23S ribosomal RNA of the bacterial ribosome 50S subunit, thus inhibiting protein synthesis by blocking elongation. The ketolides have also been reported to bind to the 23S ribosomal RNA and their mechanism of action is similar to that of macrolides (Zhanel et al, 2001).

Intrinsic resistance of Gram-negative bacilli to macrolides is probably due to relative impermeability of the cellular outer membrane to these hydrophobic compounds. However, azithromycin expands the traditional spectrum of macrolide activity: this drug contains a nitrogen inserted in the lactone ring, which contributes to improve activity against Gram-negative bacteria.

Acquired resistance to macrolide-lincosamide-streptogramine (MLS) antibiotics involves three mechanisms: modification of the target of the drugs, inactivation, and active efflux of the antibiotics (for a review, see Leclercq and Courvalin, 1993).

11.1.4. Pharmacokinetic properties

Erythromycin is characterized by poor water solubility and rapid inactivation by stomach acid, resulting in widely varying bioavailability after oral administration (for a review, see Lode et al, 1993).

Minor structural modifications of erythromycin resulted in improved pharmacokinetic properties of the newer macrolides, such as better bioavailability, increased gastro-intestinal tolerance, higher peak serum levels, longer serum half-life, and high tissue concentrations (for a review, see Lode et al, 1993). As an important characteristic, all macrolides display large volumes of distribution with excellent uptake into respiratory tissues and fluid relative to serum.

11.1.5. Toxicity and drug interactions

Macrolides are well-tolerated agents. They are divided into 3 groups for likely occurrence of drug-drug interactions: group 1 (erythromycin) is frequently involved; group 2 (clarithromycin, roxithromycin) is less commonly involved; group 3 no drug interaction has been described (azithromycin, dirithromycin) (Zhanel et al, 2001).

In conclusion, one of the more clinically important aspects of the new macrolides and azalides is the difference in pharmacology and toxicity from the earlier compounds. The new azalide/macrolide compounds are considerably better tolerated following oral administration than erythromycin and are highly tissue-active.

11.2. Azithromycin, an antibiotic with unique pharmacokinetic properties

Azithromycin is the first of the 15-membered ring azalide class of antibimicrobials. Although its mechanism of action and susceptibility to resistance are similar to those of the macrolide antibiotics, its spectrum of activity is extended (Ballow and Amsden, 1992).

However, the most important progress is the exceptionnal pharmacokinetic properties of azithromycin in comparison to erythromycin. First, azithromycin is stable at gastric pH and has an absolute bioavailability of appox. 37 % following oral administration of a single dose of 500 mg. Second, although its serum concentrations are typically low (the oral dose resulted in a mean peak concentration of about 0.4 mg/l), the drug concentrates to a high degree in tissues. Extravascular concentrations of azithromycin range between 1 and 9 mg/kg in most tissue samples obtained between 12 h and 3 days after a dose of 500 mg. Although lower in samples of fat, muscle, bone, and gastric mucosa, concentrations in all tissues are higher than those of concomitantly obtained serum samples (Foulds et al, 1990; Ballow and Amsden, 1992). Third, the high tissue levels persist for days (Foulds et al, 1990; for a review, see Lode et al, 1993).

Based on these characteristics, several clinical trials have proven that, for the treatment of upper and lower respiratory tract and skin infections, a 5-day course of azithromycin administrated once-a-day is equally efficacious to a 7-to 14-day course of other commonly used oral antimicrobials, administrated two-to-four times-a-day (Ballow and Amsden, 1992).

11.3. Contribution of cell culture model for the study of cellular pharmacokinetic properties of azithromycin

Azithromycin shows an exceptional accumulation both in phagocytic and in non-phagocytic cells. Indeed, the drug is able to concentrate in human and mouse polymorphonuclear leukocytes, murine peritoneal macrophages, mouse and rat alveolar macrophages, and J774 mouse macrophages (Gladue et al, 1989; Carlier et al, 1994; Laufen et al, 1990). It can also concentrate in human fibroblasts, rat embryo fibroblasts and NRK cells (Gladue and Snider, 1990; Carlier et al, 1994).

Studies on the accumulation of azithromycin in J774 mouse macrophages and rat embryo fibroblasts indicate that: (i) saturation of accumulation is observed only at high azithromycin concentrations; (ii) an acidic extracellular pH markedly reduces the accumulation of azithromycin; (iii) the intracellular accumulation of azithromycin is not markedly influenced by the presence of erythromycin or roxithromycin in the incubation medium (Carlier et al, 1994). Gladue and Snider showed that the characteristics of azithromycin uptake by non-phagocytic cells was also similar to those obtained with phagocytic cells (Gladue et al, 1989; Gladue and Snider, 1990). Subcellular fractionnation techniques allowed to evidence that most of the cell-associated drug distributes in the lysosomal compartment, the remaining part recovered in high speed supernatant (Carlier et al, 1994).

From these observations, i.e. the lack of competition between azithromycin and other macrolides, the lack of saturation at low concentration, the effect of pH, and the subcellular distribution of azithromycin, non-specific transport was suggested for the uptake of azithromycin by J774 macrophages and rat embryo fibroblasts (Carlier et al, 1994). This process is also proposed for the uptake of azithromycin by other cell types (Gladue et al, 1989; Laufen et al, 1990). So, the drug probably accumulates in cells by a non-specific process and is subsequently trapped in lysosomes, following the model of diffusion/segregation described by de Duve in 1974 (Gladue and Snider, 1990; Carlier et al, 1994).

Release of azithromycin from J774 macrophages preloaded for 2 h with a concentration of 10 mg/l is slow, showing a 40 % release after 4 h. From rat embryo fibroblasts preloaded for 24 h with the same concentration, 25 % is released after 3h and 75 % after 48 h (Carlier et al, 1994). Azithromycin leaks out slowly and more slowly from cells than other macrolides, suggesting that it remains more tightly associated with cellular components than other macrolides (Carlier et al, 1994).

11.4. Relation between high intracellular accumulation of azithromycin and activity against intracellular bacteria

As shown in section 11.3, macrolides are able to accumulate in lysosomes of cultured cells. Like most weak organic bases, they are susceptible to become concentrated in acidic membrane-bounded compartments in direct correlation with: (i) the pH gradient across the membrane; (ii) the

ratio permeability coefficients of the unprotonated and the protonated forms; (iii) the number of ionizable groups carried by the molecule. Azithromycin and erythromycin have log P of 4.02 and 3.06 respectively. Azithromycin has two tertiary amines (in position 9a and 3'), with a pKa value of 8.8 for the desosamine nitrogen and a pKa value of 8.1 for the azalide nitrogen, whereas erythromycin has only one amine with a pKa of 8.8. This explains why azithromycin achieves intracellular concentrations largely in excess of erythromycin and other monocationic macrolides.

Azithromycin has shown activity against a number of intracellular bacteria, such as *Staphylococcus aureus, Mycobacterium avium Complex, Chlamydia, Legionella pneumophila* and *Listeria monocytogenes*, and it has been postulated that this results from the high intracellular antibiotic concentrations reached within phagocytes and other infected cells (McDonald and Pruul, 1991). This is undoubtedly important when comparing azithromycin (or other macrolides) to other antibiotics with low or no accumulation in cells (â-lactams, aminoglycosides [at least in short term experiments]). Yet, it is also clear that the intracellular activity of azithromycin (and other macrolides) remains suboptimal. As underlined by Scorneaux et al (1996), Ouadrhiri et al (1999) and Carryn et al (submitted for publication), the activity of azithromycin in cells is probably impaired: (i) by its preferential localization in lysosomes; (ii) by the fact that part of the cell-associated drug may be bound to phospholipids; (iii) by the defeating activity exerted by the acid pH on antibiotic intrinsec activity. Moreover, time- and concentration-kill studies against both extracellular and intracellular bacteria show that azithromycin is mainly a bacteriostatic agent, with little or no activity above a concentration treshold corresponding, in broth, to its MIC. Thus, the largest accumulation of azithromycin may, *in fine*, not yield the exceptionally large activity that one may anticipate. Yet, its prolonged retention confers long-lasting protection, explaining its specific conditions of clinical use (short term [3 - 5 days] administration).

It has been suggested that the high intracellular concentration of azithromycin could be responsible for impairing the intracellular killing systems. However, this hypothesis was rejected since the high antibiotic levels found intracellularly did not appear to disrupt normal phagocytic functions: (i) there are no grounds to suggest that exposure to azithromycin alters phagocytic uptake; (ii) no studies have indicated any alteration in neutrophils chemotaxis; (iii) the *in-vitro* bactericidal activity of mouse macrophages was not altered by pre-exposure to relatively low concentrations (10 mg/l) of azithromycin, and the intracellular survival or killing of *Staphylococcus aureus* was not affected by pre-treating macrophages with azithromycin (McDonald and Pruul, 1991). Rather, it has more recently been shown that macrolide antibiotics,

used at concentrations below 2.5 mg/l, stimulate macrophage functions: (i) macrolides, except azithromycin, stimulate the growth of J774 macrophages; (ii) pretreatment with macrolides, except roxithromycin, slightly stimulates the macrophage phagocytosis of latex beads, and strongly stimulates macrophage chemotaxis to lipopolysaccharide (LPS), and macrophage cytocidal activity against *Candida albicans* (Xu et al, 1996). 10 mg/l azithromycin has been shown to induce *in vitro* a time-dependent increase in the intracellular killing of *Staphylococcus aureus* by human polymorphonuclear leucocytes without damaging phagocytes and phagocytic activity (Silvestri et al, 1995).

11.5. Contribution of cell culture model and liposomes for the study of cellular toxicological properties of azithromycin

Based on the observations that azithromycin is able to strongly accumulate in lysosomes of cultured cells, the drug could be involved in perturbations of lysosomal properties. We expose in this section, some of the toxicological properties of azithromycin.

In fibroblasts, treatment with 10 mg/l azithromycin for 2 h induces a decrease of the buoyant density of the lysosomes. This phenomenon was not observed in macrophages (Carlier et al, 1994).

In fibroblasts incubated with azithromycin, a lysosomal phospholipidosis was induced, as evidenced by biochemical and morphological analyses. After 24 h with 10 mg/l azithromycin, lysosomes were more abundant, looked enlarged and were partially filled by a mixture of concentric, osmiophilic inclusions which were of lamellar structures. This indicates that azithromycin is able to cause a phospholipidosis. Moreover, using dialysis experiments, it has been shown that azithromycin is able to interact with negatively charged liposomes at pH 5.4. Biochemical analyses have confirmed the presence of phospholipids and have shown that the drug inhibits the activity of lysosomal PLA_1 towards PC inserted in negatively charged liposomes. It has been suggested that binding of azithromycin to membrane bilayers and neutralization of their surface negative charges may provide the molecular mechanism for the inhibition of PLA_1 activity towards PC (Van Bambeke et al, 1996). A large part of the phospholipids accumulated in lysosomes of rat foetal fibroblasts has an intracellular origin. These phospholipids are normally removed by autophagy and accumulate in lysosomes because of the inhibition of PLA_1 activity.

The molecular interactions between azithromycin and acidic phospholipids were examined by biophysical and computer-aided conformational studies. ^{31}P nuclear magnetic resonance has allowed to show that azithromycin decreases the mobility of the phospholipids in negatively charged liposomes at pH 5.4. Moreover, computer-aided conformational analysis of mixed monolayers of azithromycin and PtdIns showed that the drug can be positioned largely in the hydrophobic domain, but close to the interface, with the macrocycle facing the C_1 of the fatty acids, the cladinose located on the hydrophobic side of the lipid / water interface, and the desosamine projected into the hydrophobic domain. The position of the N_{9a} endocyclic tertiary amine is such that it could interact with the phospho-groups, especially if the molecule is protonated (see Figure 16). Interestingly, both the unprotonated and the protonated forms of azithromycin could be surrounded by up to 6 molecules of PtdIns, and both forms adopt very similar conformations but also almost identical positions with respect to the interface (Montenez et al, 1996; see Figure 16). So, azithromycin is able to bind and to interact with negatively charged phospholipid bilayers at pH 5.4, by ionic as well as by hydrophobic interactions.

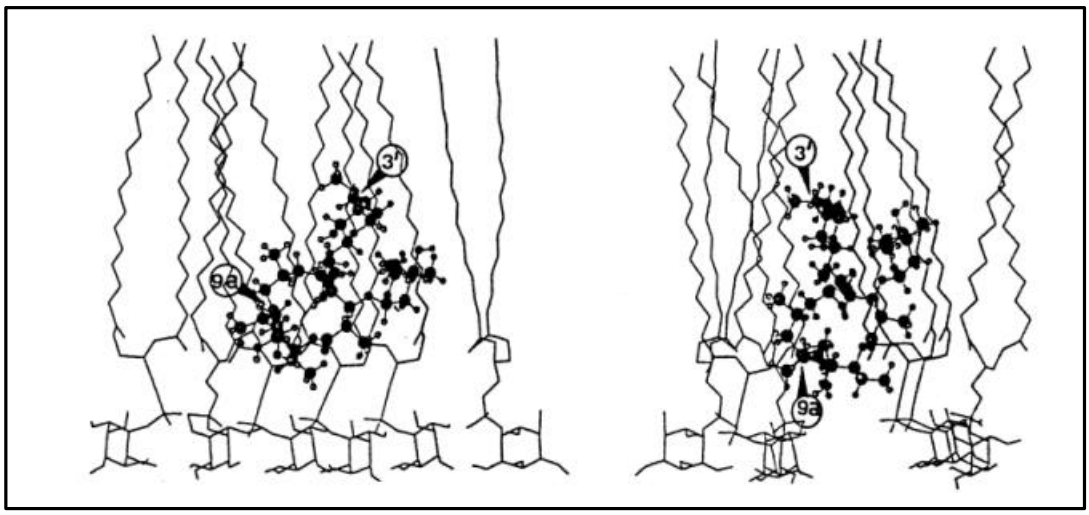


Figure 16. Views of the mode of assembly of the unprotonated (left) and protonated (right) forms of azithromycin (ball representation) and PtdIns (skeleton representation). The arrowheads point to the two amino functions of azithromycin. (From Montenez et al, 1996).

In spite of this binding, azithromycin does not induce membrane aggregation or fusion of negatively-charged liposomes, under conditions in which gentamicin causes a massive aggregation, and bis(â-diethylaminoethylether)hexestrol (a dicationic amphiphile) causes fusion. This was shown by the measurement of the dequenching rate of a lipid-soluble fluorescent probe, octadecylrhodamine B, incorporated at self-quenched concentration in membranes (Van Bambeke et al, 1996).

CHAPTER 2. AIMS OF THE STUDY, STRATEGIES AND MAJOR OBSERVATIONS

In the introduction, it is recalled that the driven endocytic pathways have been established, molecular details understood for most of these routes, and still a multitude of questions to ask and details to establish (for a review, see Marsh, 2001). One way to better understand these multiple pathways is the use of agents which inhibit selective endocytic processes. As shown in section 10, many agents indeed exist and have been used to characterize and dissect cellular and molecular mechanisms of endocytic and phagocytic pathways. However, a few are really specific of one pathway and/or one step.

In view of the lack of specific inhibitors, in particular of clathrin-independent endocytosis, we were interested by the macrolide antibiotic azithromycin, based on the facts that this drug: (i) has a marked amphiphilic character and extensively accumulates in lysosomes of cultured fibroblasts (Carlier et al, 1994; Montenez et al, 1996); (ii) induces a lysosomal phospholipidosis by inhibition of the lysosomal PLA_1 activity (Van Bambeke et al, 1996); (iii) is able to bind to negatively charged bilayers at acidic pH (Montenez et al, 1996); and (iv) is able to perturb the fusion of phospholipid-overloaded lysosomes with HRP-containing endosomes (preliminary observation in electron microscopy; Van Bambeke et al, 1996).

The first aim of the present study was to examine whether azithromycin could affect earlier steps of the endocytic apparatus, i.e. before lysosomes which are the final step of the endocytic pathway. For this purpose, we have used rat foetal fibroblasts because: (i) these cells avidly accumulate azithromycin (Carlier et al, 1994); (ii) they allow lysosomal phospholipidosis to develop (Van Bambeke et al, 1996; Montenez et al, 1996); (iii) they have been extensively used to study many aspects of endocytosis in our laboratories (Draye et al, 1988; Cupers et al, 1994; 1997). The study was focused on fluid-phase pinocytosis of HRP. We have found that azithromycin strongly impaired the uptake of HRP by rat foetal fibroblasts and we suggested that azithromycin impaired the formation of pinocytic vesicles and/or endosomes (Tyteca et al, 2001).

Based on these preliminar but encouraging results, the second aim of the study was to examine whether inhibition of fluid-phase pinocytosis did impinge on other endocytic pathways or not. In other words, we wanted to know if azithromycin could be a selective agent, or if it could

perturb many pathways and steps of endocytosis. To this aim, we selected J774 mouse macrophage cell line because: (i) these cells are less heterogeneous than primary cultures of rat foetal fibroblasts; (ii) macrophages are highly endocytic cells capable of interiorizing a broad spectrum of both soluble and particulate molecules; (iii) macrophages are cells in which kinetics of endocytosis is faster than in fibroblasts, possibly sensitising drug effects; and (iv) this cell line has been extensively used by others to study pinocytosis and phagocytosis (Mellman and Plutner, 1984; Swanson et al, 1987; Pitt et al, 1992). We systematically evaluated the effect of azithromycin on: (i) fluid-phase endocytosis using two tracers of different sizes, HRP and LY; (ii) bulk-membrane endocytosis, based on the relief of fluorescence self-quenching of *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (*N*-Rh-PE); (iii) receptor-mediated endocytosis, either by a constitutive or a ligand-induced process, using Tf and immune complexes respectively; (iv) phagocytosis, by using latex beads of two different sizes. We found that azithromycin selectively inhibited fluid-phase and bulk-membrane endocytosis without affecting phagocytosis. Moreover, we showed that the drug delayed sequestration of ligand/receptor complexes into endocytic pits and recycling vesicles (Tyteca et al, submitted).

The third aim of the study was to understand the mechanism by which azithromycin selectively impaired fluid-phase endocytosis but not phagocytosis, and delayed sequestration of ligand/receptor complexes into endocytic pits and recycling vesicles. To this aim, we used both J774 mouse macrophages and acellular membranes (liposomes and Langmuir-Blodgett monolayers). We evaluated: (i) the capacity of the drug to interact with membranes at pH corresponding to those found in endosomes and at the plasma membrane; (ii) the effect of azithromycin on the binding of three membrane probes to the plasma membrane; (iii) the capacity of azithromycin to perturb membrane fluidity and membrane organization in domains. We found that azithromycin is able to bind to and interact with negatively-charged phospholipid bilayers at pH 6.0 and 7.0, close to those of endosomes and plasma membrane. We also observed that azithromycin is able to destabilize artificial membranes, and to decrease the incorporation in the plasma membrane of three membrane tracers, C_6-NBD-SM, TMA-DPH, and *N*-Rh-PE. Finally, we found a decrease of plasma membrane fluidity induced by azithromycin, as measured by polarization fluorescence techniques, and evidenced by the absence of concanavalin A-induced patching of surface glycoproteins (Tyteca et al, in preparation).

CHAPTER 3. RESULTS

1. Paper #1 :

Azithromycin, a lysosomotropic antibiotic, impairs fluid-phase pinocytosis in cultured fibroblasts.

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Running title: Impairment of Fluid-phase Pinocytosis by Azithromycin.

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Abstract

Key words: pinocytosis, fluid-phase, horseradish peroxidase, azithromycin, macrolides.

The dicationic macrolide antibiotic azithromycin inhibits the uptake of horseradish peroxidase (HRP) by fluid-phase pinocytosis in fibroblasts in a time- and concentration-dependent fashion without affecting its decay (regurgitation and/or degradation). The azithromycin effect is additive to that of nocodazole, known to impair endocytic uptake and transport of solutes along the endocytic pathway. Cytochemistry (light and electron microscopy) shows a major reduction by azithromycin in the number of labeled-HRP endocytic vesicles at 5 min (endosomes) and 2 h (lysosomes). Within 3 h of exposure, azithromycin also causes the appearance of large and light-lucent electron-lucent vacuoles, most of which can be labeled by lucifer yellow when this tracer is added to culture prior to azithromycin exposure. Three days of treatment with azithromycin results into the accumulation of very large vesicles filled with pleiomorphic content, consistent with phospholipidosis. These vesicles are accessible to fluorescein-labeled bovine serum albumin (FITC-BSA) and intensively stained with filipin, indicating a mixed storage with cholesterol. The impairment of HRP pinocytosis directly correlates with the amount of azithromycin accumulated by the cells, but not with the phospholipidosis induced by the drug. The proton ionophore monensin, which completely suppresses azithromycin accumulation, also prevents inhibition of HRP uptake. Erythromycylamine, another dicationic macrolide, also inhibits HRP pinocytosis in direct correlation with its cellular accumulation and is as potent as azithromycin at equimolar cell contents. We suggest that dicationic macrolides inhibit fluid-phase pinocytosis by impairing the formation of pinocytic vacuoles and endosomes.

Introduction

Pinocytosis plays a central role in cell physiology by allowing for a number of cellular events such as bulk-uptake of extracellular solutes, receptor-mediated uptake of ligands, transepithelial transport of macro-molecules, and a large influx of membranes which may serve for the recycling of constituents inserted at the cell surface by exocytosis (de Duve and Wattiaux, 1966; Steinman et al., 1974; Steinman et al., 1976; Silverstein et al., 1977; Schneider et al., 1979; Draye et al., 1988; Gruenberg and Maxfield, 1995; Mukherjee et al., 1997). Impairment of pinocytosis may therefore be of importance in cellular physiopathology and toxicology.

Both basic and applied studies of pinocytosis have greatly benefited from the discovery and characterization of conditions or agents which inhibit this process and can be used to dissect its cellular and molecular mechanisms (Bond et al., 1975; Thyberg and Nilsson, 1982; Tolleshaug et al., 1982; Tartakoff, 1983; Giocondi et al., 1995; Cupers et al., 1997). Among these are cationic amphiphiles, which not only accumulate in late endosomes and lysosomes by acidotropic sequestration (de Duve et al., 1974), but also bind to biomembranes, and may thereby prevent recruitment of vesicular trafficking machineries, and induce perturbations of phospholipid catabolism (Lüllmann et al., 1978; Lüllmann-Rauch, 1979).

Azithromycin, a dicationic macrolide antibiotic derived from erythromycin A (Djokic et al., 1987; Bright et al., 1988), has a marked amphiphilic character (Montenez et al., 1996). Accordingly, it extensively accumulates in cultured cells (Gladue and Snider, 1990), where it mostly localizes in lysosomes (Carlier et al., 1994), binds to phospholipid bilayers (Montenez et al., 1996, Montenez et al., 1999), and induces a mixed lysosomal storage disorder involving phospholipids (Van Bambeke et al., 1996; Van Bambeke et al., 1998) and cholesterol (Montenez, 1996).

In this study, we have examined whether azithromycin and other dicationic macrolides could affect earlier steps of the endocytic apparatus. We selected fibroblasts because these cells avidly accumulate azithromycin (Carlier et al., 1994) and have been extensively used in our laboratories to characterize many aspects of pinocytosis, such as membrane recycling (Schneider et al., 1979), quantification of endocytic membrane traffic (Draye et al., 1988; Cupers et al., 1994), and its modulation by various conditions and agents (Cupers et al., 1997). The study focuses on fluid-phase pinocytosis, i.e. the entry of solutes without binding to the pericellular membrane (Steinman et al., 1974). To this aim, we used horseradish peroxidase (HRP), a robust enzyme which can be easily measured in biochemical assays and localized by cytochemistry at the light and electron microscope. HRP was originally used to demonstrate pinocytosis in various animal tissues (Straus, 1967; Creemers and Jacques, 1971) and has allowed to quantitate and analyze fluid-phase endocytosis in details in numerous types of cultured cells, including fibroblasts (Steinman et al., 1974; Storrie et al., 1984; Casey et al., 1986; Draye et al., 1988; Cupers et al., 1994; Cupers et al., 1997).

We found that azithromycin and erythromycylamine (another dicationic macrolide described by Massey et al. [1970]), impaired the uptake of HRP, and decreased the number of HRP-labeled endocytic vesicles at 5 min (endosomes) and 2 h (lysosomes). Azithromycin also caused swelling of endocytic structures, long before inducing a mixed lysosomal lipid storage. Inhibition of HRP uptake actually correlated with azithromycin accumulation but not with the drug-induced phospholipidosis. To further delineate the target of azithromycin, we finally investigated the influence of monensin and nocodazole, two drugs unrelated to azithromycin, known to interfere with distinct steps in the endocytic process (Tartakoff, 1983; Gruenberg et al., 1989).

Materials and methods

Cell culture, incubation with azithromycin and cell collection.

Primary cultures of rat fibroblasts were obtained by trypsinization of eviscerated 18 day-old Wistar foetuses as described earlier (Tulkens et al., 1974), and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum (complete culture medium). Cultures were seeded at a density of 10^5 cells/cm^2. After 7 days, cells were detached by gentle shaking in 0.1 % trypsin in phosphate-buffered saline (PBS) at 37°C, collected by centrifugation and frozen at - 80°C to constitute a stock of cells. For each series of experiments, a new sample was thawed. Cells were seeded in Petri dishes at a density sufficient to reach near confluency after 2 days, at which time they could be treated with azithromycin. Cells at this stage will be referred to as "2 days-cultures". In several experiments, the culture was extended to another 3 days period to yield more quiescent cells (referred to as "5 days-cultures"). Unless otherwise stated, cells were washed 5 times with ice-cold PBS, collected by gentle scraping with a rubber policeman in a small volume of 0.01 % (v/v) triton X-100, and subjected to a 30 sec sonication to achieve homogenous dispersion.

Plasma membrane integrity, DNA and protein syntheses, and ATP levels.

Plasma membrane integrity was assessed at the end of the treatments by the release of lactate dehydrogenase, assayed as described (Montenez et al., 1999). To monitor DNA and protein syntheses, cells were pulse-labeled for 3 h prior to collection with either [^{3}H]thymidine or [^{3}H]leucine (both at 5 µCi/ml). DNA- and protein-associated label was then determied as the difference between total and soluble radioactivity after precipitation in 20 % (w/v) trichloroacetic acid (TCA) and centrifugation (14,000 rpm for 10 min at 4°C [5415C centrifuge, Eppendorf, Engelsdorf, Germany]). For the measurement of cell ATP, washed cells were collected by scraping in 2 % (v/v) perchloric acid, sonicated and quickly centrifuged at 14,000 rpm for 1 min at

4°C. The supernatant was immediately neutralized to pH 7.7 with 3 M KOH / 3 M $KHCO_3$. ATP was measured by the luciferin-luciferase assay by reference to an ATP standard curve, using a commercial kit (ATP bioluminescence Assay kit CLS II, Roche Diagnostics, Mannheim, Germany).

Cell volume.

Cell volume was determined by the urea-sucrose method introduced by O'Donnell (1993). Briefly, either 1 µCi/ml [^{14}C]urea or [^{14}C]sucrose was added to the cell culture medium. After 45 min incubation at 37°C, the medium was aspired and saved, whereas cells were rapidly washed once with ice-cold PBS, scraped off in a small volume of 0.01% (v/v) triton X-100 and sonicated. The [^{14}C]urea and [^{14}C]sucrose contents of parallel cell lysates and culture media were determined with a Packard Tricarb scintillation counter (Packard, Meriden, CT, USA). Intracellular water space was then calculated by reference to cell protein as described by Oehler et al. (1996).

Cellular accumulation and decay of horseradish peroxidase (HRP).

The culture medium was replaced with a fresh medium containing 2 mg/ml HRP and uptake allowed to occur at 37°C. After appropriate times, the medium was decanted, dishes placed on melting crushed ice, and thoroughly washed with ice-cold media as follows: 3 washes of 30 sec each with 155 mM NaCl; two washes of 5 and 1 min each with complete culture medium; 5 washes of 30 sec each with 155 mM NaCl (Cupers et al., 1994). This procedure effectively removed more than 95 % of the HRP that could remain adsorbed to the cell surface. Indeed, a 2 h incubation with HRP at 4°C, a condition which prevents pinocytosis but not membrane adsorption, followed by the washing procedure described above, yielded an HRP cellular content of less than 3 % of the amount accumulated after 2 h of incubation at 37°C. To measure the decay of cell-associated tracer, cells were first exposed to HRP at 37°C for a defined interval of time (load), extensively washed by the procedure described above, then reincubated in HRP-free medium at

37°C for appropriate times. Cells were finally washed 5 times in 155 mM NaCl at 4°C (30 sec each time) prior to collection.

Biochemical assays for HRP activity, phospholipids and proteins.

HRP activity was measured in cell lysates by a stopped colorimetric assay, using hydrogen peroxide and *o*-dianisidine as substrates (Steinman et al., 1974). Neither triton X-100 nor azithromycin interfered in this assay at concentrations corresponding to those found in the samples analyzed. Phospholipids were extracted according to Bligh and Dyer (1959) and total lipid phosphorus was measured by the method of Bartlett (1959), as described (Montenez et al., 1999). The presence of 0.01 % triton X-100 in the samples interfered neither with the extraction of the lipidic phosphorus nor with the assay of phosphate. Proteins were assayed by the Lowry procedure (1951) using bovine serum albumin (BSA) as a standard. Values of all cell constituents were expressed by reference to the protein content.

Assays for azithromycin and erythromycylamine.

Azithromycin and erythromycylamine were assayed by a microbiological assay using the disc-plate technique, as described in Montenez et al. (1999). Values were expressed by reference to the cell protein content.

Peroxidase cytochemistry and microscopy.

We used the method described in details in Cupers et al. (1994), with the following minor modifications: (1) before post-fixation, cell sheets were thoroughly washed 3 times for 10 min in 50 mM Tris-HCl pH 6.0, once rapidly in distilled water, and three times for 5 min with cacodylate buffer; (2) after post-fixation, the samples were stained “en bloc” for 2 h in the dark with 0.5 % uranyl acetate. Semi-thin (approx. 1 µm) sections were cut with a glass knife, lightly stained with toluidine blue and examined in a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) with a 63 X objective.

Fluorescence microscopy of lucifer yellow, filipin, and fluorescein-labeled bovine serum albumin (FITC-BSA).

Lucifer yellow was used at 1 g /L to label the endocytic apparatus of living cells. Fibroblasts were first seeded in two-well culture coverglass (Lab-Tek, Nalge Nunc, Naperville, IL, USA) and grown for two days. After incubation for the appropriate time with the tracer in complete culture medium, cells were rapidly rinsed with ice-cold phosphate-buffered saline supplemented with 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$ (PBS-Ca^{2+}-Mg^{2+}) and immediately observed using an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to an MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA, USA). Cells were sequentially examined in bright-field phase-contrast and fluorescence, for which an excitation band of 430 nm and an emission wavelength of 540 nm were selected. Filipin was used to label free cholesterol in fixed cells in which the endocytic apparatus had been labeled with FITC-BSA. For this purpose, fibroblasts were incubated overnight with 2 g/L FITC-BSA in complete culture medium, washed with PBS-Ca^{2+}-Mg^{2+}, fixed with 4 % formaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.4, and exposed for 30 min to 50 mg/L filipin (dissolved in methanol at 10 g/L as stock solution). Coverslips were washed 5 times with PBS-Ca^{2+}-Mg^{2+}, mounted and examined with a Zeiss Axiophot microscope, using excitation and emission wavelengths of 335 and 365 nm for filipin, and 494 and 518 nm for fluorescein, respectively.

Binding and cellular accumulation of transferrin.

The techniques described by Rothenberger et al. (1987) and Cupers et al. (1994) were used. In brief, iron-saturated transferrin was labeled with ^{125}I by the iodo-beads method (Kienhuis et al., 1991) to a specific radioactivity of 800 - 1,300 cpm/ng of protein. Control and treated cells were first incubated at 4°C for 15 min in PBS, then with 50 nM ^{125}I-transferrin for 2 h at 4°C in medium

without foetal bovine serum but containing 1 % bovine serum albumin (DMEM/BSA) to prevent no-specific binding. Cells were washed 3 times with PBS-Ca^{2+}-Mg^{2+} and reincubated at 37°C for the indicated times in prewarmed DMEM/BSA. Cells were again washed 3 times with ice-cold PBS-Ca^{2+}-Mg^{2+}, exposed for 1 h at 4°C to 0.3 % (w/v) pronase in DMEM without foetal bovine serum and BSA. At the end of this incubation, cells were completely detached from the dishes by repeated pipetting and pelleted for 2 min at 14,000 rpm at 4°C. The supernatant was recovered and saved. The top of the pellet was gently washed twice without resuspension with ice-cold PBS-Ca^{2+}-Mg^{2+} and lysed in 0.01 % (v/v) triton X-100. Both the pellet and the supernatant were assayed for radioactivity using a gamma scintillation counter (1275 Mini-gamma, LKB Wallac, Sollentuna, Sweden) and were taken as representing internalized (pronase-resistant) and cell-surface bound (pronase-sensititive) transferrin, respectively.

Materials.

Azithromycin (dihydrate free base for microbiological standard; 94 % purity) was generously supplied by Pfizer s.a. (Brussels, Belgium) on behalf of Pfizer Inc. (Groton, CT, USA). The drug was dissolved in 0.1 N HCl at 30 mM (22.5 g/L) as stock solution and diluted in the culture medium to the desired final concentrations. Erythromycylamine (free base; 100 % purity) was kindly provided by E. Lilly & Co (Indianapolis, IN, USA). Nocodazole, monensin, horseradish peroxidase type II, *o*-dianisidine, diaminobenzidine, thimerosal, transferrin, lucifer yellow CH, and filipin type III were purchased from Sigma Chemical Co. (St Louis, MO, USA). Iodo-beads were from Pierce (Rockford, IL, USA). L-[4,5-^{3}H]leucine, [methyl-^{3}H]thymidine, [^{14}C]urea and [U-^{14}C]sucrose were obtained from Amersham-Pharmacia (Piscatay, NJ, USA). Pronase was from Roche Diagnostics (Mannheim, Germany). Other reagents were from Merck (Darmstadt, Germany). Pregnant Wistar rats, used for foetal fibroblast cultures, were obtained from the *Proefdieren Centrum* (*Katholieke*

Universiteit Leuven, Louvain, Belgium). All culture sera and media were supplied by Life Technologies (Paisley, UK).

Statistical analyses.

Comparisons between groups of paired data were made by the Student's t-test, and multiple comparisons between groups by one-way ANOVA using the GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA; http://www.graphpad.com).

Results

HRP is a valid tracer of fluid-phase pinocytosis in rat foetal fibroblasts.

We first verified that incubation with HRP at concentrations far above saturation of its adsorptive component (~ 30 µg/ml [Straus and Keller, 1986]), followed by an extensive washing procedure, provided appropriate tracing of fluid-phase pinocytosis in the rat foetal fibroblasts we used, namely: (i) linear uptake over a large range of concentration (0.5 - 10 mg/ml); (ii) constant rate of accumulation for several hours (1 - 4 h) after a brief initial faster rate (0 - 5 min); (iii) clearance after 5 min at ~ 125 and ~ 200 nl x mg of cell protein^{1} x h^{-1} for 2 days-cultures and 5 days-cultures, respectively. Furthermore, the initial clearance rate, taken to represent uptake without regurgitation, was ~ 250 nl x mg of cell protein $^{-1}$ x h $^{-1}$ in 2 days-cultures, close to the value we have reported in the same cells (Cupers et al., 1994). These clearance rates and general properties are essentially comparable to those found in rat foetal fibroblasts for other non-diffusible and chemically unrelated molecules such as aminoglycosides and sucrose, also thought to enter by fluid-phase pinocytosis (Tulkens and Trouet, 1978; Tulkens, 1979).

Azithromycin does not affect plasma membrane integrity, DNA and protein syntheses and cell volume and moderately decreases ATP level.

As a second preliminary verification, we examined whether azithromycin, at the concentration used, exerted major effects on the general cell integrity and functional parameters denoting unspecific toxic effects. Table 1 shows the main criteria investigated in this aim in fibroblasts incubated with azithromycin 50 mg/L (66 µM; the concentration routinely used in subsequent experiments) for up to the maxium time of observation (72 h). Under these conditions, LDH release was increased only 1.6 fold (18% release) compared to matched controls. No appreciable, consistent effect was detected on protein and DNA syntheses. ATP levels decreased to about half of control values after 48 h of treatment, but did not further decrease upon more

prolonged incuvation, nor when the drug concentration was raised to 100 mg/L. The influence of azithromycin on cell volume was assessed using [^{14}C]urea and [^{14}C]sucrose as markers of total and extracellular water spaces, respectively (O'Donnell, 1993). Variations were small (~ 25 %) and related neither to the duration of the incubation (up to 72 h), nor to the drug concentration (up to 100 mg/L).

Azithromycin slows down HRP accumulation but does not affect its decay.

In a first series of experiments, fibroblasts were incubated with 50 mg/L azithromycin for 3 h (2 days-cultures) or 3 days (5 days-cultures), then challenged by HRP uptake (2 h) either in the continuing presence of the drug, or after its removal from the culture medium. As shown in Fig. 1, accumulation of the fluid-phase tracer in control fibroblasts increased (~ 50 %) upon cell aging, as reported earlier (Steinman et al., 1974; Roederer et al., 1989). In both types of culture, azithromycin significantly decreased HRP accumulation: by approx. 35 % after 3 hours exposure and 70 % after 3 days, respectively. This decrease was found irrespective of the presence of azithromycin in the culture medium during the 2 h of HRP challenge, demonstrating that it was both independent of extracellular azithromycin and persistent within this time period. Therefore, azithromycin was subsequently removed from all culture media before challenge with HRP. We also examined whether the more severe inhibition seen in fibroblasts incubated for 3 days with azithromycin resulted from the longer exposure to the drug itself, or from a greater sensitivity of the 5-days cultures to the drug. To this aim, cells cultured for 5 days in control medium were exposed for 3 h to azithromycin. This resulted in a similar (~ 35 %) decrease of accumulation as in 2 days-cultures (data not shown). Thus, the stronger inhibition of HRP accumulation could be ascribed to the prolonged exposure to the drug.

A second series of experiments was performed to determine the dose-dependence of the inhibition of HRP uptake by azithromycin after short- and long-term exposure to the drug. As

shown in Fig. 2, inhibition was already significant at an extracellular drug concentration of 5 mg/L for 3 hours exposure, and of 2 mg/L for 3 days exposure. It levelled off at 25 mg/L (~ 40 % inhibition) and 50 mg/L (~ 80 % inhibition), for 3 h and 3 days exposure, respectively. A fixed concentration of 50 mg/L was therefore used for most subsequent experiments.

A third series of experiments addressed the influence of azithromycin on the rates of cellular uptake and decay of HRP (Fig. 3). Fig. 3A shows that preincubation of fibroblasts with azithromycin for 3 days inhibited the rate of HRP uptake to a similar extent over the entire period of pinocytosis, i.e. during both the initial period of fast clearance (first 5 min; considered as strictly reflecting pinocytic influx; Cupers et al., 1994), and the second period of slower constant accumulation (5 min to 2 h; corresponding to pinocytosis minus regurgitation). In sharp contrast to this marked inhibition of uptake, no effect of azithromycin was seen on the decay of intracellular HRP activity for up to 4 h, whether cells had been loaded with HRP for 15 min or 2 h prior transfer to HRP-free medium (Figs. 3B and 3C). This demonstrates that azithromycin did not affect HRP regurgitation (short-term decay) and suggests it had no appreciable effect on intracellular degradation (long-term decay).

Azithromycin affects neither surface binding nor internalization of transferrin in rat foetal fibroblasts.

Transferrin has proven useful to study receptor-mediated endocytosis in fibroblasts (Cupers et al., 1994). This tracer was therefore used here to examine whether the influence of azithromycin on fluid-phase pinocytosis, as evidenced by HRP uptake, also affected receptor-mediated endocytosis. Azithromycin (50 mg/L for 3 days) affected neither the surface binding (~ 400 fmol/mg cell protein), nor the internalization of 50 nM ^{125}I-labelled transferrin during a 0 to 5 min pulse (internalization efficiency of ~ 8 % per min [in % of total transferrin bound at 4°C]).

Azithromycin reduces the number of HRP-labeled endocytic vesicles and lysosomes and generates large vacuoles (3 h treatment) and thesaurismotic bodies (3 days of treatment).

Because the above experiments strongly suggested that the decreased HRP accumulation in azithromycin-treated cells specifically reflected an alteration of its influx, cytochemical studies were undertaken to examine whether the antibiotic would affect the structures involved in the early handling and intracellular routing of the endocytic tracer. Typical images are shown in Figs. 4 (light microscopy; fibroblasts treated for 3 days with azithromcyin and exposed for 5 min to HRP) and 5 (electron microscopy; cells treated for 3 h or 3 days with azithromcyin and incubated for 5 min or 2 h with HRP). In untreated cells, vesicles accessed by HRP after 5 min (as shown in Fig. 4A at the light microscope) displayed a variable size (up to 1 µm) and showed the cytochemical reaction product deposited as a thick and irregular rim of patchy electron-dense material lining the limiting membrane and surrounding an electron-lucent lumen (Fig. 5A). These features are typical of HRP-labeled endosomes, that are devoid of matrix. In untreated fibroblasts incubated for 2 h with HRP, staining was mostly seen in small round structures (maximal diameter of ~ 0.5 µm) which were frequently clustered and were homogeneously labeled (Fig. 5B). These aspects are typical of HRP-labeled lysosomes, the dense matrix of which allows the reaction product to be homogenously deposited.

In fibroblasts treated by 50 mg/L azithromycin for 3 h and then incubated for 5 min with HRP, stained vesicles were clearly less numerous (not shown). In addition, cells also contained large (~ 1 to 3 µm diameter) electron-lucent vacuoles that were not seen in controls and did not contain detectable HRP-reaction product (Fig. 5C). A similar aspect was observed in treated cells after incubation with HRP for 2 h (Fig. 5D), at which time only few lysosomes were stained, in marked contrast to control cells.

Additional striking structural changes appeared in fibroblasts after 3 days exposure to azithromycin. At the light microscope (Fig. 4B), treated cells displayed numerous granules that were stained by toluidine blue but not by the HRP-reaction product. At the electron microscope (Figs. 5E,F), these granules corresponded to very large vesicles (~ 1 to 5 µm diameter) filled with a pleiomorphic material consisting of finely granular and moderately osmiophilic structures, grossly granular clumps, layered membranous elements, and intraluminal vesicles of various size and content. Neither the matrix nor the vesicles contained in these large structures were stained by HRP at any time. Only a very small number of vacuoles with a peripheral staining (endosomes) were observed after 5 min of exposure to HRP (Fig. 5E) as well as after 2 h (Fig. 5F). Moreover, only a few stained, typical lysosomes (such as those shown in Fig. 5B) were seen at that time.

Vacuoles induced by a 3-h azithromycin treatment are endocytic structures.

The nature of the large vacuoles observed in the electron microscope after a short treatment with azithromycin was further examined using lucifer yellow. This membrane-impermeant fluorescent dye has been validated as a tracer of pinocytosis and shown to label the lysosomal compartment when maintained overnight in contact with cultured cells (Swanson et al., 1987). As shown in Fig. 6B, overnight incubation of control fibroblasts with lucifer yellow resulted in the staining of multiple discrete, small fluorescent granules which mostly coincided with refringent inclusions seen by phase-contrast microscopy (Fig. 6A). Incubation with lucifer yellow for 2 h only showed a similar labeling, albeit with a considerably weaker intensity (data not shown). When cells were incubated overnight with the dye, then treated with 50 mg/L azithromycin for the last 3 h in the continuing presence of the tracer, lucifer yellow labeled large vesicles (Fig. 6D) which corresponded to less refringent structures in phase contrast microscopy most if not all of which being labeled. (Fig. 6C). If cells were first treated with azithromycin for 3 h and then exposed to lucifer yellow for 2 h, some of the large vesicles could still be labeled, but were much

fewer in number and were considerably more faintly labeled (compare Figs. 6E and 6F). Thus, the large vacuoles generated by azithromycin are accessible to lucifer yellow and therefore belong to the endocytic apparatus.

Toluidine-blue stained granules induced by a 3-days azithromycin treatment are lysosomes and contain free cholesterol.

Previous studies have shown that azithromycin induces a mixed lysosomal storage disorder, which includes the accumulation of phospholipids (Van Bambeke et al., 1996; see also below), as well as both esterified and unesterified cholesterol (Montenez, 1996). Filipin, a fluorescent label specific for unesterified cholesterol (Sokol et al.,1988), and FITC-BSA, a common tracer of endocytosis, were therefore used to further characterize the large granules filled with a pleiomorphic material, which appear in cells after 3 days of incubation with azithromycin. Since we knew that azithromycin would impair fluid-phase endocytosis, fibroblasts were incubated with FITC-BSA for several hours to allow for a sufficient accumulation of this tracer (in the present experiments, the accumulation of FITC-BSA in azithromycin-treated cells was ~ 1/3 of the value observed in control cells). In control cells (not shown), filipin displayed a weak staining of the plasma membrane together with some concentration in small structures closely clustered around one pole of the nucleus (a pattern evocative of the Golgi complex vesicles) with no evidence of colocalization with FITC-BSA. In contrast, in azithromycin-treated fibroblasts, filipin strongly labeled numerous granules, with an apparent size exceeding 1 µm. These granules almost filled up the entire cytoplasm (Figs. 7A and C). To a large extent, this granular staining coincided with FITC-BSA labeling (Figs. 7B and D).

Effects of azithromycin on fluid-phase pinocytosis are abrogated by monensin and additive to those of nocodazole.

In an attempt to characterize the site of inhibition of HRP uptake by azithromycin, we used this antibiotic in combination with monensin or nocodazole, two drugs known to interfere with key steps in pinocytosis and lysosome functions. These experiments were performed on fibroblasts treated with azithromycin for only 3 h, both to minimize the risk toxicity by the combination of two drugs, and to achieve partial inhibition that would allow to better detect antagonistic, additive or synergic effects.

Monensin, an antifungal and antimicrobial agent which dissipates transmembrane proton gradients in lysosomes and other membrane-bound acidic compartments (Tartakoff, 1983), was used to suppress the driving force which causes azithromycin cellular accumulation. Monensin is also known to stimulate HRP regurgitation in fibroblasts without modifying its influx (Cupers et al., 1997). Table 2 shows the results of an experiment in which HRP uptake was measured in fibroblasts treated for 3 h by either azithromycin or monensin alone, or by their combination (monensin did not appreciably affect cell integrity over the duration of the experiment, as judged by LDH release). As anticipated from its effect on regurgitation (Cupers et al., 1997), monensin decreased the HRP cellular accumulation but its effect was not additive to that of azithromycin. Actually, monensin completely suppressed the cellular accumulation of azithromycin, consistent with a collapse of transmembrane pH gradients, demonstrating that only the cell-associated, and not the extracellular antibiotic, impaired HRP uptake.

Nocodazole, an inhibitor of tubulin polymerization (De Brabander et al., 1976), impairs the transfer of endocytozed material between early and late endosomes (Gruenberg et al., 1989). In our hands, nocodazole decreased HRP accumulation in a concentration-dependent manner over a 0 - 20 mg/L concentration range (causing up to ~ 40 % inhibition of a 2-h HRP uptake). However, a significant inhibition by nocodazole of pinocytosis in rat foetal fibroblasts after a brief HRP uptake (4 min) was also detected but only at a 20 mg/L (see Verrey et al., 1995; Runnegar et al., 1997). The effect of the combination of nocodazole and azithromcyin was therefore explored at

10 mg/L nodocazole for a 2 h HRP challenge, and at 20 mg/L for a 4-min HRP challenge. Table 2 shows that the effects of nocodazole and azithromycin were additive under both conditions. In contrast to monensin, nocodazole did not modify azithromycin accumulation.

Inhibition of pinocytosis correlates with azithromycin accumulation, but not with phospholipidosis and ATP level.

We further examined whether the inhibition of HRP uptake by azithromycin was related to drug accumulation *per se*, to phospholipidosis induced by the drug (Van Bambeke et al., 1996), or to the decrease in ATP levels it causes (see above). To assess the influence of phospholipid overload, cells were treated with 50 mg/L azithromycin for increasing periods of time (0 - 3 days), after which HRP uptake, drug accumulation and total cell phospholipid content were measured. In separate experiments, cells were preloaded with azithromycin, then transferred to drug-free medium for increasing intervals during which HRP uptake as well as drug and phospholipid contents were measured. Azithromycin, indeed, accumulates very rapidly in fibroblasts (Carlier et al., 1994), whereas a prominent phospholipidosis requires at least 1 to 2 days to develop (Gerbaux et al., 1996). Likewise, a substantial efflux of azithromycin may occur without concomitant regression of the phospholipidosis (Montenez et al., 1999).

Fig. 8 shows the results of a typical experiment in which fibroblasts were treated with azithromycin for 3 days, then some cultures were maintained in azithromycin-free medium for 2 additional days. In both groups, the level of inhibition of HRP uptake closely matched the corresponding changes in drug cellular levels, but were independent of the variations in phospholipid contents. Interestingly, the phospholipid cell content eventually continued to rise after drug removal because of unsufficient drug wash-out. Incubating fibroblasts for 5 days in azithromycin-free medium (after a 3 days load), allowed for only a 50 % drug release, underlining

the exceptionally high cell retention properties of this drug (Foulds et al., 1990). Unfortunately, cells could not be maintained for more than 8 days without subculture.

A series of additional, independent experiments were then performed to better assess the relationship between inhibition of HRP pinocytosis and either azithromycin or phospholipid cell content, by manipulating the drug concentrations and times of exposure, together with variable withdrawals periods. The results of all these experiments are combined in Fig. 9. Inhibition of HRP uptake closely correlated with azithromycin cellular level, but did not correlate at all with total phospholipid content.

Since azithromycin caused a moderate, albeit significant decrease in cellular ATP levels (see Table 1), we also examined whether a relationship could be established between the loss of ATP and the inhibition of HRP pinocytosis. No correlation was seen ($r^2 = 0.47$) for a set of 12 independent conditions, in which HRP uptake impairment varied from ~ 20 to ~ 75 %, and ATP levels varied from ~ 40 to ~ 110 % of control values.

At equimolar cell content, erythromycylamine inhibits pinocytosis like azithromycin.

Erythromycylamine, another dicationic macrolide, also accumulates in fibroblasts (although less avidly than azithromycin), and causes a similar phospholipidosis when equimolar cellular drug contents are achieved by adjusting the extracellular concentrations (Montenez et al., 1999). In the present study, the capacity of erythromycylamine to inhibit HRP uptake was compared to that of azithromycin, using increasing extracellular concentrations. Analysis of data presented in Table 3 shows that the inhibition of HRP uptake is directly related to the actual molar cell content of either drug (~ 1 % inhibition per nmol of drug/mg cell protein), suggesting that both act by the same mechanism.

Discussion

The fine regulation of pinocytosis remains poorly understood and its pharmacological control is therefore elusive. The present study shows that two dicationic macrolides, azithromycin and erythromycylamine, cause a concentration-dependent impairment of fluid-phase pinocytosis, as assessed by HRP uptake in cultured rat foetal fibroblasts. HRP is constitutively internalized via clathrin-coated and non clathrin-coated invaginations of the plasma membrane to reach endosomes within approx. 5 min (Casey et al., 1986; Cupers et al., 1994). From this compartment, about 20 - 40 % is regurgitated (Adams et al., 1982; Cupers et al., 1994), the remainder being transferred to, and sequestered in lysosomes. The latter are fully accessed after about 45-60 min and are responsible for HRP degradation (with a half-life of approx. 7 h; Steinman et al., 1974; Steinman et al., 1976; Storrie et al., 1984). In fibroblasts, this process of fluid-phase endocytosis increases upon cell aging (Steinman et al., 1974; Roederer et al., 1989). Several of these features were confirmed in the present study, validating the use of HRP to assess the potential effects of macrolides on fluid-phase pinocytosis.

As a key observation, azithromycin was found to inhibit HRP accumulation from the very beginning of pinocytosis, being detected during the first 2 min which essentially correspond to the formation of primary pinocytic vesicles. Impairment of pinocytosis by azithromycin could not be attributed to a general toxic effect on rat foetal fibroblasts, since no major change in cell viability, protein and DNA syntheses, cell volume and receptor-mediated endocytosis of transferrin in these cells could be observed. Ultrastructure of organelles also appeared normal except for endosomal and lysosomal compartments. The observed changes in cellular ATP levels could also be ruled out as the primary cause of pinocytosis impairment, since they did not correlate with the extent of the inhibition of HRP uptake.

Azithromycin did not alter the rate of HRP loss from fibroblasts upon reincubation in HRP-free medium, whether loading was brief (15 minutes, at which time HRP was mainly localized in endosomes) or prolonged (2 h, a period over which the tracer has had access to lysosomes). This implies that azithromycin decreases HRP accumulation by specifically slowing down its influx, i.e. the endocytic process itself, and not by accelerating its efflux from endosomes nor its intracellular breakdown. Cytochemistry further revealed that azithromycin decreases the number of endosomes that are accessed by HRP. Thus, the combination of the biochemical and morphological data strongly suggests that azithromycin slows down the rate of the formation of the primary endocytic vesicles budding from the pericellular membrane and passively filled by HRP, which normally fuse to generate endosomes (Ward et al., 1995). Inhibition of fluid-phase pinocytosis in azithromycin-treated fibroblasts did not impinge on the clathrin-dependent receptor-mediated endocytosis of transferrin (Hanover et al., 1985), indicating that dicationic macrolides could selectively abrogate the clathrin-independent pathway of fluid-phase pinocytosis (see Lamaze and Schmid, 1995 for a discussion on the respective importance of these two pathways).

The impairment of pinocytosis seen after 3 h of azithromycin treatment could be related to the intense vacuolation caused by the drug at this stage. These vacuoles must be considered as being part of a dynamic late endosomal-lysosomal compartments, since they were stained with lucifer yellow when fibroblasts had been preloaded with the dye. In contrast, when the tracer was given for a short period to azithromycin-treated cells, fewer such vacuoles were stained, and considerably more weakly. This is entirely consistent with the pinocytosis inhibition detected by HRP uptake experiments. Thus, it appears that the large vacuoles seen in cells after incubation with azithromycin for 3 h are either swollen lysosomes or swollen late endosomes readily accessible on retrograde fashion from lysosomes (Jahraus et al., 1994), and in both cases poorly accessible from the extracellular space.

Therefore, a simple hypothesis is that azithromycin impairs fluid-phase pinocytosis because it accumulates in preexisting endosomes and lysosomes and causes their dilatation to an extent where they can no longer fuse and/or accept material from newly formed incoming primary endocytic vesicles. Accumulation of dicationic drugs such as azithromycin or erythromycylamine in endosomes and lysosomes may be explained by their acidic pH (~ 6 or lower [Mukherjee et al., 1997]; see de Duve et al., 1974, for a general discussion of the accumulation of basic drugs in acid, membrane-bounded cellular compartments). Decreased accumulation of a tracer taken up by fluid-phase pinocytosis due to the swelling of endosomes and lysosomes has already been observed with other unrelated compounds such as sucrose (Montgomery et al., 1991), nicotine (Thyberg and Nilsson, 1982) and procaine amide (Bond et al., 1975). However, these studies did not critically assess whether the decreased accumulation of the endocytic tracer by these compounds reflected impaired influx or increased regurgitation.

Impairment of pinocytosis through endosome/lysosome swelling would still hold true for cells treated with azithromycin for longer periods of time (3 days). At this stage, however, the cell morphology upon treatment with azithromycin becomes very different, since we are then dealing with a mixed situation of vacuolation and lysosomal storage of undigested material. This was demonstrated by the simultaneous appearance of lamellar bodies, reflecting the accumulation of polar lipids such as phospholipids, and of filipin-positive structures, revealing large amounts of free cholesterol. Since the latter structures also accumulated FITC-BSA, they are presumably lysosomes. Yet, no correlation was found between phospholipid overload and the inhibition of HRP pinocytosis, suggesting that the storage of undigested material does not *per se* impairs pinocytosis.

Accepting the hypothesis of pinocytosis impairment through early endosomal swelling leads one to place the target of azithromycin upstream of that of monensin, which decreases

HRP accumulation by stimulating its regurgitation (Cupers et al., 1997). It could also explain additivity with nocodazole, which at low concentrations impairs the transfer of HRP from early to late endosomes and to lysosomes (Gruenberg et al., 1989). Indeed, nocodazole primarily acts on tubulins, an effect not reported so far for azithromycin, and does not influence the early uptake and transfer of solutes to endosomes. The mechanism of the effect of nocodazole on the initial fluid-phase uptake reported by some but not all investigators (Verrey et al., 1995) has not been elucidated. Our data would suggest that, also at this level, it could be different from that of azithromycin since both drugs show additive effects.

Other mechanisms of pinocytosis impairment, also related to azithromycin accumulation, may, however, be envisaged. As described earlier, about 30 to 40 % of cell-associated azithromycin is not sedimentable and therefore presumably found in the cytosol (Carlier et al., 1994). Azithromycin is a highly diffusible drug but also binds to phospholipids (Montenez et al., 1996). Due to its presence in the cytosol, azithromycin could have access to and bind to the inner leaflet of the pericellular and endosomal membranes. This might therefore influence their fluidity and reduce their fusion capabilities. A similar effect on the biophysical properties of membranes has been documented for benzyl alcohol and local anesthetics, such as dibucaine and procaine, resulting into impaired fluid-phase and receptor-mediated endocytosis (Tolleshaug et al., 1982; Giocondi et al., 1995). Alternatively, the membrane-bound azithromycin could also interfere with the function of various proteins involved in the control of vesicle fusion and movement (for reviews, see Gerke and Moss, 1997; Allan and Schroer, 1999 and Chavrier and Goud, 1999).

The significance of the alterations reported here should be integrated in the overall context of the structural and functional organization of pinocytosis and its regulation or perturbation by drugs. First, the similarity of effects between azithromycin and erythromycylamine suggests that the impairment of pinocytosis may be a general feature of

dicationic macrolides antibiotics when they reach a sufficiently large intracellular concentration. Second, other major cellular alterations of the vacuolar system have been described for other macrolides and macrolide-related antibiotics. These include structural alterations of the Golgi apparatus and impairment of exocytosis (Bonay et al., 1996), inhibition of the lysosomal H^+ - ATPase (Tapper and Sundler, 1995), impairment of the formation of vesicular intermediates between early and late endosomes (Clague et al., 1994), and a reduction of the delivery of internalized molecules from mature endosomes to lysosomes (van Deurs et al., 1996). We suggest that macrolide antibiotics may prove useful drugs to further dissect the mechanism(s) of pinocytosis by perturbing different steps of intracellular trafficking.

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Tables and Figures

Table 1: Influence of 50 mg/L azithromycin on plasma membrane integrity, DNA and protein syntheses and ATP level.

	% of matched controls (no azithromycin) after			
parameter	8 h	24 h	48 h	72 h
LDH release [a]	88.3 ± 25.0 [NS]	N.D.	N.D.	165.3 ± 16.8 [*]
^{3}H-leucine incorporation [b]	97.2 ± 0.8 [NS]	116.1 ± 7.9 [NS]	111.7 ± 6.8 [NS]	118.1 ± 10.4 [NS]
^{3}H-thymidine incorporation [b]	98.8 ± 0.5 [NS]	94.5 ± 1.1 [NS]	94.4 ± 3.2 [NS]	93.3 ± 2.5 [NS]
ATP content [c]	82.7 ± 17.1 [NS]	74.1 ± 12.4 [*]	51.9 ± 9.7 [**]	57.7 ± 15.5 [*]

Values are means ± SD of 3 dishes (except for ATP where n=6).

[a] control value at t=0: 4.0 % (activity released in medium in % of total activity), with a steady increase of up to 11 % over the next 72 h;

[b] control value at t=0 for ^{3}[H]leucine and ^{3}[H]thymidine incorporation respectively: 56.7 ± 2.6 and 91.6 ± 0.4 (TCA-insoluble label in % of total cell associated label);

[c] control value at t=0: 38.9 ± 6.6 nmol/mg cell protein with a steady decline of up to 29 % over the next 72 h.

N.D. not determined

Statistical analysis (Student's t-test):

* significantly different from the matched control (***** $p<0.05$; ****** $p<0.01$)

^{NS} not significant from the matched control

Table 2: Inhibition of HRP accumulation by azithromycin, monensin and nocodazole.

treatment	HRP challenge [1]	inhibition of HRP accumulation (%) [+]	azithromycin accumulation (µg/mg protein) [++]
azithromycin [a]	4 min	27.3 ± 8.8	62.5 ± 2.8
	2 h	26.8 ± 4.7	20.3 ± 4.9
monensin [b]	2 h	53.1 ± 3.2 [A]	
monensin [b] + azithromycin [a]	2 h	53.1 ± 7.7 [A]	n.d.
nocodazole [c]	4 min	41.4 ± 5.3 [NS]	
nocodazole [c] + azithromycin [a]	4 min	68.2 ± 11.9 [B,C]	61.7 ± 9.5 [ns]
nocodazole [d]	2 h	32.1 ± 5.0 [NS]	
nocodazole [d] + azithromycin [a]	2 h	55.3 ± 3.4 [B,C]	25.1 ± 0.9 [ns]

Values are means ± SD (n=3 dishes, except for controls and azithromycin-treated cells where n=6).

[1] cells were pretreated for 3 h with the indicated drugs, then incubated for 4 min or 2 h with HRP in the absence of drugs.

[a] 50 mg/L; [b] 13.9 mg/L; [c] 20 mg/L; [d] 10 mg/L; n.d., not detectable (< 2.5 µg/mg cell protein).

Statistical analysis:

[+] one way ANOVA:

[A] monensin *vs* azithromycin, or monensin combined with azithromycin *vs* azithromycin alone ($p < 0.01$);

[B] nocodazole combined with azithromycin *vs* azithromycin alone ($p < 0.001$);

[C] nocodazole combined with azithromycin *vs* nocodazole alone ($p < 0.001$);

[NS] not significant; nocodazole *vs* azithromycin.

Note that the sum of the inhibitions caused by azithromycin alone and by nocodazole alone (68.7 and 58.9 % for a 4 min and a 2 h challenge with HRP respectively) is not significantly different from the value obtained for the combination of both drugs (68.2 and 55.3 % for both challenges). Note also that the slightly lower impairment of HRP accumulation in these experiments as compared to Fig. 2 can be explained by the lower accumulation of azithromycin (see Fig. 9).

[++] Student's t-test:

[ns] not significant; azithromycin accumulation in cells treated with nocodazole and azithromycin vs azithromycin alone. Note that the lower accumulation of azithromycin in cells challenged with HRP for 2 h vs those challenged for 4 min is due to continuous drug leakage in its absence in the extracellular medium during HRP challenge.

Table 3: Inhibition of HRP accumulation by azithromycin and erythromycylamine.

drug	extracellular concentration (mg/L) [µM]	drug accumulation (µg/mg cell protein) [µmol/mg cell protein]	inhibition of HRP accumulation	
			%	% per nmol of accumulated drug
azithromycin	50 [66]	61.6 ± 4.8 [82.2 ± 6.4] 4	80.0 ± 3.1 ***	1.0
erythromycylamine	250 [340]	8.0 ± 5.7 $^{+}$ [65.3 ± 7.7 $^{+}$]	51.3 ± 6.7 ***, $^{++}$	0.8

Values are means ± SD of 3 dishes.

Statistical analysis (Student's t-test):

* significantly different from controls (*** $p<0.001$)

$^{+}$ significantly different from 50 mg/L azithromycin ($^{+}$ $p<0.05$; $^{++}$ $p<0.01$)

Captions to figures

Figure 1. Effect of azithromycin on the accumulation of HRP in rat foetal fibroblasts cultured for 2 days (A) or 5 days (B). Cells were either untreated (controls; open bars) or incubated with 50 mg/L azithromycin for the last 3 h (A) or 3 days of culture (B) prior to a 2 h HRP challlenge. In each panel, treated cells were challenged with HRP either in the continuing presence of the drug (hatched bars) or in the absence of azithromycin (filled bars). Values are means ± S.D of 3 dishes. Statistical analysis (Student's t test): NS, non significantly different; *, $p < 0.05$; ***, $p < 0.001$. Control values after 2 days of culture are significantly different ($p < 0.01$) from those after 5 days.

Figure 2. Effect of azithromycin concentration on the inhibition of HRP accumulation in rat foetal fibroblasts cultured for 2 days (A) or 5 days (B). Cells were treated with azithromycin for the last 3 h (A) or 3 days of culture (B) prior to a 2 h HRP challenge. Values are means ± S.D. of 3 dishes, expressed as % of decrease with respect to control values (577 ± 82 and 1446 ± 62 ng/mg cell protein after 2 and 5 days respectively). After 3 h of preincubation (A), all, but the lowest concentrations of azithromycin are significantly different ($p < 0.05$) from controls (no drug). After 3 days of preincubation (B), all concentrations of azithromycin are significantly different from controls ($p < 0.05$ for 2 and 5 mg/L; $p < 0.01$ for 10 mg/L; and $p < 0.001$ for 25 to 100 mg/L).

Figure 3. Effect of azithromycin on the kinetics of HRP cellular uptake (A) and decay (B,C) in rat foetal fibroblasts cultured for 5 days. Cells were either untreated (control; open circles) or exposed to 50 mg/L azithromycin for the last 3 days of culture (filled circles) prior to HRP challenge. Values are means ± S.D of 3 dishes. Panel A (uptake), cells were challenged with HRP for the indicated periods of time after drug exposure. All values of treated cells are

significantly different ($p < 0.05$) from the corresponding controls. Panels B and C (decay), cells were preloaded with HRP for 15 min (B) or 2 h (C), then transferred to HRP-free medium. Control and treated cells do not significantly differ at any time point.

Figure 4. Light microscopic analysis after peroxidase cytochemistry (semi-thin plastic sections). Control cells (A) and cells treated with 50 mg/L azithromycin for 3 days (B) were exposed to HRP for 5 min prior to fixation, processed for HRP cytochemistry (brownish staining) and slightly counterstained with toluidine blue. Bars are 10 µm.

Figure 5. Ultrastructural peroxidase cytochemistry. Control and azithromycin-treated cells were challenged with HRP for 5 min (A,C,E) or 2 h (B,D,F). Upper panels: control cells. At 5 min (panel A), typical pinocytic vesicles and early endosomes are stained by the reaction product at the luminal side of their limiting membrane. At 2 h (panel B), typical lysosomes filled with homogenous staining are clearly visible. Central panels: cells pretreated with 50 mg/L azithromycin for 3 h. In cells exposed to HRP during 5 min (panel C), large electron-lucent HRP-negative vacuoles (asterisk) coexist with typical HRP-positive endosomes. No detectable change is seen when cells are exposed to HRP for 2 h (panel D). Lower panels: cells pretreated with 50 mg/L azithromycin for 3 days. Note the appearance of very large membrane-bounded structures containing a pleiomorphic material suggestive of a mixed storage disorder. HRP staining remains essentially limited to endosomes at 5 min (panel E) as well as at 2 h (panel F). Bars are 0.5 µm.

Figure 6. Phase contrast (A,C,E) and confocal fluorescence microscopy (B,D,F) of cells exposed to lucifer yellow (1 g/L). A,B, control cells; C- F, cells treated with azithromycin (50 mg/L). A,B, cells incubated with lucifer yellow for 14 h; C,D, cells incubated with lucifer

yellow for 14 h, then with azithromycin for 3 h in the continuing presence of lucifer yellow; E,F, cells treated with azithromycin for 3 h, then exposed to lucifer yellow for 2 h. Bar is 10 µm.

Figure 7. Fluorescence microscopy of cells treated with 50 mg/L azithromycin for 3 days, incubated overnight with FITC-BSA, and reacted with filipin (50 mg/L) after fixation. A,C, fluorescence of filipin; B,D, fluorescence of FITC-BSA. Bar is 10 µm.

Figure 8. Comparison of the kinetics of cellular accumulation and retention of azithromycin, upon pulse and chase (A,B), of inhibition of HRP accumulation (C,D; 2 h challenge) and of total cell phospholipid content (E,F). After 2 days of culture, cells were treated with 50 mg/L azithromycin for up to 3 days (A,C,E), or further transferred to fresh medium and incubated in the absence of drug for up to 2 additional days (B,D,F). All values are means ± SD of 3 dishes. Control values of HRP accumulation after 5 days were 755 ± 108 and 762 ± 11 ng/mg cell protein in the pulse and chase experiments, respectively. Control values of phospholipid accumulation after 5 days were 233 ± 2 and 243 ±6 nmol/mg cell protein. At left (azithromycin pulse), all values of azithromycin accumulation and of inhibition of HRP uptake are significantly different ($p < 0.001$) from controls (no azithromycin); phospholipids show a significant difference from the 48 h time point ($p < 0.01$). At right (azithromycin chase), values of drug cellular contents and of inhibition of HRP accumulation are significantly different ($p < 0.05$) from the 0 h time point for reincubation times in azithromycin-free medium of at least 24 h.

Figure 9. Correlation between the inhibition of HRP uptake versus the cellular azithromycin content (A) or the cellular total phospholipid content (B). Compilation of a series of

independent experiments in which the extracellular concentrations of azithromycin were varied from 0 to 100 mg/L and the incubation times from 0 to 3 days (n = 51).

Figure 1

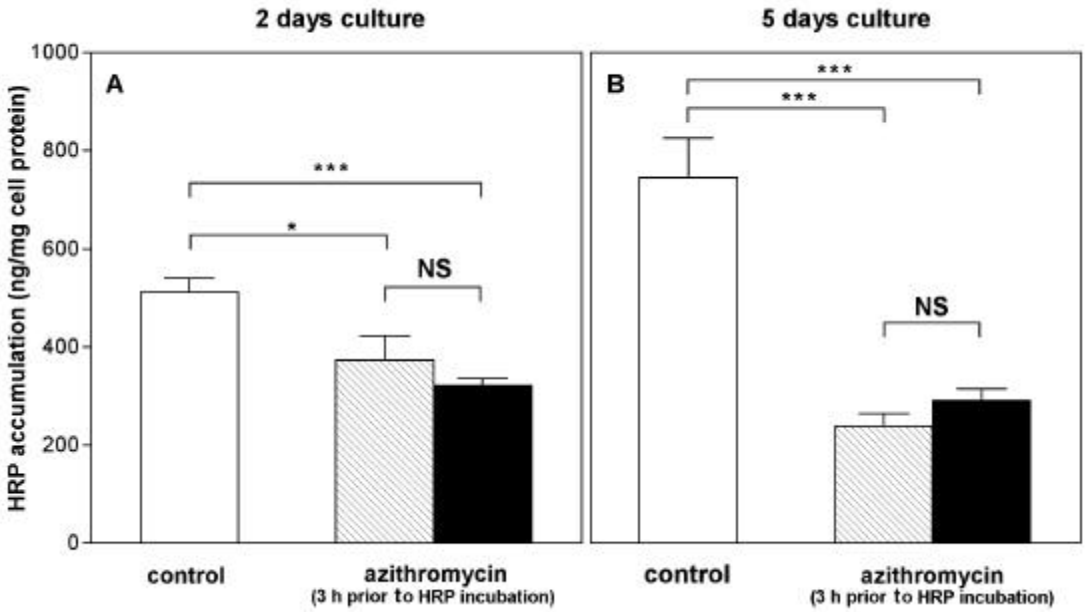

2 days culture
5 days culture
A
B
HRP accumulation (ng/mg cell protein)
1000
800
600
400
200
0
***
*
NS
***
***
NS
control
azithromycin
(3 h prior to HRP incubation)
control
azithromycin
(3 h prior to HRP incubation)

Figure 2

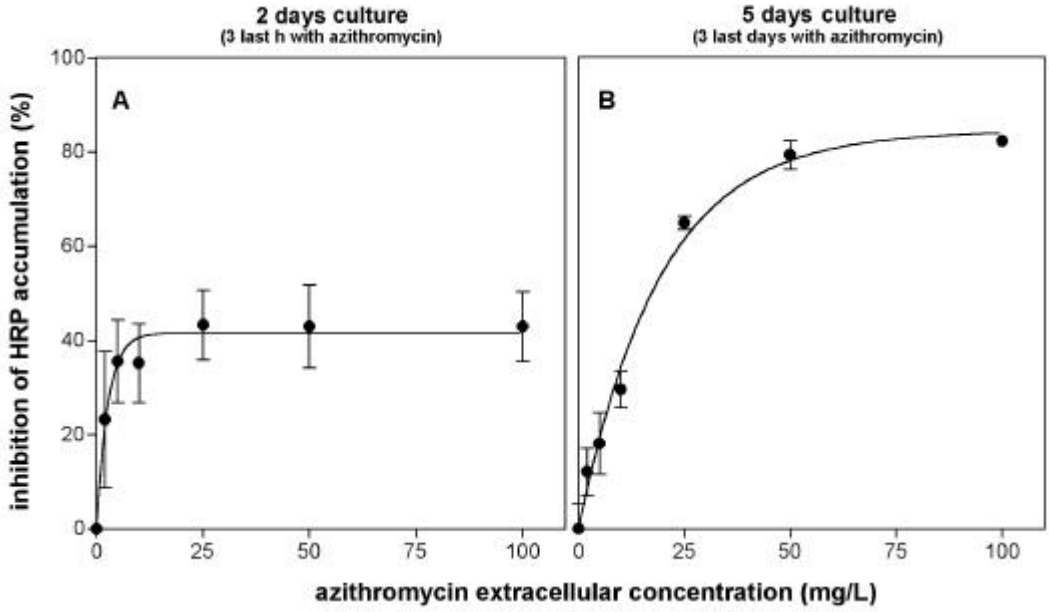

2 days culture
(3 last h with azithromycin)
5 days culture
(3 last days with azithromycin)
A
B
inhibition of HRP accumulation (%)
azithromycin extracellular concentration (mg/L)
0
20
40
60
80
100
0
25
50
75
100

Figure 3

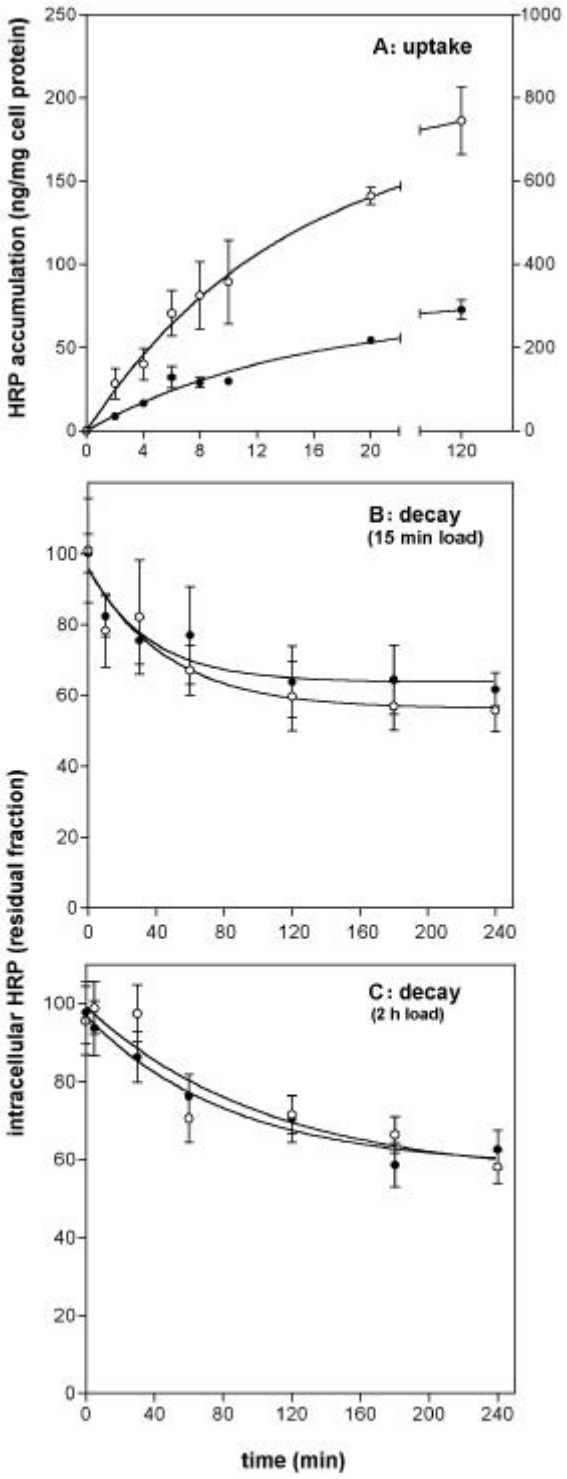

A: uptake
HRP accumulation (ng/mg cell protein)
B: decay
(15 min load)
C: decay
(2 h load)
intracellular HRP (residual fraction)
time (min)

Figure 4

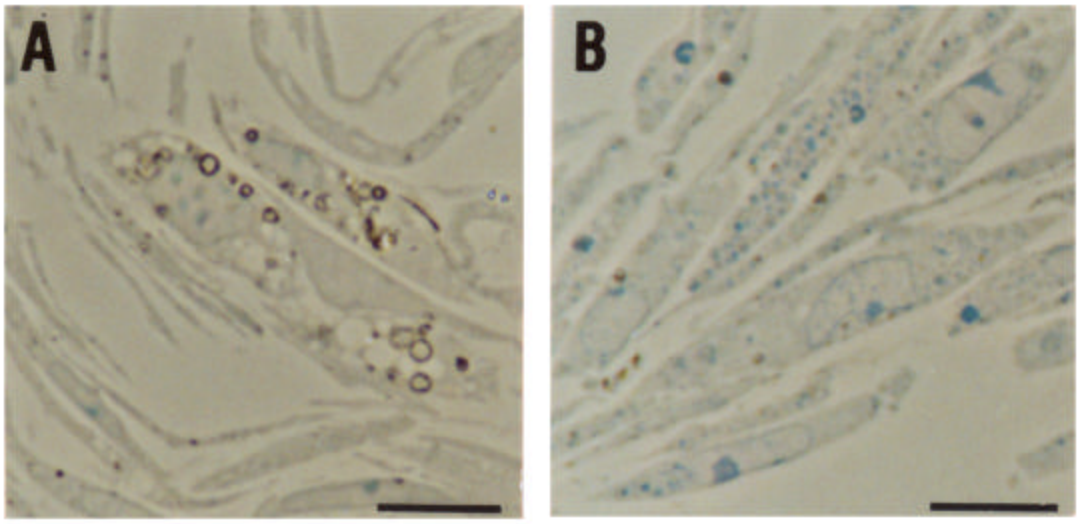


Figure 5

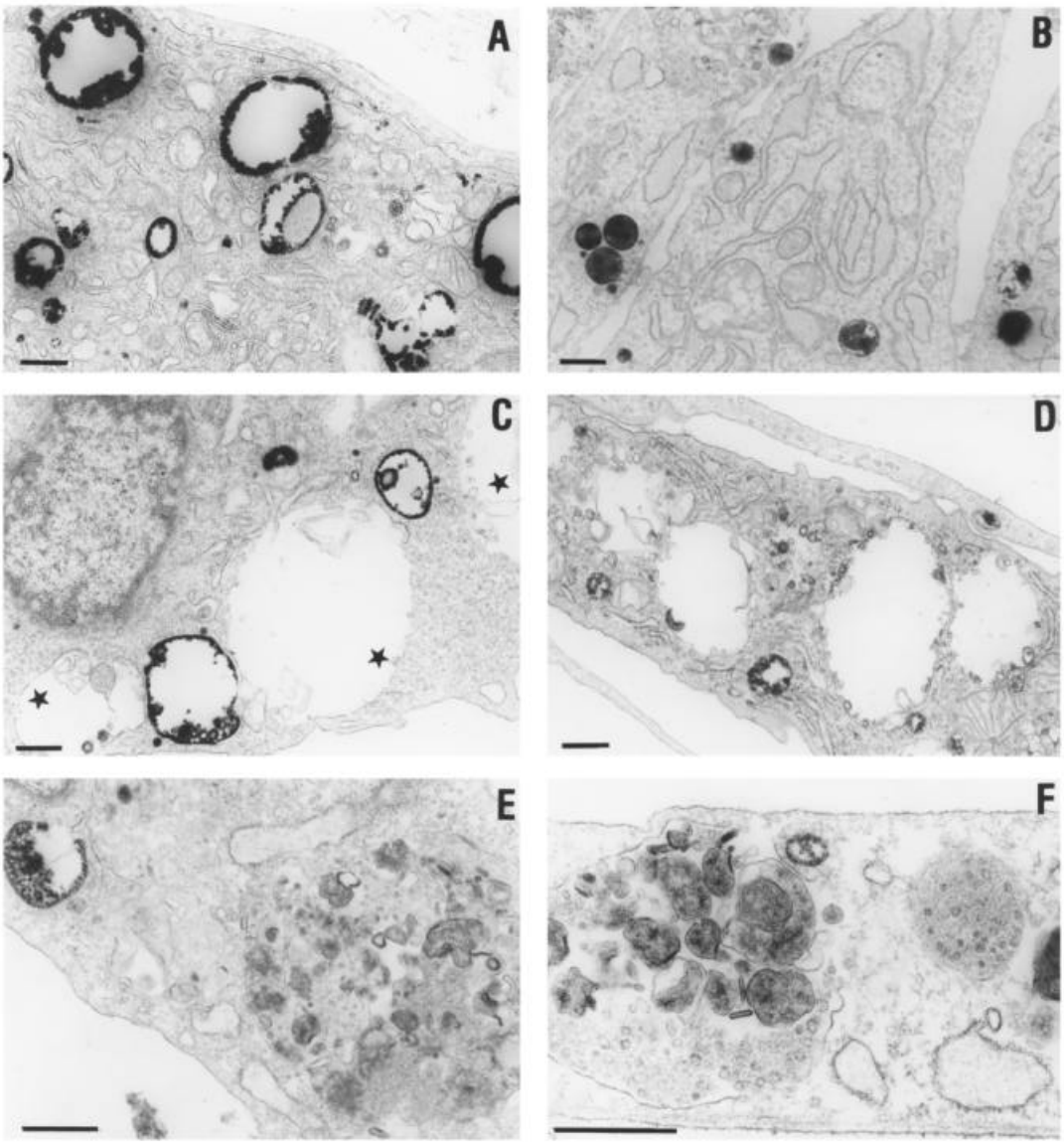


Figure 6

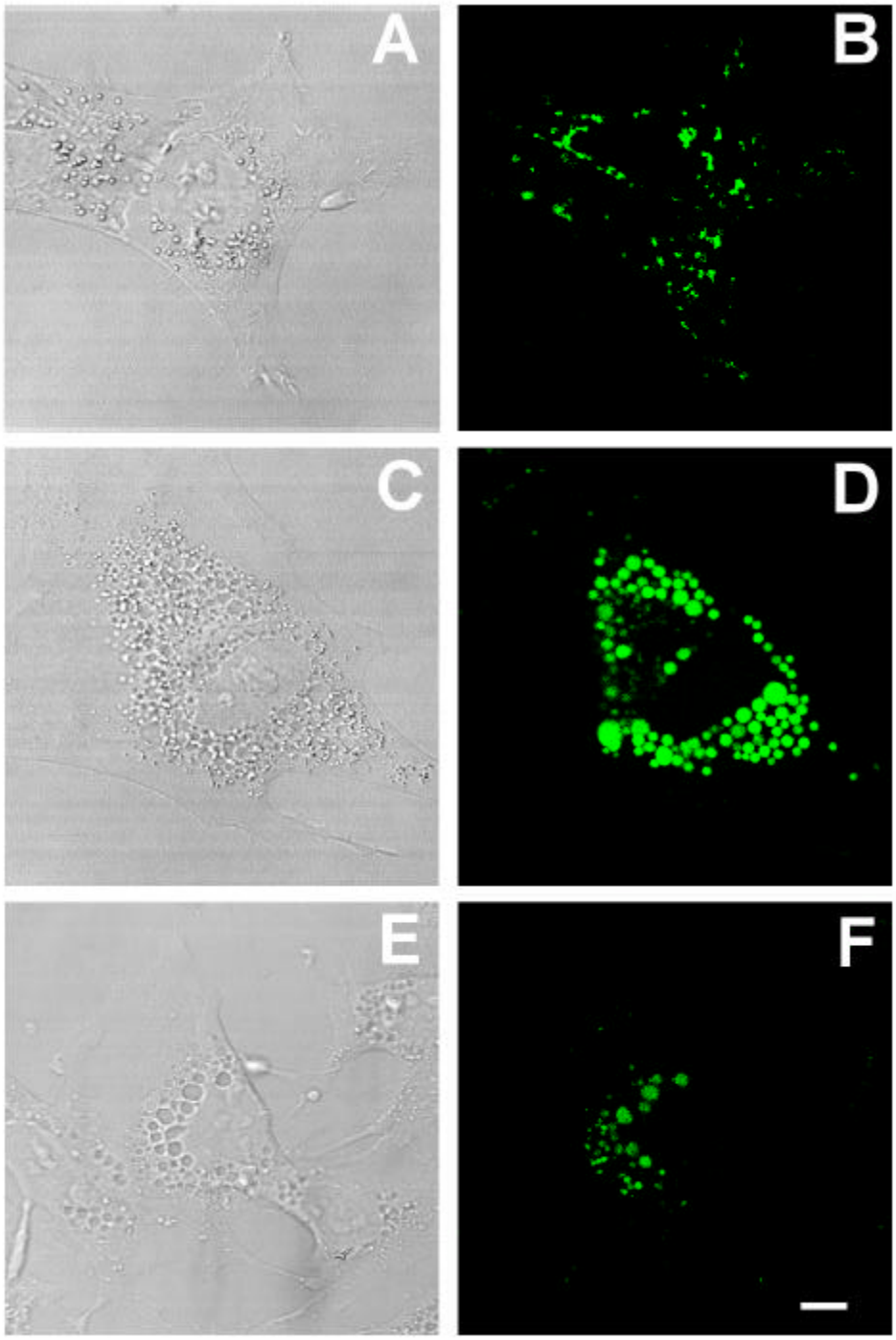


Figure 7

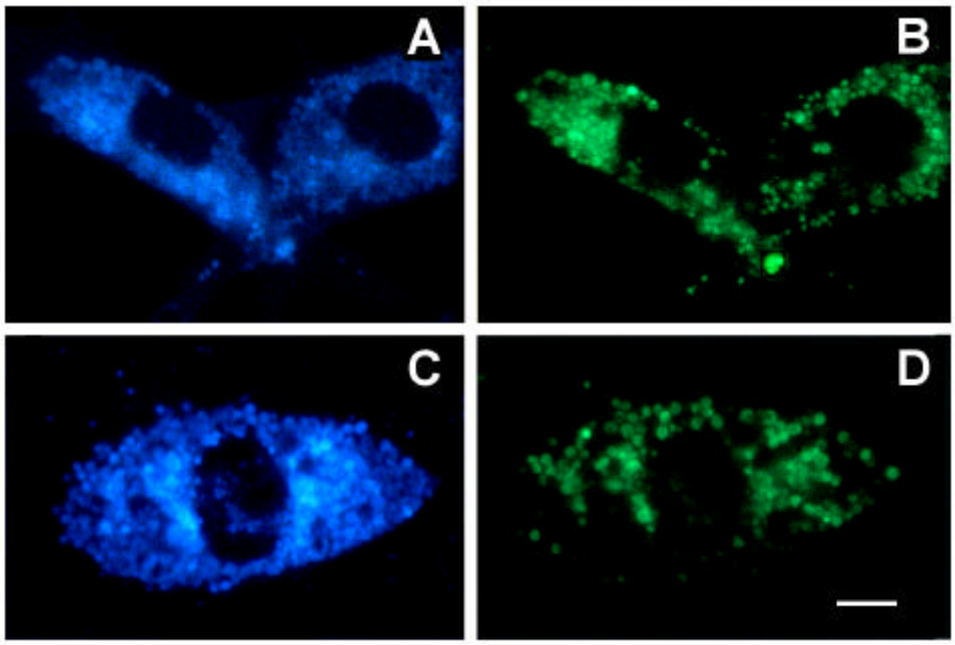


Figure 8

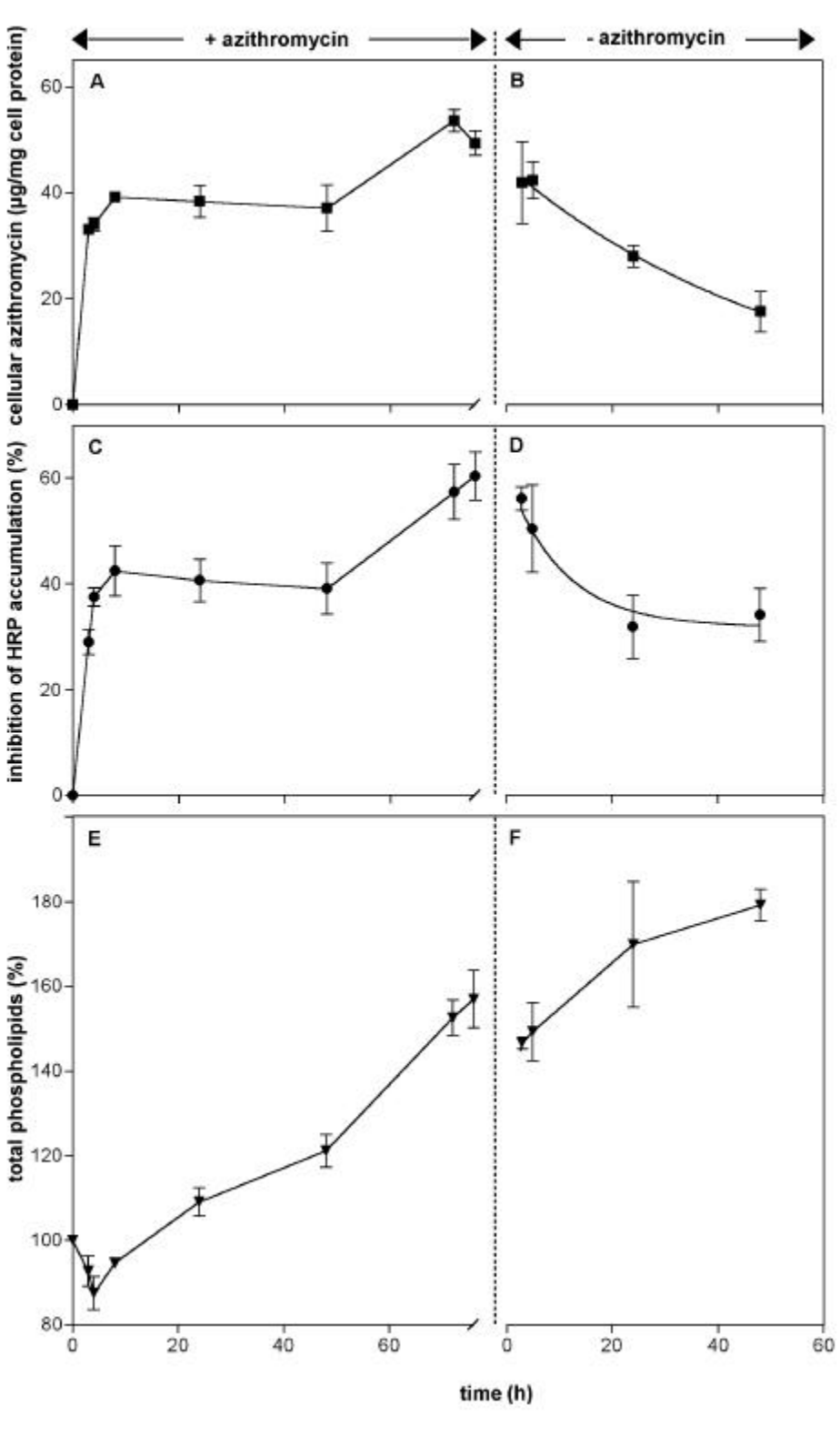
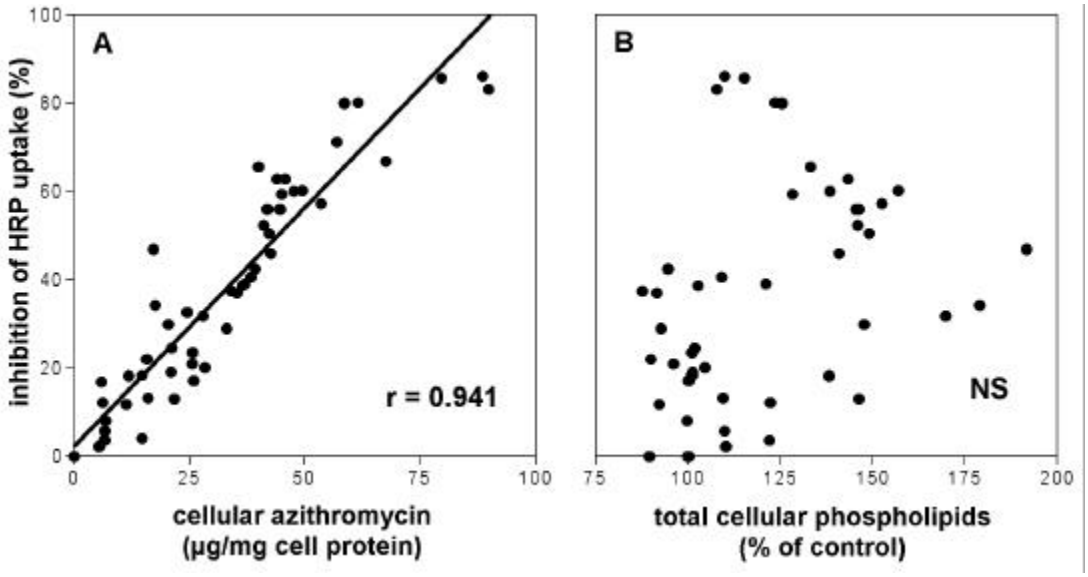

+ azithromycin
- azithromycin
A
B
C
D
E
F
cellular azithromycin (µg/mg cell protein)
inhibition of HRP accumulation (%)
total phospholipids (%)
time (h)

Figure 9


A
B
inhibition of HRP uptake (%)
r = 0.941
NS
cellular azithromycin
(µg/mg cell protein)
total cellular phospholipids
(% of control)
0
20
40
60
80
100
0
25
50
75
100
75
100
125
150
175
200

2. Paper #2 :

Azithromycin inhibits clathrin-independent pinocytosis and slows down sequestration of ligand-receptor complexes into endocytic and recycling vesicles of J774 macrophages.

(submitted for publication)

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Running title: Azithromycin inhibits pinocytosis

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Summary

Pretreatment of J774 macrophages by the macrolide antibiotic, azithromycin (AZ), selectively inhibited fluid-phase pinocytosis of horseradish peroxidase and lucifer yellow as well as bulk-membrane endocytosis of *N*-rhodamine-phosphatidylethanolamine, but not phagocytosis of latex beads. AZ delayed sequestration of receptor-bound transferrin and immune complexes into inaccessible endocytic pits and vesicles, but did not slow down the subsequent rate of receptor-mediated endocytosis. AZ down-regulated cell surface transferrin-receptors, but not Fcγ-receptors, by causing a major delay in the accessibility of internalized transferrin-receptors in the recycling route, without slowing down subsequent efflux. Acidotropic accumulation of AZ led to more extensive vacuolation of late endosomes/lysosomes than chloroquine and strongly inhibited their accessibility to peroxidase and immune complexes, but not to latex beads. The inhibitory profile of AZ was distinct from that of chloroquine and can thus not be solely accounted for by acidotropism. We propose that AZ may help dissecting various steps of the endocytic apparatus: lateral mobility at the plasma membrane, formation of clathrin-independent pinocytic vesicles, orientation of transferrin-receptors into the recycling route, and fusogenicity with lysosomes.

Introduction

Endocytosis plays a central role in cell physiology and pathology (for reviews, see Mukherjee et al., 1997; Marsh, 2001). The pathways followed by materials internalized by the four main modes of endocytosis, namely fluid-phase endocytosis, bulk-membrane endocytosis, receptor-mediated endocytosis and phagocytosis, have been amply delineated, clathrin-dependent and -independent pinocytosis can be distinguished, and the complexity of the underlying protein machineries and their interaction motifs are increasingly unravelled. In contrast, although rate-limiting interactions between molecular actors and subcellular compartments are crucial for cell homeostasis and disease, kinetic aspects of endocytosis have received comparatively less attention, yet are often illuminating (e.g. Cupers et al., 1994; Berthiaume et al., 1995; Van Weert et al., 1995; Hao and Maxfield, 2000). More recently, the role of general physical properties of the membrane such as lateral tension and fluidity (Rauch and Farge, 2000) and the participation of specific phosphoinositides (for a review, see Simonsen et al., 2001) became increasingly realized.

Dissection of endocytosis has greatly benefited from a variety of drugs or mutations. For example, drugs interfering with vacuolar acidification (e.g. acidotropic amines such as chloroquine, the proton ionophore monensin and vacuolar ATPase inhibitors such as bafilomycin A1) helped dissecting exit routes from endosomes towards lysosomes (Stenseth and Thyberg, 1989; Clague et al., 1994; Van Weert et al., 1995; van Deurs et al., 1996; Cupers et al., 1997) or the recycling pathway (Cupers et al., 1997; Presley et al., 1997).

However, drugs selectively inhibiting the earliest step of pinocytosis, that is the pinching of primary pinocytic pits, in particular those supporting clathrin-independent pinocytosis, have long been lacking (see Dautry-Varsat, 2001). This explains why temperature shifts, metabolic poisoning (energy depletion), general surface modifications (phenylarsine oxide) or empirical perturbations such as cytosolic acidification, potassium depletion or hypertonic media have been extensively used. With the two latter perturbations, it was found that whereas rapid receptor-mediated endocytosis is efficiently blocked, fluid-phase and bulk-membrane endocytosis is fully preserved, at least in some cells (Cupers et al., 1994). These perturbations also arrest transfer to lysosomes (Cupers et al., 1997). The recently introduced cholesterol-complexing agent, methyl-β-cyclodextrin, inhibits receptor-mediated endocytosis and marginally bulk-phase endocytosis (Rodal et al., 1999; Subtil et al., 1999), but also impairs non-opsonic phagocytosis (Peyron et al., 2000).

Mutations affecting endocytic proteins (Robbins et al., 1983; Guo et al., 1994) show pleiotropic effects and none of these has been found so far to exclusively inhibit the formation of

primary endocytic pits supporting fluid-phase endocytosis in mammalian cells. For example, dominant-negative dynamin efficiently blocks formation of clathrin-coated vesicles in mammalian cells, but only transiently affects fluid-phase endocytosis (Damke et al., 1994, 1995). In addition, dynamin was shown to have several other effects, including on phagocytosis (Gold et al., 1999), on the recycling route (van Dam and Stoorvogel, 2001) and at distal stages of the degradation pathway (e.g. Nicoziani et al., 2000). Rab5 dominant-negative mutation slows down fluid-phase and receptor-mediated endocytosis to a comparable extent and causes major endosomal alterations, indicating that its effect on the formation of primary endocytic vesicles could be indirect (Bucci et al., 1992).

Two agents have recently been reported to inhibit clathrin-independent but not clathrin-dependent endocytosis, at least in some conditions: poly(ethylene glycol) cholesteryl ether (PEG-cholesterol), in the K562 and A431 cell lines (Ishiwata et al., 1997; Baba et al., 2001) and azithromycin in primary cultures of rat foetal fibroblasts (Tyteca et al., 2001). We serendipitously found that this dicationic acid-stable amphiphile, widely used in clinical practice as a macrolide antibiotic (Djokic et al., 1987), markedly inhibited at supratherapeutic concentrations fluid-phase endocytosis of horseradish peroxidase in fibroblasts without affecting its regurgitation, and apparently did not impair receptor-mediated endocytosis of transferrin. Azithromycin also caused vacuolation of the endocytic apparatus and blocked pinocytic transfer to lysosomes (Tyteca et al., 2001). This prompted us to evaluate more thoroughly whether this inhibitor was specific for clathrin-independent pinocytosis, or might also affect bulk-membrane endocytosis, receptor-mediated endocytosis and phagocytosis. To this aim, we selected the established macrophage-like cell line J774, where pinocytosis and phagocytosis have been extensively studied (Mellman and Plutner 1984; Swanson et al., 1987; Pitt et al., 1992) and because kinetics of endocytosis are expected to be faster in macrophages than in fibroblasts (Steinman et al., 1976), possibly sensitizing drug effects.

We report here on a comprehensive study of azithromycin effects on (i) fluid-phase endocytosis, using two classical tracers of different sizes (Berthiaume et al., 1995), horseradish peroxidase (Steinman and Cohn, 1972) and lucifer yellow (Swanson et al., 1987); (ii) bulk-membrane endocytosis, based on a relief of fluorescence-self-quenching of *N*-rhodamine-phosphatidylethanolamine; (iii) two classical tracers taken up by receptor-mediated endocytosis, either by a constitutive (transferrin: Watts, 1985) or ligand-induced process (immune complexes; Mellman and Plutner, 1984; Kiss and Rohlich, 1984), and respectively addressed to the recycling and degradation pathways; and (iv) phagocytosis of latex beads of two different sizes.

Azithromycin was found to (i) selectively inhibit fluid-phase and bulk-membrane endocytosis; (ii) delay sequestration of receptor-bound ligands into primary endocytic and recycling vesicles, presumably reflecting a decrease in membrane fluidity, but not impair the rate of subsequent endocytosis and recycling steps; (iii) inhibit transfer of solutes to lysosomes but not affect phagocytosis nor fusion of phagosomes with lysosomes. This drug may thus be useful to dissect clathrin-dependent and -independent pinocytosis, evaluate the role of membrane fluidity in endocytosis, identify rate-limiting step(s) in the recycling route, and distinguish fusogenicity of endosomes and phagosomes.

Materials and methods

Cell culture and viability

The J774 macrophage cell line, derived from a mouse reticulosarcoma (Snyderman et al., 1977), was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). Unless stated otherwise, cells were seeded at 5.5 $x10^4$ cells per cm^2, and grown until confluency (2 days) before exposure to azithromycin (100 mg/l for 3 hours). Cells were then washed and transferred to fresh medium containing the appropriate tracer but no azithromycin, since its effect was lasting for at least 3 hours after drug withdrawal. Toxicity was evaluated by cellular ATP level, plasma membrane integrity (release of lactate dehydrogenase) and DNA and protein syntheses as described (Tyteca et al., 2001).

Fluid-phase endocytosis

Horseradish peroxidase (HRP) uptake and regurgitation were studied exactly as described (Tyteca et al., 2001). The same procedure was used for lucifer yellow (LY), except that cells were washed 3 times for 30 seconds with PBS supplemented with 1 mg/ml bovine serum albumin (BSA) and 4 times with PBS (yielding a 99% detachment of LY bound to cells at 4°C). Assay of LY was made on sonicated cell lysates prepared in 0.05% (v/v) triton X-100 by fluorimetry (Perkin-Elmer LS-30 Fluorescence Spectrophotometer [Perkin-Elmer, Beaconsfield, U. K.], λ_{exc} 430 nm, λ_{em} 540 nm). Since uptake of both tracers proved strictly linear with their extracellular concentration in the range used, uptake was performed at higher concentration for short-term than long-term kinetics, then values were normalized to both cell protein and to a nominal 1 mg/ml tracer concentration, to allow for comparison and provide equivalents to clearance values.

Relief of *N*-rhodamine-phosphatidylethanolamine self-quenching

N-(lissamine rhodamine B sulfonyl) diacylphosphatidylethanoalmine (*N*-Rh-PE), at 5 μM in FCS-free medium, was allowed to bind to cells pre-cooled at 4°C for 30 minutes. After a rapid wash, cells were reincubated at 37°C in FCS-free medium for the indicated intervals, washed twice with PBS for 30 seconds each, briefly (~20 seconds) treated with 1% (w/v) trypsin in PBS, and gently recovered in PBS. Self-quenching was immediately determined by measuring fluorescence before (F1) and after (F2) addition of 0.5% (w/v) SDS, and calculated as [(F2 - F1)/F2] x 100%.

Receptor-mediated endocytosis

Iron-saturated transferrin was labelled by ^{125}I with iodo-beads to a specific radioactivity of 800-1300 cpm/ng of protein. Surface binding isotherm was determined after incubating cells pre-cooled at 4°C with increasing concentrations of ^{125}I-transferrin in FCS-free medium supplemented with 1% (w/v) BSA for 1 hour, followed by 3 washes in ice-cold PBS-Ca^{2+}-Mg^{2+} (PBS supplemented with 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$). Radioactivity was measured in cell lysates prepared in 0.01% (v/v) triton X-100 (1275 Mini-gamma counter; LKB Wallac, Sollentuna, Sweden). Non-specific binding, measured by competition by a 300-fold excess of cold transferrin, was subtracted to yield specifically bound values. For internalization, 50 nM ^{125}I-transferrin was allowed to bind at 4°C for 1 hour, then cells were reincubated in medium without FCS but containing 1% (w/v) BSA for the indicated intervals, followed by 3 washes at 4°C with PBS-Ca^{2+}-Mg^{2+}. Sensitivity to pronase (1 hour at 4°C; 0.3% [w/v] in RPMI) was used to distinguish between surface-accessible *vs* intracellular transferrin as described (Cupers et al., 1994); this method removed ~97% of the amount bound at 4°C in untreated cells. Recycling of ^{125}I-transferrin was studied on cells after binding at 4°C, internalization for 3 minutes at 37°C, washing at 4°C and reincubation at 37°C for up to 60 minutes, as described (Cupers et al., 1997).

For binding and internalization of peroxidase-antiperoxidase (PAP) immune complexes, the same procedure was used, except that (i) binding at 4°C was extended for 2 hours; (ii) non-specific binding at 4°C was measured by competition with a 100-fold mass (~250-fold molar) excess of mouse IgG; and (iii) surface-accessible was distinguished from internalized ligand upon subsequent displacement by excess mouse IgG at 4°C for 1 hour (yielding 90% removal of the material initially bound at 4°C). Attempts of surface digestion with pronase (3 mg/ml), trypsin (1 mg/ml), bromelain (3 mg/ml) or acid-stripping at pH 3 proved ineffective if performed at 4°C.

Phagocytosis of latex beads

Cells were incubated at 37°C with 1 µm and 0.1 µm carboxylate-modified latex beads covalently coupled to Texas red. After 5 washes for 30 seconds with PBS at 4°C (removing > 90% of the beads bound at 4°C), cell content was measured on sonicated cell lysates by fluorimetry (λ_{exc} 575 nm; λ_{em} 610 nm).

Other assays

All tracer values were expressed by reference to the cell protein content, measured by the Lowry assay. Azithromycin was assayed by a disc-plate microbiological technique, as described in Montenez et al. (1999). A 10-fold excess of azithromycin in the final assay mixtures over that

contributed by treated cell lysates caused no interference. Chloroquine was assayed on sonicated cell lysates by fluorimetry (λ_{exc} 335 nm; λ_{em} 378 nm), after precipitation of proteins with trichloroacetic acid and addition of NaOH to reach pH 10.

Western blotting

Washed cells were lysed in 0.5% (w/v) sodium deoxycholate, 0.1% SDS (w/v) and 1% (v/v) of the non-ionic detergent Igepal CA-630 in the presence of protease inhibitors (phenylmethylsulfonylfluoride, aprotinin and sodium orthovanadate), resolved by SDS-PAGE under denaturing and reducing conditions (NuPAGE antioxidant), and analysed by standard Western blotting procedure, using blocking in 1% non-fat milk and 0.025% Tween 20, overnight incubation at 4°C with mouse anti-human transferrin-receptor IgG (cross-reacting with the murine transferrin-receptor; 1:250), followed by anti-mouse HRP-conjugated secondary antibody (1:750) and revealed by chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

Immunolabelling of transferrin- and Fcg-receptors

To analyze well-defined cells, confocal microscopy studies were performed on J774 macrophages seeded at lower density (1.3×10^4 cells per cm^2). Cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4 for 20 minutes at 4°C, washed with PBS-Ca^{2+}-Mg^{2+} and permeabilized with 0.05% saponin in PBS for 10 minutes. Non-specific sites were blocked with 1% BSA, 1 mg/ml lysine, 0.01% saponin and 0.02% azide in PBS (Q-PBS) for 30 minutes. Cells were then incubated for 1 hour with mouse anti-human transferrin- and rat anti-mouse Fcγ-receptors monoclonal antibodies (0.5 µg/ml and 5 µg/ml respectively). After 6 washes of 5 minutes each with Q-PBS, cells were incubated for 30 minutes with 5 µg/ml of the appropriate anti-IgG antibodies (Alexa fluor 488-anti-mouse IgG and Alexa fluor 568-anti-rat IgG), washed again 6 times for 5 minutes each in PBS, post-fixed for 5 minutes with 4% formaldehyde in 0.1 M phosphate buffer, washed 3 times in PBS and mounted in polyvinyl alcohol/diazabicyclo[2.2.2]octane (mowiol/DABCO) overnight. Immunofluorescence localization was performed with a MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA, USA) mounted on a Zeiss Axiovert confocal microscope (Zeiss, Oberkochen, Germany; λ_{exc} 495 and λ_{em} 519 nm for transferrin-receptors; λ_{exc} 578 nm, λ_{em} 603 nm for Fcγ-receptors).

Peroxidase cytochemistry and ultrastructural microscopy

This was performed exactly as reported in Tyteca et al. (2001).

Endocytosis of HRP, PAP immune complexes and latex beads, and vital labelling of lysosomes

Fluid-phase endocytosis, receptor-mediated endocytosis and phagocytosis were tracked in living cells using 1 mg/ml rhodamine-HRP, 10 µg/ml Alexa fluor 568-PAP immune complexes, and 2.5/nl Texas red-latex beads (0.1 µm), respectively. Vital labelling of acidic organelles was obtained upon incubation with 75 nM lysoTracker (Green DND-26). Cells were washed in ice-cold PBS-Ca^{2+}-Mg^{2+} containing lysoTracker and immediately examined by confocal microscopy (with the following λ_{exc} λ_{em}: lysoTracker green, 504 and 511 nm; rhodamine-HRP, 580 and 605 nm; Alexa 568-PAP, 578 and 603 nm; Texas-red latex beads, 575 and 610 nm).

Materials

Azithromycin (dihydrate free base for microbiological standard; 94% purity) was generously supplied by Pfizer s.a. (Brussels, Belgium) on behalf of Pfizer Inc. (Groton, CT, USA). The drug was dissolved in 0.1 N HCl at 30 mM (22.5 mg/ml; stock solution) and thereafter diluted in the culture medium to the desired final concentrations. HRP type II, LY CH, carboxylate-modified polystyrene latex beads covalently coupled to Texas red, holo-transferrin, *o*-dianisidine, diaminobenzidine, thimerosal, mouse IgG, rhodamine-HRP, phenylmethylsulfonylfluoride, aprotinin, sodium deoxycholate, Igepal CA-630, sodium orthovanadate and DABCO were from Sigma-Aldrich (St Louis, MO, USA); lysoTracker Green DND-26, Alexa fluor 568 protein labelling kit, Alexa fluor 488-anti-mouse IgG and Alexa fluor 568-anti-rat IgG antibodies from Molecular Probes (Eugene, OR, USA); pronase and ATP bioluminescence assay kit CLSII from Roche Diagnostics (Mannheim, Germany); PAP immune complexes and anti-mouse HRP-conjugated secondary antibodies from Dako (Glostrup, Denmark); *N*-Rh-PE from Avanti polar lipids (Alabaster, AL, USA); monoclonal mouse anti-human transferrin-receptor antibodies from Zymed (South San Francisco, CA, USA); rat anti-mouse Fcγ III/II receptor monoclonal antibodies from Pharmingen (San Diego, CA, USA); NuPAGE reagents for Western blotting from Invitrogen/Life Technologies (Carlsbad, San Diego, CA, USA); mowiol 4-88 from Calbiochem (La Jolla, CA, USA); iodo-beads for ^{125}I-labelling from Pierce (Rockford, IL, USA); and Na^{125}I, L-[4,5-^{3}H]leucine, [methyl-^{3}H]thymidine from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Other reagents were from Merck (Darmstadt, Germany). Culture sera and media were supplied by Life Technologies (Paisley, UK).

Reproducibility and statistical analyses

All values shown are means ± SD of three dishes. Where not visible, error bars are included in the symbols. The number of experiments is indicated under each figure legend. Statistical comparisons of experimental values, slopes and curvilinear parameters were made by the Student's *t*-test using the GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA (http://www.graphpad.com). Differences were considered significant at $p < 0.05$.

Results

Azithromycin inhibits fluid-phase endocytosis in J774 mouse macrophages

When J774 mouse macrophages were incubated for 3 hours with 100 mg/l (132 µM) azithromycin, drug accumulation reached 60-80 µg/mg cell protein, corresponding to ~100-150-fold cellular concentration with respect to the extracellular medium. Azithromycin accumulation was decreased by >90% in the presence of 20 µM monensin, demonstrating acidotropic concentration. For comparison, 132 µM chloroquine, known to decrease by half the acidification of endocytic structures (Maxfield, 1982), was also concentrated ~100-fold in J774 cells.

Azithromycin pretreatment markedly impaired the endocytic accumulation of the fluid-phase tracer, HRP, in J774 cells. Inhibition increased linearly with drug concentration over a 0-150 mg/l range (Fig. 1A), reached a plateau after 1 hour and remained constant for up to 24 hours of pretreatment, but was abolished if J774 macrophages were pretreated with azithromycin in the presence of monensin. Moderate variations in the inhibition of HRP accumulation between experiments correlated with corresponding levels of cellular azithromycin concentration (data not shown). Whereas azithromycin was readily released with a t ½ of 2.9 hours, the inhibition of the accumulation of HRP persisted for at least 3 hours, then progressively vanished (half-relief time of 4.0 hours) and was almost completely reversible after 1 day (Fig. 1B). After 3 hours of exposure at 100 mg/l azithromycin, despite spectacular vacuolation (see below), there was no alteration in ATP content, LDH release, as well as DNA and protein syntheses (Table 1). Therefore, this treatment was selected for all future experiments, unless stated otherwise, and endocytic assays were performed in the absence of azithromycin, to exclude possible quantitative interference due to direct interaction between the drug and tracers in the extracellular medium.

Since the rate of fluid-phase endocytosis varies according to the size of the tracer (Berthiaume et al., 1995), we next compared the effect of azithromycin on HRP (44 kDa) and lucifer yellow uptake (457 Da). In control cells, the net accumulation of HRP and LY after a 2-hours pulse was strictly linear with concentration up to 4 mg/ml and intersected the ordinate exactly at its origin (data not shown). Hence, for subsequent studies, concentrations were adapted to yield sufficient sensitivity, and for comparison purposes, data were presented after normalization to 1 mg/ml (i.e. equivalent to clearance values). As shown at Fig. 1C, both tracers showed a linear and undistinguishable accumulation after relatively long pulses (15 minutes-4 hours), corresponding to a clearance of 11.2 ± 1.6 and 11.4 ± 1.3 nl/min/mg cell protein for HRP and LY, respectively (means ± SD of slopes from 4 experiments; NS). These values are comparable to those reported for LY uptake in the same cell line (Swanson et al., 1987). At all

tracer concentrations tested, the long-term linear endocytic accumulation of the two tracers was similarly inhibited by 50-70% upon azithromycin treatment (Fig. 1C). At comparable cellular accumulation, this inhibition was 1.5-fold higher than in rat foetal fibroblasts (Tyteca et al., 2001).

After very short intervals of tracer uptake, when the regurgitated fraction is still incomplete, the initial rate of endocytic accumulation of LY was slightly faster than for HRP, measured strictly in parallel, and its inhibition by azithromycin was lower (~40% *vs* ~65%; Fig. 1D). Inhibition of HRP and LY accumulation was observed as early as after 2 minutes of uptake, arguing against an indirect effect due to accelerated regurgitation. At all time points, the difference in accumulation of both tracers between control *vs* treated cells was statistically significant. In pulse-chase experiments, regurgitation from untreated J774 cells was ~2-fold slower than in rat fibroblasts, limiting the accuracy of this analysis. Regurgitation seemed nevertheless ~2-fold faster for LY than for HRP, and the rate of efflux did not differ significantly between control and treated cells (data not shown). When azithromycin was replaced by the same concentration of chloroquine, a comparable inhibition of the fluid-phase endocytosis of LY was observed (data not shown).

Azithromycin inhibits bulk-membrane endocytosis

Next, the effect of azithromycin on the rate of membrane internalization was evaluated by using a phospholipid derivative bearing a fluorescent head group, *N*-Rh-PE. This tracer forms microaggregates in the plane of the plasma membrane (Kok et al., 1990), resulting in fluorescence self-quenching, which is relieved by endocytosis and fusion with pre-existing endosomes due to dilution with unlabelled membrane. As shown in Fig. 2, the rate of self-quenching relief was slowed down by ~50% upon azithromycin treatment. Preliminary results indicate that azithromycin inhibits to the same extent endocytosis of the B subunit of cholera toxin (data not shown).

Azithromycin delays transfer of receptor-bound ligands into clathrin-coated pits, but does not slow down subsequent rate of receptor-mediated endocytosis

To specifically address the kinetics of clathrin-coated pits, receptor-mediated endocytosis of both transferrin and immune complexes was studied in detail. Endocytosis and efflux of transferrin reflect the constitutive internalization and the recycling of transferrin-receptors, whereas immune complexes induce the internalization of Fcγ-receptors and lead to the delivery of ligand/receptor complexes to lysosomes for degradation and generation of antigenic peptides.

In untreated J774 cells, ^{125}I-transferrin binding at 4°C reached saturation at 1,200 fmoles/mg cell protein (Fig. 3A), corresponding to ~75,000 binding sites/cell, with a single class

of receptors exhibiting an apparent K_D of 15 nM (Fig. 3B). To study endocytosis with maximal sensitivity, cells were incubated at 37°C with 50 nM ^{125}I-transferrin, then surface was digested with pronase to distinguish surface-accessible (sensitive) from surface-sequestered (Schmid and Smythe, 1991) and intracellular tracer (resistant; Fig. 3C). To correct for variations in surface binding, the kinetics of internalization was plotted as ratio of pronase-resistant *vs* -sensitive tracer (Fig. 3D). ^{125}I-transferrin endocytosis was extremely rapid in J774 cells (> 30% of surface-bound being internalized in 1 minute), which is about 4-fold faster than in rat foetal fibroblasts (8% x min^{-1}; Tyteca et al., 2001), and approached a plateau at 4-5 minutes, when the early accessible intracellular pool corresponded to 1.2-fold of the surface pool.

Azithromycin treatment caused a dramatic decrease of the surface pool of transferrin-receptors, down to 20% of control, without detectable difference in apparent affinity (Fig. 3B). This disappearance increased linearly with azithromycin concentration (Fig. 3F, inverted filled triangles). Remarkably, after ^{125}I-transferrin binding at 4°C for 1 hour, increasing azithromycin concentrations enhanced the pronase-sensitive fraction from 96.6% to 99.7% (Fig. 3F, filled diamonds), indicating that sequestration into pronase-inaccessible pits was slowed down (lateral movement along the plasma membrane into coated pits still occurs at 4°C: Van Der Smissen et al., 1996). Although receptor-mediated endocytosis of ^{125}I-transferrin at 37°C was proportionally decreased in absolute terms (Fig. 3C), the efficiency of internalization of surface-bound tracer was not affected after 5 minutes, but showed a significant delay of about 1-2 minutes (Fig. 3D). This emphasizes that single-time values can be misleading and that early time-point kinetics are essential to evidence effects on endocytosis. The delay in sequestration at 37°C, as reflected by the inhibition of endocytosis efficiency after 2 minutes, was less sensitive to increasing concentrations of azithromycin than at 4°C, compatible with a lesser effect on fluidity at this temperature (Fig. 3F, filled squares).

The dynamics of Fcγ-receptor was followed in parallel by mouse peroxidase-antiperoxidase immune complexes (PAP), assayed by peroxidase activity. Specific values were obtained after subtraction of the activity that could not be removed by a 250-fold molar excess of mouse IgG. Saturation of binding at 4°C was reached at 850 ng/mg cell protein, corresponding to ~220,000 binding sites on an average untreated cell and was marginally decreased to ~185,000 sites per treated cell (Fig. 4A). There was no effect of azithromycin treatment on affinity, with a single class of receptors exhibiting an undistinguishable apparent K_D of 40 nM, close to the values previously reported for the same cells (Mellman and Plutner, 1984). In untreated cells, endocytosis of PAP at 37°C proceeded rapidly, then levelled off after 1 hour. In azithromycin-treated cells, PAP sequestration was clearly delayed by about 8 minutes, after which the rate of

internalization became comparable to that of control cells (Fig. 4B) and reached comparable values after 1 hour (data not shown).

Azithromycin causes a major redistribution of transferrin- but not of Fcg-receptors by delaying access of internalized transferrin-receptor complexes into the recycling route

An obvious explanation for the major decrease of the surface pool of transferrin-receptors is intracellular sequestration. This possibility was first suggested by pulse-chase experiments that demonstrated a remarkable initial delay of efflux of ~10 minutes (Fig. 3E). After this delay, recycling of internalized transferrin was essentially complete after 1 hour in treated cells, as in control cells, and its rate of efflux was even moderately accelerated, from a t ½ of 18 minutes in control cells to 12 minutes in treated cells. Remarkably, efflux was not only delayed, but there was even an increase in the pronase-resistant pool of ^{125}I-transferrin at the beginning of the chase, indicating that more surface-bound tracers entered this pool than the amount concomitantly lost by recycling. Thus, it appeared that entry of transferrin-receptors into endocytic clathrin-coated pits at the cell surface was delayed by ~1-2 minutes upon azithromycin treatment, while orientation of internalized receptors into the recycling route was delayed by ~10 minutes, after which vesicular trafficking back to the plasma membrane was not impaired, to the contrary. This contrasts with the lack of delay but the slower kinetics of efflux of transferrin-receptors upon inhibition of acidification by monensin or bafilomycin A1 (Cupers et al., 1997; Presley et al., 1997). Interestingly, bafilomycin A1 was found to decrease internalization and recycling of transferrin to the same extent, resulting in unaltered transferrin-receptor expression at the cell surface (Van Weert et al., 1995). The much longer delay of transferrin-receptors recycling than in endocytosis readily explains why azithromycin causes its major relocation into intracellular compartments at the expense of the surface pool. There was no such effect in rat foetal fibroblasts where receptor-mediated endocytosis of transferrin proceeded at a 4-fold lower rate (Tyteca et al., 2001).

Direct demonstration of quantitative relocation of transferrrin-receptors inside azithromycin-treated cells was provided by the combination of two additional lines of evidence. First, by Western blotting of total cell extracts, the abundance of transferrin-receptors was not significantly decreased in treated cells (Fig. 5A; 94 ± 6% of control; mean ± SD; n = 4, NS). Second, by confocal microscopy, transferrin-receptors were relocalized deeper in the cells, into large aggregates including vacuolated structures, where signal appeared altogether more intense than in control cells (Figs 5B,C). Consistent with comparable binding isotherms, there was no detectable difference in the immunofluorescence pattern of Fcγ-receptors between control and azithromycin-treated cells (data not shown).

Azithromycin does not affect phagocytosis of latex beads

The effect of azithromycin on phagocytosis was investigated by the accumulation of carboxylate-modified polystyrene latex beads of large (1 μm) and small (0.1 μm) size, covalently coupled with Texas red (Fig. 6). In both control and azithromycin-treated cells, accumulation of large beads presented at 270 particles/nl increased linearly during at least 4 hours (clearance of ~3,000 nl/min/mg cell protein). Similarly, small beads presented at 230,000 particles/nl for equal sensitivity, showed comparable level of uptake in control and treated cells, as well as similar partial saturation during this period (clearance fell from ~6,000 nl/min/mg cell protein at 15 minutes to ~2,000 nl/min/mg cell protein at 4 hours). In contrast to azithromycin, when J774 cells were pretreated for 3 hours with 132 μM chloroquine, phagocytosis of 1-μm latex beads was inhibited by 37% (control cells: 18.8 ± 1.8 beads/cell *vs* 11.9 ± 1.2 in treated cells, means ± SD, n = 3, $p < 0.01$).

Azithromycin causes vacuolation of phagosomes and late endosomes/lysosomes and inhibits their accessibility to pinocytosed but not phagocytosed tracers

By electron microscopy, azithromycin induced the disappearance of classical lysosomes together with the appearance of very large electron-lucent vacuoles containing multiple membrane-bound profiles including narrow tubular invaginations. The Golgi apparatus was moderately swollen, but there was no other noticeable ultrastructural alteration induced by the drug. Whereas HRP had largely access to lysosomes after 2 hours of pulse in control cells, it remained in azithromycin-treated cells in non-dilated tubulospherical endosomes. Induced vacuoles were consistently devoid of HRP labelling (Fig. 7).

The nature of the large vacuoles induced by azithromycin was further examined using lysoTracker green (DND-26), a fluorophore which concentrates in deeply acidified cellular compartments such as the numerous late endosomes and lysosomes of untreated cells (Figs 8A,D,G). As shown in Figs 8B,E,H, lysoTracker green clearly labelled azithromycin-induced vacuoles, that were much less numerous but could exceed 10 μm in diameter, indicating that azithromycin accumulation caused the dilation by fusion without proportionate fission of late endosomes/lysosomes. Vacuolated structures could thus still acidify to the extent of showing detectable lysoTracker concentration, sometimes with decreasing intensity as vacuoles size increased (see Fig. 8H). Azithromycin decreased the net accumulation of 1.5 μM lysoTracker (from 410 ± 19 pmoles/mg cell protein in untreated cells, i.e. a ~50-fold concentration over that in the extracellular medium, to 218 ± 18 pmoles/mg cell protein in treated cells; means ± SD; n = 3; p

< 0.01). These vacuoles disappeared after overnight incubation in drug-free medium, at which time essentially all cell-associated drug had been released (Fig. 1B). Incubation with 132 µM chloroquine for 3 hours caused much less intensive vacuolation with homogenous lysoTracker labelling (Figs 8F,I), and the net accumulation of 1.5 µM lysoTracker did not decrease (404 ± 34 pmoles/mg cell protein), suggesting that enlargement of the acidified compartment compensated for its decreased acidification.

In control cells, the many small lysosomes labelled by lysoTracker were fully (rhodamine-HRP) or largely (Alexa fluor 568-PAP and 0.1 µm Texas-red latex beads) accessible to endocytic tracers after 1 hour of pulse followed by 2 hours of chase (Figs 8A,D,G). In azithromycin-treated cells, fluorescent HRP and PAP remained in many small punctated structures and showed an almost complete segregation from the large vacuoles labelled by lysoTracker, in good agreement with ultrastructural cytochemistry (Figs 8B,E). Upon time-lapse imaging, the small fluorescent vesicles containing either HRP or PAP were not infrequently seen to dock to the large vacuoles, without subsequent fusion and content mixing. In contrast, the majority of latex beads occurred within the large lysoTracker-positive vacuoles (Fig. 8H). The presence of latex beads within these structures was further demonstrated by observing movement therein by time-lapse imaging (Fig. 9). Like azithromycin, chloroquine impaired fusion of PAP into lysoTracker-labelled compartments, but, unlike azithromycin, also greatly impaired their accessibility by latex beads (Figs 8F,I).

Whereas the large, lysoTracker-positive vacuoles induced by azithromycin were thus essentially inaccessible to HRP or PAP when the tracer was added to cells after their formation, the converse was not true. Indeed, these two endocytic tracers having ended up in lysosomes after overnight chase before exposure to azithromycin clearly localized within all large vacuoles upon drug treatment (shown for HRP at Fig. 8C).

Discussion

This study discloses several striking findings: (i) azithromycin inhibits to a comparable extent fluid-phase and bulk-membrane endocytosis in J774 macrophages; (ii) while it delays sequestration of transferrin/ and especially PAP/receptor complexes into inaccessible clathrin-coated pits/vesicles, the drug does not slow down the subsequent rate of internalization *per se*; similarly, azithromycin delays recycling of internalized transferrin, without impairing its subsequent rate of efflux; (iii) azithromycin causes a major vacuolation of the late, acidified endocytic apparatus and severely inhibits its accessibility by fluid-phase endocytic tracers and immune complexes; and (iv) azithromycin does not slow down phagocytosis nor impede access of phagosomes to swollen late endosomes/lysosomes. Incidentally, from a clinical perspective, concentration in lysosomes, as well as lack of interference with phagocytosis and transfer of particles to lysosomes, are three interesting properties for an antibiotic aiming at lysosomal bacterial killing. How can we explain and integrate all these effects and how useful is this drug for cell biologists ?

The first obvious explanation is that azithromycin, a lactonic macrocycle bearing an endocyclic tertiary amine (pKa, 8.1) and an aminated sugar (pKa, 8.8), behaves as a dicationic amphiphile, like chloroquine (de Duve et al., 1974). Indeed, azithromycin accumulates by acidotropism (abolished by monensin), reaching a cellular concentration that exceeds by 100-fold the extracellular concentration and causes vacuolation of closed acidified compartments, as directly evidenced by lysoTracker vital labelling. The stronger vacuolating effect of azithromycin, as compared with chloroquine, correlates with a stronger inhibition of acidification, as indicated by a lower net accumulation of lysoTracker green in a biochemical assay and fainter green fluorescence by confocal microscopy. Like azithromycin, chloroquine or its congener, primaquine, inhibits fluid-phase endocytosis, causes the intracellular sequestration of transferrin-receptors (Octave et al., 1982; Stoorvogel et al., 1987) and severely impairs accessibility of fluid-phase tracers to lysosomes (Stenseth and Thyberg, 1989). The latter effect could be explained by defective recruitment due to lesser acidification of the COP machinery required for the budding of endocytic carrier vesicles (Aniento et al., 1996).

However, interference with acidification does not directly account for three distinct properties of azithromycin that, it should be noted, could be evidenced even several hours after drug withdrawal: (i) azithromycin delays sequestration in clathrin-coated pit structures of receptor-bound ligands, as was most clearly evidenced for PAP/Fcγ-receptor complexes (~8 minutes), but did not appreciably affect the subsequent rate of endocytosis *per se*; (ii) similarly, azithromycin

causes a major delay of transferrin-receptors recycling (~10 minutes) without impairing the subsequent rate of efflux. This contrasts with the absence of reported delay but the prolonged half-life of efflux of transferrin-receptors in monensin- and bafilomycin A1-treated cells (Van Weert et al., 1995; Cupers et al., 1997; Presley et al., 1997); (iii) in opposition to chloroquine, but like in ε-COP-defective cells, azithromycin did not inhibit phagocytosis nor altered the accessibility of phagocytosed latex beads into lysosomes.

The striking delay induced by azithromycin for Fcγ-receptor sequestration and for transferrin-receptor recycling appears to be novel findings. In retrospect, and although this data was interpreted in a different way, a delay of ~2 minutes in the sequestration of α_2-macroglobulin-trypsin/receptor complexes is apparent in the original report of the effects of the weak base methylamine in macrophages (Kaplan and Keogh, 1981). This delay suggests interference with lateral mobility and/or availability of a rate-limiting factor for coat recruitment (Stoorvogel et al., 1996), e.g. a Rab member acting as a timer (Rybin et al., 1996), due to intracellular trapping on the vacuolated structures. In addition, it was recently shown that transferrin recycling implies transfer between a Rab5 and a distinct Rab4 domain within the same endosome (Sönnichsen et al., 2000). This, or a similar later step, could be selectively affected by azithromycin. Direct evidence that azithromycin decreases membrane fluidity has been recently obtained in our laboratories, based on biophysical methods; in good agreement, azithromycin was also found to prevent patching induced by concanavalin-A at 4°C (Tyteca et al., manuscript in preparation).

Interference with three coat components can be envisaged. Dynamin is regarded as a "pinchase" (McNiven, 1998), but it is unlikely that azithromycin acts by interfering with its interaction with the plasma membrane, since dominant-negative dynamin causes a stable inhibition in the rate of receptor-mediated endocytosis and phagocytosis, and is rapidly compensated for with respect to fluid-phase endocytosis (Damke et al., 1994, 1995; Gold et al., 1999). Dominant-negative Rab5 causes fragmentation of the endocytic apparatus and slows down fluid-phase endocytosis, and it inhibits to a comparable extent the rate of receptor-mediated endocytosis of transferrin (Bucci et al., 1992). Conversely, dominant-positive Rab5 accelerates phagocytosis (Duclos et al., 2000; Roberts et al., 2000). In contrast, some features in azithromycin-treated cells are reminiscent of ε-COP inactivation in CHO cells, which (i) selectively inhibits fluid-phase endocytosis when measured after long intervals; (ii) may inhibit receptor-mediated endocytosis (Daro et al., 1997; but it does not delay the process, and see also Gu et al., 1997); (iii) does not inhibit phagocytosis; and (iv) blocks transfer of a pinocytosed tracer to lysosomes, but not maturation of phagosomes (Aniento et al., 1996; Gu et al., 1997; Botelho et al., 2000).

Although lacking the level of selectivity offered by protein-protein interactions that support preferential inclusion in, or exclusion from budding vesicles or tubules, bulk-endocytic processes do not necessarily occur at the same kinetics for every single constituent. For soluble tracers, size-fractionation may also operate at every step of vesicular exchange, so that iterative minor selection bias may result into major long-term effects on transfer and retention. Size selectivity of soluble tracers has been elegantly demonstrated and discussed by Swanson and coworkers (Berthiaume et al., 1995). Bias may be due to either relative space restriction or unequal diffusion coefficients, causing decreased availability into narrow recycling tubules or during the brief interval of continuity between fusion and fission, referred to as "kiss and run model". "Kiss and run" exchange probability should be greatly affected by membrane fluidity. Indeed, unequal sensitivity to azithromycin during the early fluid-phase endocytosis could be evidenced between two tracers of unequal size, the major inhibition being observed for the larger tracer, HRP, as expected.

Likewise, for membrane trafficking, lateral diffusion (e.g. Draye et al., 1988) or membrane partitioning (see Mukherjee and Maxfield, 2000) must also be taken into account. Decreased lateral motility could explain the delay in ligand-induced sequestration of Fcγ-receptors into clathrin-coated vesicles and in transferrin-receptors incorporation into recycling vesicles. Extensive intracellular sequestration of transferrin-receptors as compared to Fcγ-receptors accounts for the much more severe decrease of receptor-mediated endocytosis of transferrin in absolute terms than for PAP immune complexes. The difference in sensitivity of receptor-mediated endocytosis of transferrin to azithromycin between J774 cells (major inhibition in absolute terms) and rat foetal fibroblasts (no detectable effect, at similar vacuolating concentrations) could be explained by the slower rate of endocytosis in these cells.

It is now well accepted that pinocytosis involves both clathrin-dependent and clathrin-independent pathways (e.g. van Deurs et al., 1989; Sandvig and van Deurs, 1994; Dautry-Varsat, 2001). In this context, and restricting the term "endocytosis" to the generation of primary endocytic vesicles, azithromycin was found to affect neither the formation of phagosomes, nor the budding of clathrin-coated pits into clathrin-coated vesicles, but to decrease by about half the rate of fluid-phase and bulk-membrane endocytosis. Since the two latter modalities of endocytosis reflect the sum of both clathrin-associated and clathrin-independent bulk-flow endocytosis, and since the rate of receptor-mediated endocytosis *per* se is not affected, one is led to conclude that azithromycin selectively inhibits clathrin-independent pinocytosis. Phagosomes and clathrin-coated vesicles are clearly surrounded by a cytoplasmic coat that imposes a local constraint on membrane fluidity. For example, this coat protects clathrin-coated structures against "corrugation" by the cholesterol complexing agent, filipin (Steer et al., 1984). In contrast, no such coat has been

reported so far for clathrin-independent pinocytic vesicles. Bulk-flow endocytosis may be affected not only by local lipid asymetry across the bilayer, but also by physical forces such as tension and fluidity (Farge et al., 1999; Rauch and Farge, 2000). Combined with indirect (this paper) and direct evidence (Tyteca et al., in preparation) that azithromycin decreases membrane fluidity, one can thus propose that clathrin-independent pinocytosis critically depends on such biophysical parameters. This interpretation is supported by the selective inhibition of clathrin-independent pinocytosis by PEG-cholesterol at low concentration and impairment of incurvation of clathrin-coated pits at higher concentration (Ishiwata et al., 1997; Baba et al., 2001)

Does azithromycin find its place as a tool in the armamentarium to dissect the endocytic apparatus ? After a period of extensive use of non-specific perturbations, several laboratories have developed or applied genetic approaches based on dominant-negative actors, with the hope to get a specific grip on well-defined steps. These systems proved very informative, yet several dominant-negative constructs show multiple effects, possibly reflecting the facts that (i) the overall function of the endocytic apparatus is coordinated; (ii) the same actors are involved at many steps; or (iii) interacting motifs are being shared by more than one actor. More recently, interest was raised again to the use of generic approaches, such as cholesterol depletion by methyl-β-cyclodextrin.

Despite all of their limitations, pharmacological agents immediately and equally affect all cells in a population, are generally reversible, and are more amenable to titration. Pleiotropic effects can thus be differentially modulated. In the case of azithromycin, it was found that interference with accessibility of transferrin/receptor complexes to surface digestion (presumably reflecting lateral mobility at the plasma membrane at 4°C), delayed receptor-mediated endocytosis at an early time point, and intracellular sequestration of transferrin-receptors (leading to down-regulation of the surface pool), showed distinctive concentration dependence. Whereas it is clear that rigourous studies with pharmacological agents require several controls, it is suggested that azithromycin, or related agents, could be useful for kinetics studies addressing the contribution of clathrin-independent pinocytosis (see Dautry-Varsat, 2001), sorting steps in the recycling pathway (Sönnichsen et al., 2000), fusogenicity of phagosomes (Jahraus et al., 1998) and possibly cell signalling (Lamaze et al., 2001).

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Tables and Figures

Table 1. Assessment of cell integrity and overall functionality

	Control	Azithromycin
ATP content [1] (nmol/mg cell protein)	22.8 ± 3.0	24.9 ± 2.5
LDH release in medium [1] (% of total)	1.3 ± 0.5	1.2 ± 0.2
^{3}H-leucine incorporation [2] (TCA-insoluble; % of total cell counts)	95.9 ± 0.3	95.3 ± 0.2
^{3}H-thymidine incorporation [2] (TCA-insoluble; % of total cell counts)	98.4 ± 0.0	97.0 ± 0.2

[1] cells were pre-treated with 100 mg/l azithromycin (132 µM) for 3 hours; [2] cells were further pulse-labelled with 70 nM ^{3}H-leucine or 120 nM ^{3}H-thymidine for 3 hours in the presence of azithromycin; total cell associated label was 21.8 ± 0.5 *vs* 19.4 ± 1.3 pmol of ^{3}H-leucine /mg cell protein, and 324 ± 7 *vs* 297 ± 17 pmol of ^{3}H-thymidine /mg cell protein, in control *vs* treated cells respectively.

Captions to figures

Fig. 1. Effect of azithromycin (AZ) on fluid-phase endocytosis of horseradish peroxidase (HRP, squares) and lucifer yellow (LY, triangles). J774 mouse macrophages were either left untreated (open symbols) or pre-treated for 3 hours with 100 mg/l AZ (except in A; filled symbols) prior to HRP or LY uptake in the absence of AZ. A, effect of AZ concentration on HRP accumulation after 2 hours of uptake; B, reversibility of the inhibition of HRP accumulation (filled squares) and cellular retention of AZ (filled circles): treated cells were chased in AZ-free medium for the indicated times, then incubated for 2 hours with HRP, after which HRP and AZ were assayed in cell lysates; C,D, effect of AZ on long-term (C) and short-term (D) kinetics of HRP and LY accumulation. For all figures, values are means ± SD of 3 dishes. All these experiments were reproduced at least 3 times.

Fig.2. Effect of azithromycin on bulk-membrane endocytosis. Cells were either untreated (open squares) or exposed to 100 mg/l AZ for 3 hours (filled squares), labelled at 4°C with 5 μM *N*-Rh-PE for 30 minutes, then reincubated at 37°C for the indicated times. Relief of self-quenching was expressed as percentage of self-quenching measured at time zero (81.8 ± 1.0% in control cells; 82.2 ± 2.1% in treated cells; NS). Experiment was reproduced 4 times.

Fig. 3. Effect of azithromycin on receptor-mediated endocytosis of ^{125}I-transferrin. Cells were either untreated (open squares) or pre-treated for 3 hours with 100 mg/l AZ (except in F; filled squares), prior to incubation with the tracers. A, specific binding isotherms of ^{125}I-transferrin at 4°C (non-specific binding, measured with excess cold transferrin, was subtracted). B, corresponding Scatchard plot; C, kinetics of transferrin internalization: cells were exposed to 50 nM ^{125}I-transferrin at 4°C, washed and incubated at 37°C in transferrin-free medium for the indicated times, after which pronase-resistant counts were taken as a measure of intracellular transferrin; D, efficiency of ^{125}I-transferrin internalization, expressed as ratio of pronase-resistant (surface-sequestered and intracellular) *vs* sensitive counts (surface-accessible) with increasing times of incubation at 37°C; notice the delayed and parallel (couple of arrowheads) curve in AZ-treated cells; E, recycling: after 3 minutes of internalization as in C, cells were reincubated for the indicated times in fresh medium and ^{125}I pronase-resistant material was measured; F, effect of AZ concentration on pronase-accessibility after incubation at 4°C (filled diamonds), total pronase-sensitive transferrin-receptor pool at 4°C (filled inverted triangles) and endocytosis efficiency after

2 minutes at 37°C (filled squares), all expressed as percentage of untreated cells. Effect of drug concentration and delay in sequestration and recycling were reproduced 3 times.

Fig. 4. Effect of azithromycin on receptor-mediated endocytosis of PAP immune complexes. Cells were either untreated (open squares) or pre-treated with 100 mg/l AZ for 3 hours (filled squares). A: specific binding of PAP immune complexes. Control and treated cells were exposed to the indicated concentrations of PAP at 4°C for 2 hours, then cell-associated peroxidase was assayed. Non-specific binding, measured in presence of 250-fold molar excess mouse IgG, was subtracted. Insert: Scatchard plot where abscissa values are expressed as ng/mg cell protein x 10^{-2} and ordinate as µl/mg cell protein x 10^{-1}; B, endocytosis: cells were similarly exposed to 20 µg/ml PAP at 4°C, and reincubated at 37°C in tracer-free medium for the indicated times, after which peroxidase activity resisting surface displacement by added excess mouse IgG was measured. Experiments were reproduced twice.

Fig. 5. Total content and subcellular localization of transferrin-receptors. Cells were either untreated or pre-treated with 100 mg/l AZ for 3 hours. A: Western blotting (one major band of identical intensity is observed at 107,000 Da; experiments were reproduced three times); B,C: immunolocalization of transferrin-receptors in control (B) and treated cells (C). Bar: 10 µm.

Fig. 6. Effect of azithromycin on phagocytosis of texas red-labelled latex beads. Control (open squares) or pre-treated cells (filled squares) were incubated with 1-µm latex beads (A, 270/nl) or 0.1-µm latex beads (B, 230/pl) for the indicated times. After washing, cell-associated fluorescence was measured and translated into number of beads per cell. Differences between control and treated cells are not significant. Experiments were reproduced 2-7 times.

Fig. 7. Ultrastructural peroxidase cytochemistry of J774 cells. Untreated (A) or pre-treated cells (B,C) were incubated with HRP for 2 hours. Several lysosomes are labelled by HRP in control cells (A). Vacuoles in pre-treated cells (asterisks) contain multiple membranous inclusions or tubular invaginations (C), suggesting they arise from dilated lysosomes. Notice also in azithromycin-pre-treated cells the normal appearance of endoplasmic reticulum and mitochondria, the absence of dilation of HRP-labelled endosomes which are frequently tubular (B), the much reduced accessibility of multivesicular bodies (arrow at C) and residual bodies (arrowhead at C), and the lack of HRP labelling in dilated vacuoles (B,C). Bars: 0.5 µm.

Fig. 8. Accessibility of late endocytic structures to soluble and particulate tracers. Control (A,D,G) or azithromycin-pre-treated cells (B,E,H) were incubated for 1 hour with rhodamine-HRP (A,B), Alexa fluor-PAP (D,E) or 0.1 μm latex beads (G,H), then chased for 2 hours in the presence of 75 nM lysoTracker green. As a control (C), cells were first loaded with rhodamine-HRP for 1 hour, chased overnight in tracer-free medium, then treated with azithromycin for 3 hours in the presence of lysoTracker. For comparison purposes (F,I), cells were pre-treated for 3 hours with 132 μM chloroquine instead of 132 μM azithromycin, then incubated with Alexa-PAP (F) or Texas-red latex beads (I). Merged images are shown. Bar: 10 μm. Experiments were reproduced twice.

Fig. 9. Dynamics of latex beads in vacuolated phagosomes/lysosomes. Same protocol as at Fig. 8H. The same field was photographed at 10 s intervals. Notice that all beads in this confocal section appear yellow and that several are differently located in the three images, demonstrating rapid intravesicular movement. Experiments were reproduced twice.

Figure 1

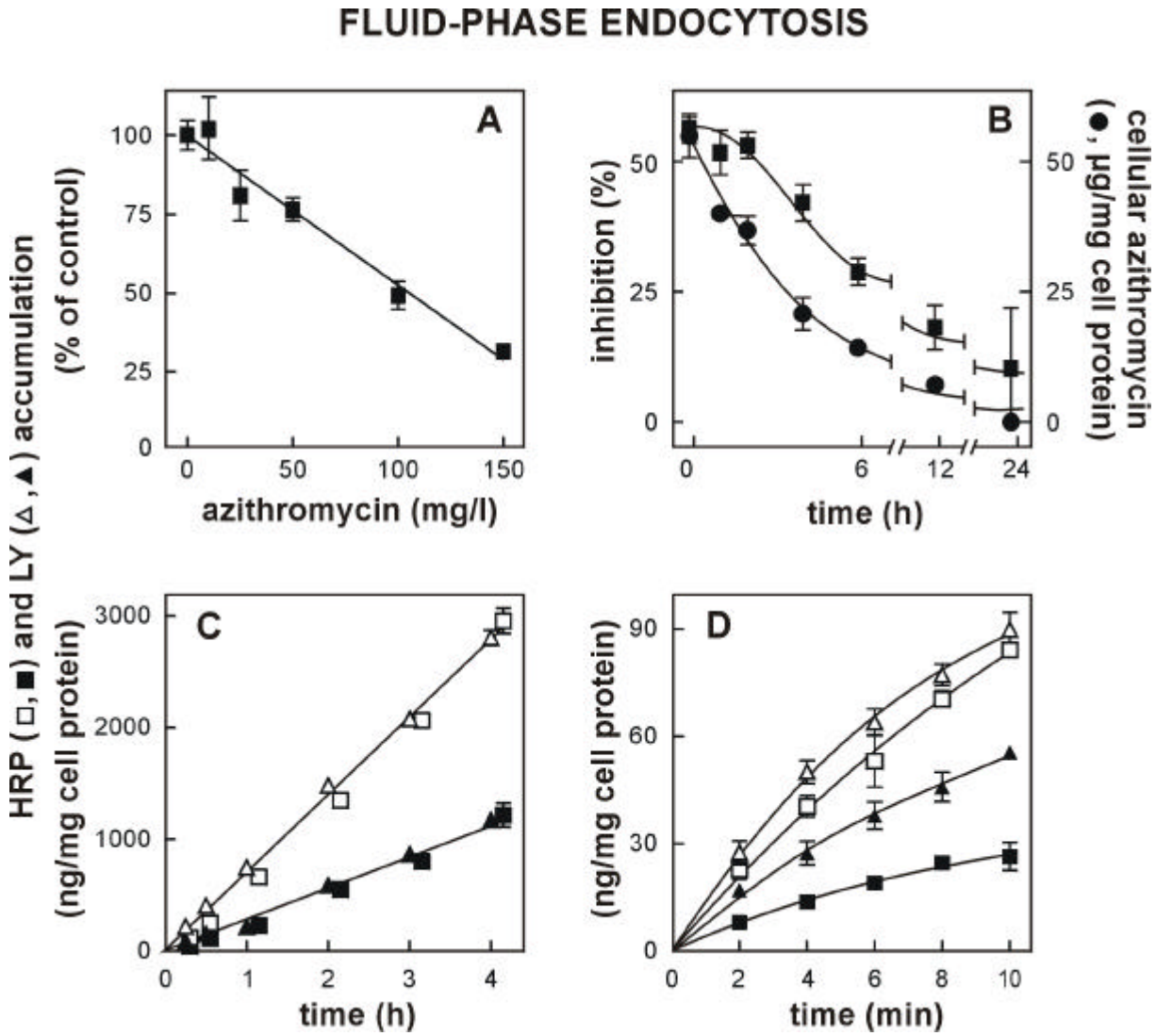

FLUID-PHASE ENDOCYTOSIS
A
azithromycin (mg/l)
HRP (□,■) and LY (△,▲) accumulation (% of control)
B
inhibition (%)
cellular azithromycin (●, μg/mg cell protein)
time (h)
C
(ng/mg cell protein)
time (h)
D
(ng/mg cell protein)
time (min)

Figure 2

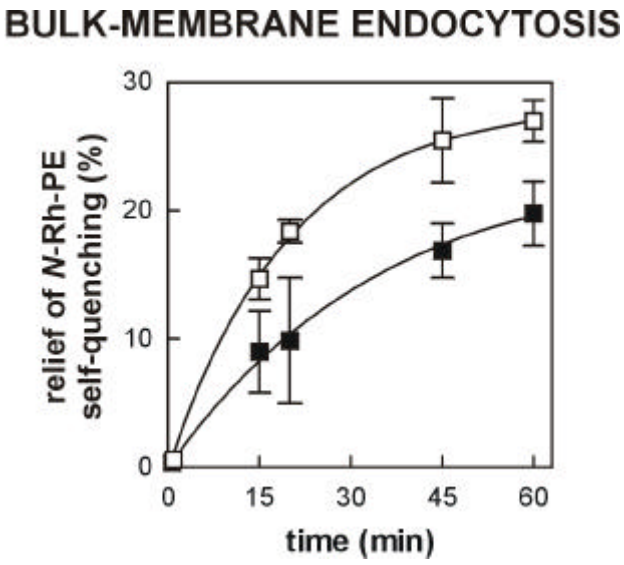

BULK-MEMBRANE ENDOCYTOSIS
relief of N-Rh-PE self-quenching (%)
30
20
10
0
0
15
30
45
60
time (min)

Figure 3

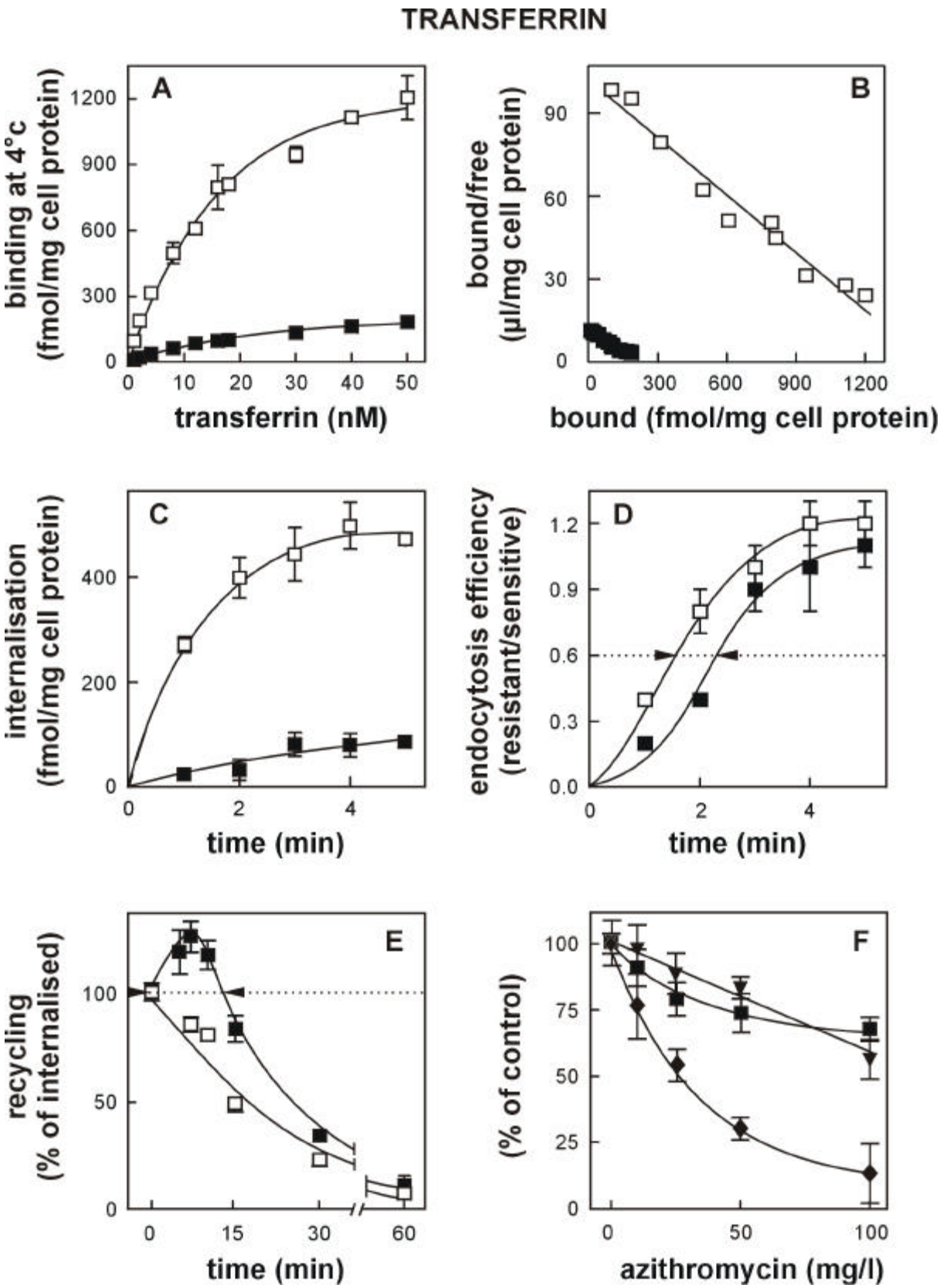

TRANSFERRIN
A
binding at 4°c (fmol/mg cell protein)
transferrin (nM)
B
bound/free (µl/mg cell protein)
bound (fmol/mg cell protein)
C
internalisation (fmol/mg cell protein)
time (min)
D
endocytosis efficiency (resistant/sensitive)
time (min)
E
recycling (% of internalised)
time (min)
F
(% of control)
azithromycin (mg/l)

Figure 4

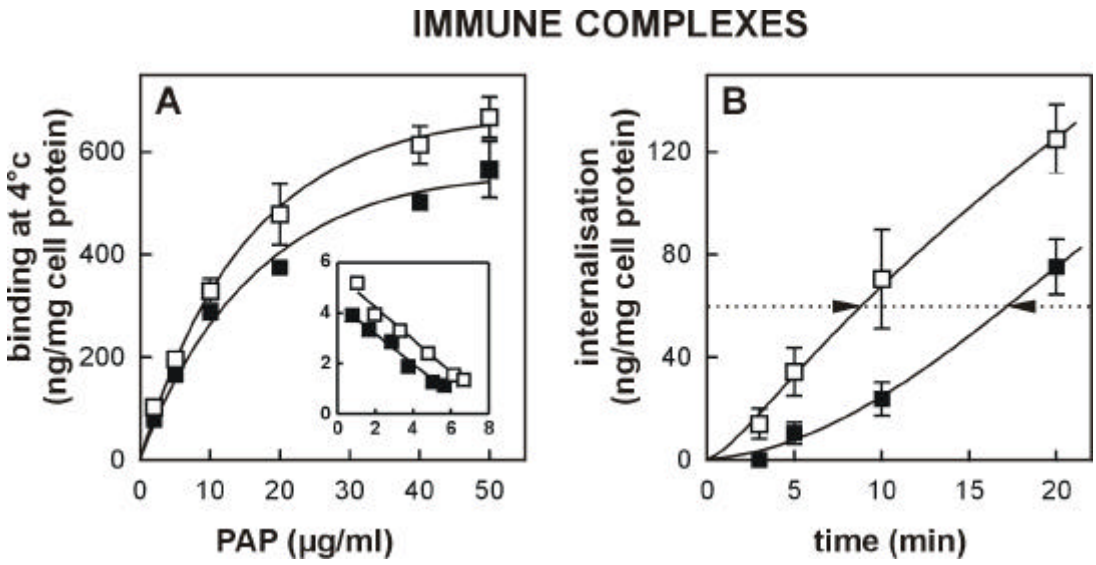

IMMUNE COMPLEXES
A
binding at 4°c
(ng/mg cell protein)
0
200
400
600
0
10
20
30
40
50
PAP (µg/ml)
0
2
4
6
0
2
4
6
8
B
internalisation
(ng/mg cell protein)
0
40
80
120
0
5
10
15
20
time (min)

Figure 5

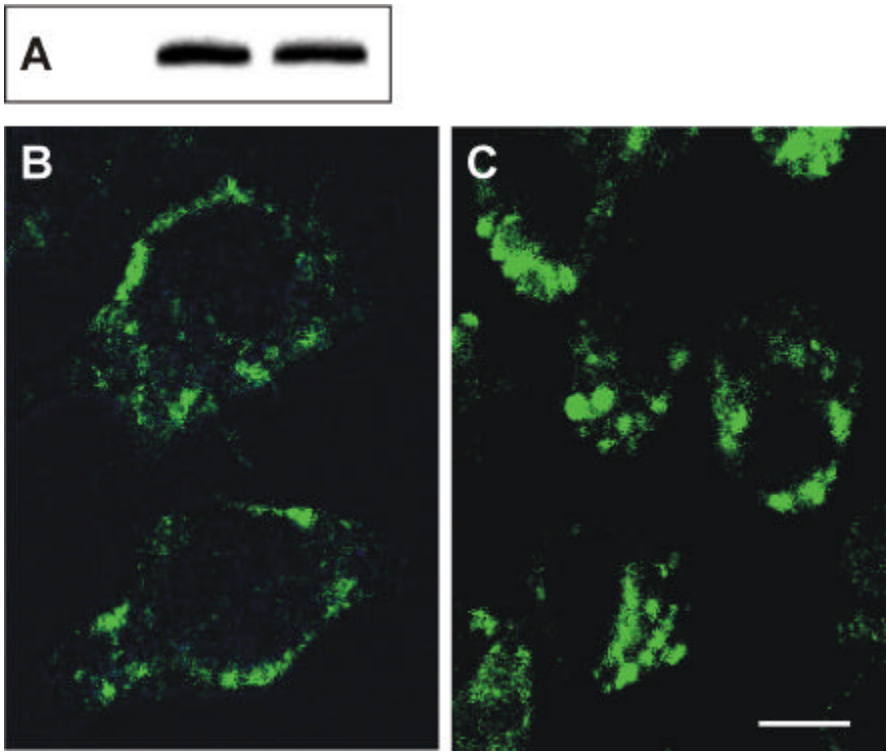


Figure 6

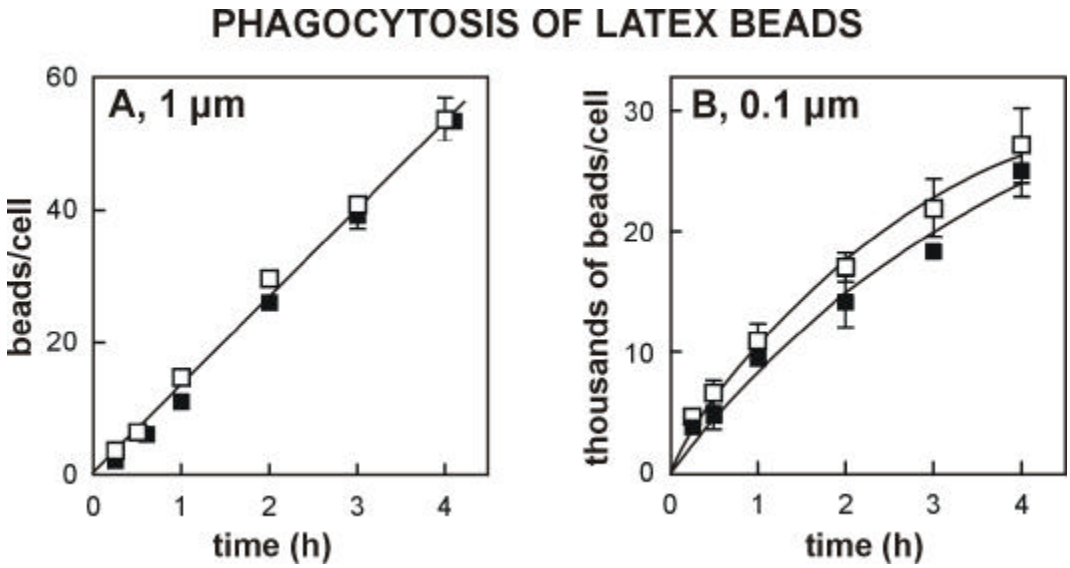

PHAGOCYTOSIS OF LATEX BEADS
A, 1 µm
beads/cell
time (h)
B, 0.1 µm
thousands of beads/cell
time (h)

Figure 7

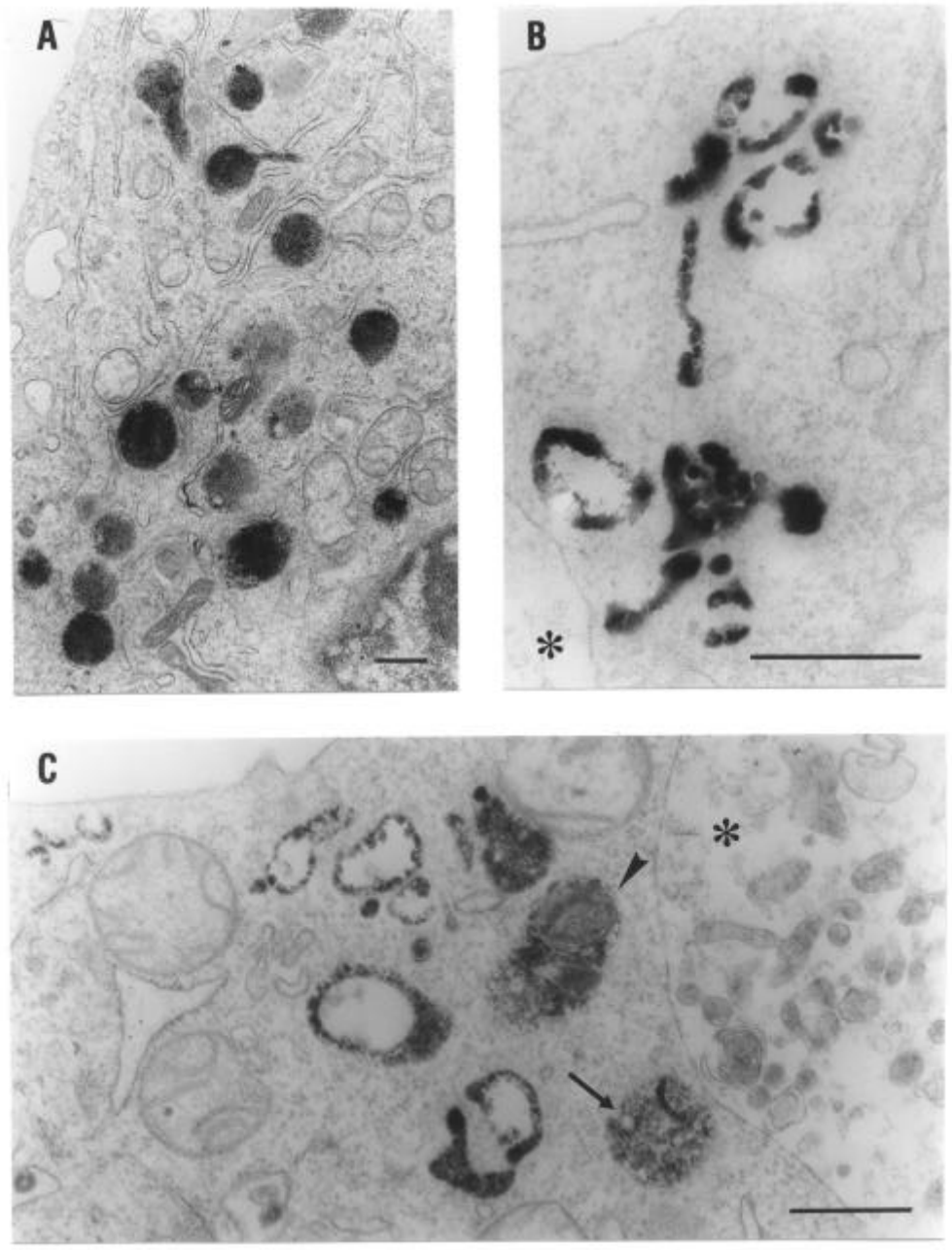


Figure 8

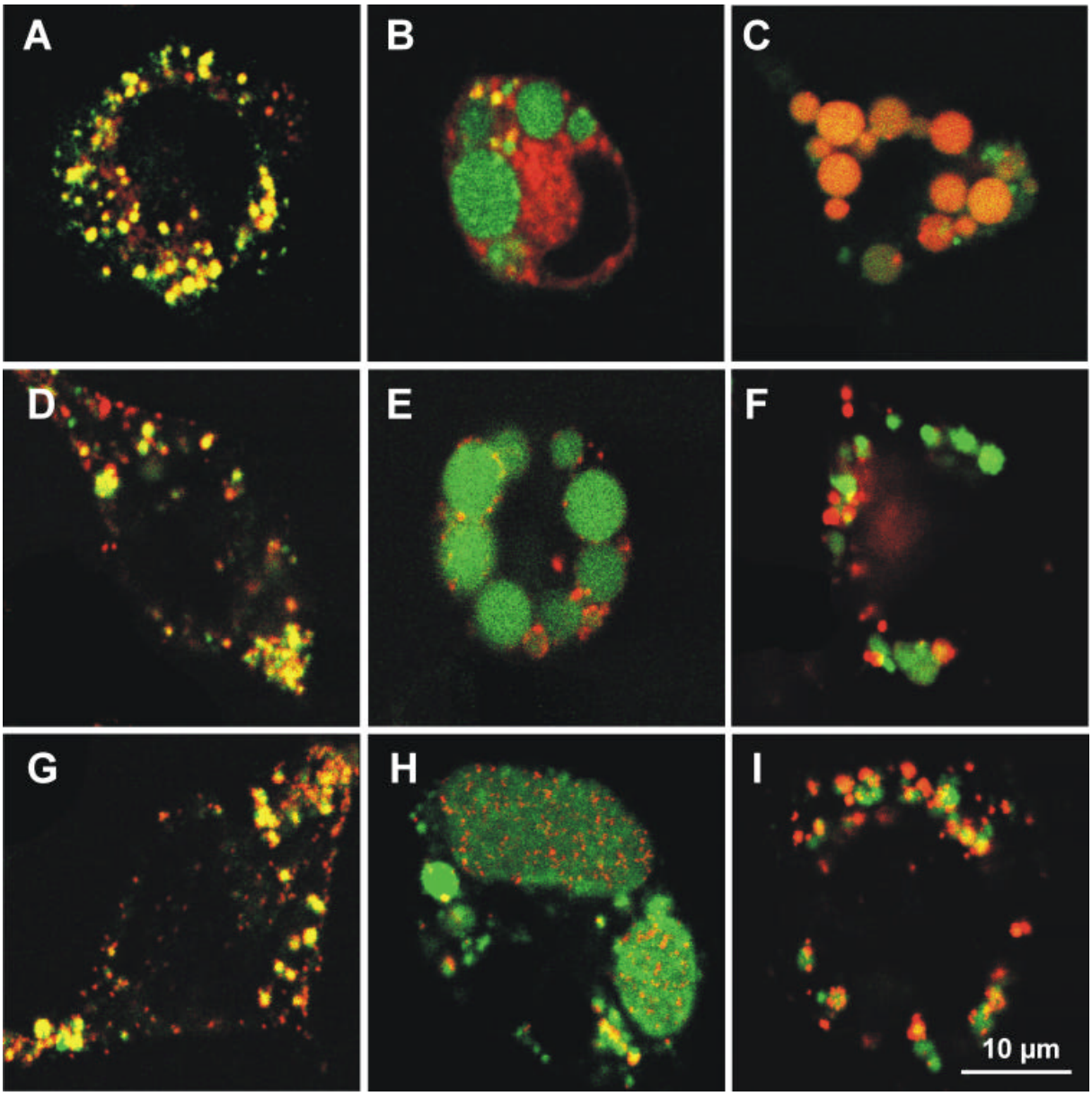
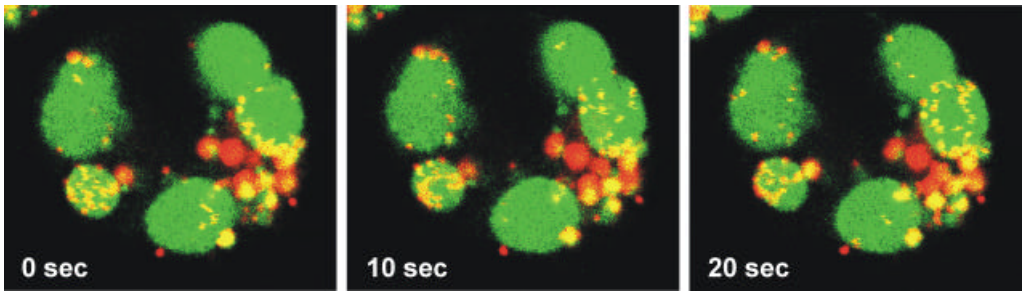


Figure 9



3. Paper #3 :

Azithromycin, a macrolide antibiotic able to impair endocytosis, interacts with lipids, decreases the insertion of membrane tracers and affects membrane fluidity.

(in preparation)

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Running title: Interaction between azithromycin and membrane.

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Introduction

Endocytosis is well-known to play a key role in cell physiopathology (Silverstein et al, 1977; Mukherjee et al, 1997; Marsh, 2001). This process involves the formation of invaginations in the plasma membrane that bud to form vesicles. The budding and fission force leading to membrane vesiculation in endocytosis is classically thought to be generated by the polymerization of clathrin-coat at the plasma membrane and by the severing of the neck. Recently, more attention is given to lipid and metabolism in the regulation of intracellular membrane trafficking events and in particular the budding force (Farge et al, 1999; Huijbregts et al, 2000). Among these, are the increase in the net surface area of the plasma membrane, the local segregation of phospholipid domains, and a difference in the surface area between the inner and the outer layers of the membrane (Dai and Sheetz, 1995; Farge et al, 1999).

Our previous studies (Tyteca et al, 2001; Tyteca et al, submitted) have shown that azithromycin, a dicationic macrolide antibiotic (Djokic *et al.*, 1987; Bright *et al.*, 1988), markedly inhibits fluid-phase and bulk membrane endocytosis and induces a delay between ligand binding to the receptor and subsequently internalization into clathrin-coated pits without affecting the subsequent rate of endocytosis (Tyteca et al, 2001; Tyteca et al, submitted). This suggests that the effect of azithromycin could occur at an early stage of the endocytic process. We also showed that a part of cell-associated azithromycin is not sedimentable and therefore presumably found in the cytosol (Carlier et al., 1994) and that azithromycin is able to interact *in vitro* with negatively-charged bilayers at pH 5.4 (Van Bambeke et al, 1996; Montenez et al, 1996). This prompted us to investigate if azithromycin is able to interact with biological membranes and so impair the budding process.

We have evaluated the capacity of the drug to bind to negatively-charged bilayers at pH 6.0 and pH 7.0, corresponding to what is found in endosomal compartments and plasma membrane respectively, and compared these results with those obtained at pH 5.4 (late endosomes and lysosomes; Mukherjee et al, 1997). We particularly have characterized azithromycin binding to liposomes by equilibrium dialysis, explored the interaction of the drug with phosphate groups by nuclear magnetic resonance (^{31}P-NMR spectroscopy) and investigated the effect of azithromycin on the flat monolayer structure by atomic force microscopy (AFM). In parallel, we have examined on a cellular model whether interaction of the drug with membranes impairs the

incorporation of three fluorescent membrane probes (C_6-NBD-SM, 1-((4-trimethylamino)phenyl)-6-phenyl-hexa-1,3,5-triene [TMA-DPH] and *N*-Rh-PE). Results are discussed in relation with the effect induced by azithromycin on plasma membrane fluidity.

Materials and Methods

Studies with liposomes and Langmuir-Blodgett monolayers.

Liposome preparation.

Unilamellar vesicles (~ 100 nm) and multilamellar vesicles (MLV; ~ 500 nm) were used for binding experiments and ^{31}P-NMR studies. Dry lipid films made of cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol (5.5:4:4:3, molar ratio) were hydrated for 1 h under nitrogen at 37°C in 40 mM Tris maleate buffer, pH 5.4, 6.0 or 7.0. The composition of liposomes containing phosphatidylethanolamine was: cholesterol, phosphatidylcholine, sphingomyelin, phosphatidylinositol and phosphatidylethanolmaine, 5.5: 4: 1.7: 3: 2.3, molar ratio. The resulting suspension was thereafter either sonicated to prepare unilamellar vesicles (Montenez et al, 1999) or submitted to 5 successive cycles of freezing and thawing to obtain MLV. The actual phospholipid content of each preparation was determined by phosphorus assay (Bartlett, 1959) and the concentration of liposomes was adjusted accordingly for each type of experiment.

Binding of azithromycin to phospholipids.

Binding of azithromycin to unilamellar vesicles (10 mM in phospholipids) was investigated by equilibrium dialysis using a Dianorm apparatus as described (Van Bambeke et al, 1996; Montenez et al, 1999), except that the molecular weight cut off was 10,000 and that dialysis was performed overnight at 4°C under constant rotation at 8 rpm.

The initial concentration of drug was set up at 132 µM (100 mg/L) in all cases. The method was validated to ensure binding equilibrium and full drug recovery at the three pH used. Azithromycin was assayed in the left chamber (containing no liposomes; D_{free}) and the apparent concentration of the total drug in the right chamber (D_{total}= D_{free}+D_{bound}) was calculated as $D_{initial}$ – D_{free} (Van Bambeke et al, 1996).

^{31}P NMR.

The effect of azithromycin on lipidic organization was studied on MLV. These liposomes give a bilayer signal due to their organization in concentric bilayers, where rapid motion of phospholipid molecules along their long axis results in axial symmetry and partially averages the chemical shift anisotropy ($\Delta\sigma$). The chemical shift anisotropy can be measured on the spectra by taking the difference of chemical shifts between the low field shoulder ($\sigma_{//}$) and the high field peak

($\sigma_\perp$). Since the shoulder at low field was not well defined, we deduced the $\Delta\sigma$ value by measuring the difference between the high field maximum chemical shift ($\sigma_\perp$) and the isotropic shift (σ_i) which corresponds to one third of the $\Delta\sigma$ value. Indeed, since the isotropic shift (σ_i) corresponds to the trace of the effective anisotropy tensor: (σ_i) = 1/3 (2 $\sigma_\perp$ + $\sigma_{//}$), the anisotropic part of this tensor is $\Delta\sigma = \sigma_{//} - \sigma_\perp$ and it comes $\Delta\sigma = 3 (\sigma_i - \sigma_\perp)$ (Seelig, 1978).

Control samples of 3 ml of phospholipids (25 mM) were prepared from concentrated MLV suspension (100 mM) by addition of buffer and 500 µl of D_2O for locking on the deuterium signal. The samples were obtained by adding concentrated drug solutions up to final concentrations of 5 mM for azithromycin and 10 mM for glucosamine (used as negative control; Mingeot-Leclercq et al, 1989).

Proton-decoupled ^{31}P NMR spectra were acquired on a Bruker AC 250 spectrometer. A 10 mm broad-banded probe was used to acquire data at 101.3 MHz. Typical Fourier transform parameters were: 2500 scans, 45°(12 µs) flip angle, 25 kHz spectral width, 4K data points, 1.2 s repetition time. A line broadening of 50 Hz was applied to the free induction decay before Fourier transformation. Spectra were recorded upon warming of the sample from 33°C to 70°C with an equilibration time of 15 min before data acquisition at a new temperature.

Langmuir-Blodgett (LB) monolayer preparation.

LB monolayers of DPPC: cholesterol (2:1) (1 mM) were prepared at 25°C with an automated system (LFW2 3''5, Lauda, Königshofen, Germany) in presence or not of azithromycin (0.2 mM) dissolved at 1 mM in chloroform:methanol (2:1). A molar mixture of DPPC:cholesterol:azithromycin (4:2:1.2) was spread on 10 mM Tris subphase adjusted at pH 7.1 with HCl. After evaporation of the solvent (30 min), monolayers were compressed at a rate of 150 cm^2/min. They were deposited at a constant surface pressure of 30 mN/m by raising vertically freshly cleaved mica through the air-water interface at a rate of 10 mm/min. The transfer ratio was always close to 0.9.

Atomic force microscopy (AFM).

AFM imaging was performed at room temperature (20°C) using a commercial optical lever microscope (Nanoscope III, Digital Instruments, Santa Barbara, CA). Contact mode topographic and friction images were recorded in air using oxide-sharpened microfabricated Si_3N_4 cantilevers (ThermoMicroscopes, Sunnyvale, CA) with typical radius of curvature of 20 nm and spring

constants of 0.01-0.03 N/m. The scan rate was 2-6 Hz. Unless otherwise stated, the applied force was maintained below 1 nN.

Studies with cultured cells.

Cell culture and incubation with agents.

J774 mouse macrophages, a continuous cell line derived from a mouse reticulosarcoma (Snyderman et al, 1977), were maintained in RPMI 1640 medium supplemented with 10 % foetal calf serum (FCS). For each series of experiments, cells were seeded at a density of 5.5 $x10^4$ cells/cm^2 and grown until confluency (2 days). At this time, they were incubated for 3 h with the indicated concentration of azithromycin before addition of the membrane-tracers, *N*-Rh-PE [*N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine], C_6-NBD-SM [6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosyl phosphocholine], TMA-DPH [1-((4-trimethylamino) phenyl)-6-phenyl-hexa-1,3,5-triene].

Insertion of C_6-NBD-SM and TMA-DPH.

To insert the fluorescent lipid analogs into the plasma membrane, appropriate amounts of C_6-NBD-SM and TMA-DPH were solubilized in absolute ethanol and dimethylformamide respectively. An aliquot of each solution was added to the medium or phosphate-buffered saline (PBS) respectively, under vigorous vortexing. Cooled cells were incubated at 4°C with C_6-NBD-SM in FCS-free medium for 30 min or with TMA-DPH in PBS for 2 min. To measure insertion of C_6-NBD-SM, cells were rapidly washed 5 times at 4°C in PBS supplemented with 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$ (PBS-Ca^{2+}-Mg^{2+}) and lysed in 0.05% (v/v) Triton X-100. Cell-associated C_6-NBD-SM was measured by fluorimetry (λ_{exc} 465; λ_{em} 530 nm). Results were determined by reference to a standard curve generated from known amounts of C_6-NBD-SM in 0.05% Triton X-100. We verified that azithromycin did not interfere with the fluorescence of C_6-NBD-SM. To measure insertion of TMA-DPH, cells were washed once with PBS and then once with 1% trypsin in PBS at room temperature. After 30 sec, cells were recovered in PBS and gently removed from the plate to give a perfectly homogeneous suspension. Cell-associated TMA-DPH was assessed on intact cells recovered in PBS by fluorimetry (λ_{exc} 362; λ_{em} 435 nm).

Insertion of N-Rh-PE.

To insert *N*-Rh-PE into the plasma membrane, appropriate amounts of the tracer were solubilized in absolute ethanol. An aliquot of this solution was added to the FCS-free medium,

under vigorous vortexing. Insertion of *N*-Rh-PE was made at 4°C during 30 min using pre-cooled cells. Cells were washed twice for 30 sec with PBS and then once with 1% trypsin in PBS at room temperature. Cells were recovered as described for TMA-DPH. To quantify the amount of membrane-inserted lipid, sodium dodecylsulphate (SDS) was added to a final concentration of 0.5% (w/v) and *N*-Rh-PE was assessed by fluorescence (Perkin Elmer LS-30 fluorimeter; λ_{exc} 560 nm and λ_{em} 590 nm). Results were determined by reference to a standard curve generated from known amounts of the probe in 0.5 % SDS (w/v). We verified that azithromycin did not interfere with the fluorescence of *N*-Rh-PE in SDS.

Treatment with methyl-β-cyclodextrin.

Cells were preincubated or not with 100 mg/L azithromycin for 3 h at 37°C and thereafter incubated in FCS-free medium but containing methyl-β-cyclodextrin for 30 min at 37°C. At this time, cells were washed with PBS and incubated 30 min with cooled PBS before incubation with *N*-Rh-PE, C_6-NBD-SM or TMA-DPH.

Labeling of the J774 macrophages cell surface by concanavalin A.

To facilitate the analysis of well-defined cells, confocal microscopy studies were performed on J774 macrophages seeded at lower density (1.3 x10^4 cells per cm^2). After washing, cells were incubated at 4°C in PBS-Ca^{2+}-Mg^{2+} for 30 min and then with concanavalin A tetramethylrhodamine isothiocyanate (20 µg/ml) in PBS-Ca^{2+}-Mg^{2+} supplemented with 1 % bovine serum albumin (BSA) for 1 h at 4°C. Cells were then fixed with 4 % formaldehyde in 0.1 M phosphate buffer pH 7.4 for 20 min at 4°C. Analysis was performed with a MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA, USA) mounted on a Zeiss Axiovert confocal microscope (Zeiss, Oberkochen, Germany) using λ_{exc} 555 and λ_{em} 580 nm).

TMA-DPH fluorescence-anisotropy measurements.

Membrane fluidity was assessed, as described in Coupin and Kuhry (1999), from fluorescence anisotropy measurements (r), which are given by : $(I_{par} - I_{per})/(I_{par} + 2\ I_{per})$ where I_{par} and I_{per} represent the components of the light intensity emitted respectively parallelly and perpendicularly to the direction of the vertically polarized excitation light. Anisotropy represents the extent of depolarization of the emitted light that accounts for membrane fluidity : a high r value corresponds to a low membrane fluidity. For fluorescence anisotropy measurements at the plasma membrane, cells were incubated for 30 sec at 37°C with 5 µM TMA-DPH. To evaluate changes in

membrane fluidity during endocytosis, cells were incubated at 37°C for 3 min with TMA-DPH (pulse), then washed 7 times at 4°C with PBS plus 5% (w/v) BSA and 4 times with PBS (to extract the peripheral labeling), and reincubated for various times in TMA-DPH-free culture medium at 37°C. At the end of the incubation, cells were washed once with 1% trypsin in PBS at room temperature. After 30 sec, cells were recovered in PBS and gently removed from the plate to give a perfectly homogeneous suspension. Cells were immediately assayed at room temperature for fluorescence anisotropy (Perkin Elmer LS50B spectropolarofluorimeter; λ_{exc} 362 nm and λ_{em} 435 nm). Each sample was measured 6 times for 15 sec.

Azithromycin assay.

Azithromycin was assayed by a microbiological assay using the disc-plate technique, as described in Montenez et al (1999).

Protein assay.

Proteins were assayed by the method of Lowry (1951) using BSA as a standard. Values of all cell constituents were expressed by reference to the protein content, except for membrane fluidity measurements.

Materials.

Azithromycin (dihydrate free base for microbiological standard; 94 % purity) was generously supplied by Pfizer s.a. (Brussels, Belgium) on behalf of Pfizer Inc. (Groton, CT, USA). Azithromycin was dissolved in 0.1 N HCl and thereafter diluted to the desired final concentrations. Glucosamine, sphingomyelin, cholesterol, methyl-β-cyclodextrin and concanavalin A-tetramethylrhodamine isothiocyanate (TRITC) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). C_6-NBD-SM and TMA-DPH were obtained from Molecular Probes (Eugene, OR, USA). *N*-Rh-PE was obtained from Avanti polar lipids (Alabaster, AL, USA). Egg phosphatidylcholine, phosphatidylinositol, and dipalmitoylphosphatidylcholine were purchased from Lipid products (Redhill, United Kingdom). All culture sera and media were supplied by Life Technologies (Paisley, UK). Other reagents were from Merck (Darmstadt, Germany).

Results

Azithromycin interacts with lipidic membranes.

Interaction between azithromycin and lipids was first investigated by equilibrium dialysis using an initial drug concentration of 100 mg/L and unilamellar vesicles in 40 mM Tris maleate buffer. Fig. 1 shows that approx. 25 % of azithromycin was bound to liposomes and that this binding was independent of the pH investigated. Similar results were obtained if phosphatidylethanolamine was added to liposomes to better mimic the lipid composition of cellular membranes (data not shown).

To further evaluate the capacity of azithromycin to interact with phospholipids, we measured by ^{31}P NMR spectroscopy the decrease in effective chemical shift anisotropy ($\Delta\sigma$) upon warming. Typical spectra obtained at pH 5.4 and 7.0 are shown in Fig. 2. In controls, signal profiles at acidic pH are characteristic of a bilayer organisation with a high field maximum and a low field shoulder. By warming, we observed a decrease of the effective chemical shift anisotropy, $\Delta\sigma$, i.e. the difference between the high-field peak and the low-field shoulder and the appearance of an isotropic signal (Fig. 2). In the presence of azithromycin, the $\Delta\sigma$ values also decreased upon warming but were higher than for control liposome spectra. The isotropic signal was less important as compared to control liposomes (Fig. 2). At neutral pH, the $\Delta\sigma$ values were not significantly different from those obtained at acidic pH but the isotropic signal was reduced (Fig 2). Additional experiments were performed with liposomes containing phosphatidylethanolamine. The $\Delta\sigma$ values also decreased upon warming and, by taking the standard deviation of $\Delta\sigma$ (1.2 ppm) into account, the results were not significantly different from those obtained with liposomes without phosphatidylethanolamine (data not shown). Again, in the presence of azithromycin, the $\Delta\sigma$ values were higher than in controls. Glucosamine, a compound which does not interact with liposomes and used as negative control (Mingeot-Leclercq et al, 1989), had no effect as compared to control liposomes at the three pH investigated (data not shown). Figure 3 shows the calculated values of the effective chemical shift anisotropy ($\Delta\sigma$) in the different conditions tested. A sharp decrease was seen when liposomes were warmed from 33°C to 70°C and the values of $\Delta\sigma$ were systematically higher when liposomes were incubated with azithromycin. No major effect of the pH was observed.

Atomic force microscopy was used to investigate the effect of azithromycin on the organization of mixed lipid Langmuir-Blodgett monolayers transferred on mica. Fig. 4A shows a representative topographic image of mixed DPPC/cholesterol monolayers. Phase-separation was observed, the step height measured between the lower and higher domains being 1.9 ± 0.1 nm. The topographic and friction contrasts always showed a negative correlation, higher friction being associated with the lower domains (data not shown). As shown in Fig. 4B-D, addition of azithromycin to the lipid mixture yielded very different morphologies. In most areas, monolayer domains were no longer seen and aggregated structures of about 1-5 nm height were distributed across the mica surface (Fig. 4B). Furthermore, while monolayers, in the absence of azithromycin, were stable during consecutive scanning, the aggregates formed in the presence of the drug were easily displaced by the scanning probe even under minimal load (Fig. 4C). The low stability of the system is further illustrated in Fig. 4D in which a 2 µm x 2 µm image was first recorded at large force (several nN), followed by imaging a 4 µm x 4 µm image of the same area under normal load (< 1 nN). The image shows that most of the aggregates have been pushed aside by the probe and have accumulated on the edges of the 2 µm area. This result also confirms the absence of a continuous film on the mica substrate.

Azithromycin differently affects the insertion of three membrane tracers in the plasma membrane and slightly impairs its fluidity.

Because azithromycin is able to interact with membranes, notably in conditions mimicking binding to the plasma membrane, we evaluated its ability to perturb the insertion and the cell-association of the head-group fluorescent-labeled phospholipid derivative, *N*-Rh-PE, a tracer which forms small lateral agregates in the plane of the plasma membrane (Kok et al, 1990). We showed that the amount of tracer incorporated into the plasma membrane of J774 increased linearly with its extracellular concentration. This was also observed in azithromycin-treated cells but with a reduction of approx. 40% over the entire range tested, i. e. between 2 and 8 µM of *N*-Rh-PE (Fig. 5A). A fixed concentration of 5 µM of *N*-Rh-PE was therefore used for subsequent experiments.

To gain more insight on the effect of azithromycin on membrane tracer insertion, we also evaluated the effect of azithromycin on the insertion of two other tracers, C_6-NBD-SM and TMA-DPH, which are distinct from *N*-Rh-PE by their organization and location after insertion into the membrane. Plasma membrane fluorescence increased proportionaly to C_6-NBD-SM concentration in the medium, as described by Mayor et al (1993) (Fig. 5B). When TMA-DPH concentration has

raised from 0.5 to 4 µM (Fig. 5C), the fluorescence intensity increased proportionally, reflecting further insertion into the plasma membrane. From 4 µM (Fig. 5C), the fluorescence continued to increase linearly fashion but at a much lower rate, which could be explained by the self-quenching of the probe at high concentration (Illinger et a, 1990; 1991). Concentrations of 4 µM C_6-NBD-SM and 2 µM TMA-DPH were therefore selected for subsequent experiments. Fig. 5B and C also show that azithromycin significantly decreased surface-inserted C_6-NBD-SM and TMA-DPH respectively by 15% and 25% over the entire range of extracellular tracer concentrations.

In parallel, we investigated the effect of azithromycin concentration on the cell surface insertion of the three tracers. The decreasing effect of azithromycin on the insertion of C_6-NBD-SM and *N*-Rh-PE to J774 macrophages increased with the concentration of azithromycin (20% for 50 mg/L and 40% for 100 mg/L for *N*-Rh-PE *vs* 10 and 20% for C_6-NBD-SM). In contrast for TMA-DPH the rate of decrease is about 25% for both 50 and 100 mg/L of azithromycin (data not shown).

To study if this decrease of membrane-tracer insertion induced by azithromycin is related to an effect of this antibiotic on membrane fluidity, we evaluated the effect of methyl-β-cyclodextrin on the plasma membrane insertion of *N*-Rh-PE, C_6-NBD-SM and TMA-DPH in control and azithromycin-pretreated cells. Methyl-β-cyclodextrin is known to remove cholesterol from cells, an important lipid for membrane fluidity (Yeagle et al, 1990). Low concentration of methyl-β-cyclodextrin (10 mM) had no major effect for the insertion of *N*-Rh-PE in control and pre-treated cells (Fig. 6A). At 20 mM, a 25% decrease of the insertion of *N*-Rh-PE was observed for control cells. This effect reached 70% for pre-treated cells with azithromycin (Fig. 6A). A different pattern was observed for control cells incubated with C_6-NBD-SM and TMA-DPH. Methyl-β-cyclodextrin decreased the insertion of C_6-NBD-SM in a concentration-dependent fashion over the entire range of concentrations tested (0 - 20 mM) reaching a 40% decrease at 20 mM (Fig. 6B) and had no significant effect on the insertion of TMA-DPH (Fig. 6C). No effect of azithromycin was observed with C_6-NBD-SM and TMA-DPH (Fig. 6B and C).

In parallel, we directly determined the potential of azithromycin to alter membrane fluidity. For this purpose, we examined the effect of azithromycin on the degree of fluorescence anisotropy (r) of TMA-DPH. Cells were preincubated with azithromycin or benzyl alcohol (a local anesthetic known to alter membrane fluidity [Friedlander et al, 1987] and used as a positive control) and

thereafter incubated for 30 sec at 37°C with TMA-DPH in the absence of drug. In the plasma membrane of control J774 cell, we found a r value of 0.268 ± 0.001. This value is similar to that found by Coupin and Kuhry (1999) and corresponds to a well organized plasma membrane. In cells pre-treated with azithromycin at increasing concentrations, the TMA-DPH fluorescence anisotropy values increase to reach a plateau value of about 0.282 ± 0.002 at 50 mg/L of azithromycin. These values were, however, only significantly different from that observed in control cells at 150 mg/L of azithromycin (Fig. 7). In contrast, in cells preincubated with 30 mM benzyl alcohol for 30 min, the r value was significantly different from that observed in control cells ($r = 0.291 \pm 0.004$; $p < 0.001$).

To gain more insight on the effect of azithromycin on membrane fluidity, we also evaluated by confocal microscopy the property of clustered glycoproteins to be mobile in the plane of the plasma membrane by labeling J774 macrophages with concanavalin A. In control cells, lectin-induced clusters become patched on the cell surface. In contrast, in azithromycin pre-treated cells, these patches disappeared, suggesting a reduced membrane fluidity which inhibit the capacity of glycoproteins to move in the lateral plane of the plasma membrane and therefore to aggregate (Fig 8).

This effect of azithromycin on membrane fluidity prompted us to investigate the evolution of TMA-DPH fluorescence anisotropy during membrane internalization. Experiments were performed during 0 to 10 min chase times, after a 3-min pulse with the tracer and a subsequent back-exchange. Anisotropy started out with a high value of 0.296 and then decreased to reach a constant value of 0.286 after approx. 5 min. In contrast, in cells pre-treated for 3 h with 100 mg/L azithromycin, r remained constant during the entire range of time (r of 0.293 at time 0 as well as at time 10 min) (Fig.9).

Discussion

The present study was performed on acellular membranes (liposomes and Langmuir-Blodgett monolayers) and on cellular model (J774 mouse macrophages) to further characterize the interactions of azithromycin with lipidic membrane and to delineate if interaction between azithromycin and phospholipid could explain its inhibitory effect on endocytosis (Tyteca et al, 2001; Tyteca et al, submitted). We focused on the effect of azithromycin on membrane-tracer organization and fluidity and will discuss the results in the context of the role played by lipids and lipid membrane properties in the budding from plasma membrane and endosomes.

Azithromycin, a dicationic weak base characterized by a partition coefficient (log P) of 4.02, has been shown by subcellular fractionation to largely co-distribute with lysosomal hydrolases where it accumulates by diffusion-sequestration process. After fractionation by isopycnic centrifugation, about 30 to 40 % of cell-associated azithromycin is not sedimentable and therefore presumably found in the cytosol (Carlier et al, 1994). Being diffusible, azithromycin could interact with the plasma membrane as well as with the cytoplasmic leaflet of the endocytic compartments. Two arguments allow us to suggest that azithromycin could perturb endocytosis by interacting with the inner leaflet of the plasma membrane rather than with the outer leaflet: (i) the inhibition of HRP accumulation is abolished if both fibroblasts and macrophages are pretreated with azithromycin in the presence of 20 μM monensin; and (ii) azithromycin preferentially interacts with acidic phospholipids which are mostly located in the inner leaflet of the plasma membrane. Likewise, acidic phospholipids are also mostly located in the cytosolic leaflet of the endocytic compartments. It is also possible that azithromycin causes impairment of endocytosis through an interaction with the luminal leaflet of membranes of the vacuolated endosomes. The data presented here establish that azithromycin is able to bind to and interact with negatively-charged phospholipid bilayers at pH 6.0 and 7.0, corresponding to that of the inner leaflet of plasma membrane and endosomes, as it was observed for experiments at pH 5.4 (Van Bambeke et al, 1996). The proportion of azithromycin bound to lipids, which never exceeded 30 %, probably reflects the fact that experiments have been performed at a relatively large ionic strength. This interaction of azithromycin with lipids is also clearly revealed by ^{31}P-NMR spectroscopy. In the presence of the drug, the chemical shift anisotropy is increased in the whole range of measured temperatures indicating that the antibiotic interacts at the level of the phosphate groups and slightly reduces their motional freedom. The comparable binding of azithromycin at the three pH examined

as observed both by equilibrium dialysis and ^{31}P-NMR spectroscopy, strongly supports conclusion that azithromycin interacts not only with phosphate groups but also with the lipophilic zones of the bilayer (Montenez et al, 1996).

With the aim to gain direct insight into the molecular interactions between azithromycin and model membranes, we used atomic force microscopy (AFM), a high resolution imaging technique which is now well-established in biophysics for characterizing lipid films on the nanometer scale (for a review, see Dufrêne and Lee, 2000). However, applications dealing with drug-membranes interactions are scarce (Onoa et al, 1998; Rotsch and Radmacher, 2000; Cazzalini et al, 2001). For mixed DPPC/cholesterol monolayers, the lower and higher levels in the topographic image can be assigned to cholesterol-rich and DPPC-rich domains, respectively, in agreement with previous data obtained for palmitic acid/cholesterol monolayers (Sparr et al., 1999). The step height, 1.9 $\pm$ 0.1 nm, measured between the two phases is in qualitative agreement with the film thickness, expected to be about 3 nm and 1.5 nm, for DPPC and cholesterol, respectively. However, the measured step height (1.9 nm) is larger than the film thickness difference (1.5 nm) which may indicate deformation of the lower cholesterol-rich domains by the AFM probe, an interpretation consistent with the friction data. Addition of azithromycin to the DPPC/cholesterol mixture dramatically alters the film properties since we observed aggregates at the mica surface, rather than a continuous film and a destabilization of the lipid system when the material is reorganized perpendicularly to the scanning direction. These results suggest thus that after spreading the DPPC/cholesterol/azithromycin monolayer at the air/water interface, the drug interacts with the DPPC molecules. Upon transfer, this binding might inhibit interactions with the mica substrate and promote aggregate formation.

Membrane destabilizing effect of azithromycin as observed on lipidic models of membranes could explain the decrease of insertion in the plasma membrane of three membrane tracers (15, 25 and 40 % inhibition of incorporation for C_6-NBD-SM, TMA-DPH and *N*-Rh-PE, respectively) induced by the drug. Specific interaction of azithromycin with some domains of the plasma membrane, in an asymmetric fashion across the bilayer and/or in its lateral plane, could be related to the differential effect observed for C_6-NBD-SM, TMA-DPH and *N*-Rh-PE. First, the lesser decrease of the incorporation of C_6-NBD-SM induced by azithromycin compared to that of TMA-DPH could be explained by the fact that C_6-NBD-SM labels the outer interfacial zone of the bilayer and does not translocate to the cytoplasmic surface during the endocytic process (Koval and Pagano, 1989). In contrast, TMA-DPH is a synthetic compound labeling the deep domain of

the bilayer (Kaiser and London, 1998). The effect of azithromycin on the incorporation of *N*-Rh-PE in the plasma membrane is more difficult to understand since the behavior of *N*-Rh-PE is not so well characterized. However, we know from our studies and from the litterature that *N*-Rh-PE is organized as small aggregates in the bilayer (Kok et al, 1990) and is not exchangeable. This organization could explain the more important effect of azithromycin on the plasma membrane insertion of *N*-Rh-PE, in comparison with C_6-NBD-SM and TMA-DPH, but also the marked effect of methyl-β-cyclodextrin on *N*-Rh-PE incorporation in cells pre-treated with azithromycin. Second, in term of lateral organization, the fact that methyl-β-cyclodextrin decreased the insertion of C_6-NBD-SM without affecting the insertion of TMA-DPH to control J774 is probably related to the fact that C_6-NBD-SM, but not TMA-DPH, is localized in cholesterol-rich microdomains. Azithromycin would not interact with these specific lateral microdomains since this drug did not change the effect of methyl-β-cyclodextrin on the insertion of C_6-NBD-SM and TMA-DPH. The interpretation of the effect of methyl-β-cyclodextrin could be, however, more complicated. Indeed, it is well known that this compound induces a depletion of cholesterol, a lipid known to alter the membrane fluidity (Yeagle et al, 1990) which in turn could explain its perturbating effect on pinocytosis and phagocytosis (Subtil et al, 1999; Rodal et al, 1999; Peyron et al, 2000). This explanation could have a great significance since we show in this study a decrease tendency of membrane fluidity induced by the interaction of azithromycin with lipid membranes and the disappearance of concanavalin A receptor patching after treatment with azithromycin. Consistently, azithromycin maintained *N*-Rh-PE in a more aggregate state upon tracer internalization (Tyteca et al, submitted).

In conclusion, we have shown that azithromycin is able to interact with lipids, to destabilize membranes and to modify their biophysical properties at selective sites of the plasma membrane as well as on endosomes. The probleme is now to reconcile the interaction of azithromycin with lipids, and its effect on membrane fluidity with the inhibition of fluid-phase and bulk-membrane endocytosis and the decreased mobility of receptors at the plasma membrane and in endosomes (Tyteca et al, submitted). We know that endocytosis is initiated by the budding of vesicles from living cell plasma membranes (Farge et al, 1999). This process is considered as a transition of a relatively planar structure to a more curved structure. Two major driving forces are involved for the vesicle formation *in vivo*. The first is the polymerization of proteins, like clathrin, onto the cytoplasmic phospholipid leaflet aided by an array of mostly cytosolic proteins, often referred as “accessory proteins” (Takei and Haucke, 2001; Huttner and Zimmerberg, 2001). The second,

observed in absence of clathrin, is the active generation of phospholipid asymmetry between the two monolayers of the plasma membrane (Farge and Devaux, 1992; Farge, 1994 ; Devaux and Zachowski, 1995; Zha et al, 1998). Both driving forces locally induce the curvature of the membrane which then generates buds. This process would be altered by the interaction of azithromycin whith specific sites of the membrane, especially those generating uncoated vesicles, characterized by a low lipid order compared to those involved in the formation of coated vesicles (Coupin and Kuhry, 1999). Accordingly, we suggest that selective perturbation due to azithromycin may help to determine the physico-chemical properties involved in endocytosis and to further explore the membrane properties involved in budding processes.

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Figures

Captions

Figure 1: Influence of pH on azithromycin binding to negatively-charged unilamellar vesicles, as determined by equilibrium dialysis. Azithromycin (initial concentration 132 µM, 100 mg/L; corresponding to a drug: phospholipid ratio of 0.013) in 40 mM Tris maleate buffer, pH 5.4, 6.0 or 7.0 was placed in the left chamber. Buffer (control) or liposomes (cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol: 5.5:4:4:3 molar ratio), prepared in the same buffer, were placed in the right chamber. The abcissa shows the azithromycin concentrations measured in the left chamber, which represents the free drug (D_{free}), and the calculated concentrations of the total drug (bound and free drug; D_{total}) in the right chamber (D_{total} = 120 – D_{free}) at the end of the experiment. Results are means ± SEM of 3 independent experiments, each made of 3 measurements at each pH.

Figure 2: ^{31}P nuclear magnetic resonance (NMR) spectra of large multilamellar vesicles (25 mM in phospholipids) obtained at pH 5.4 and 7.0 both at 33°C and 70°C, in the absence or in the presence of 5 mM azithromycin (corresponding to drug:phospholipid molar ratio of 0.2). Liposomes were made as in Fig 1. The isotropic signal was set at 0 ppm as a reference and the figures show the chemical shift values of the peak at high field.

Figure 3: Variation of the effective chemical shift anisotropy ($\Delta\sigma$) in ^{31}P NMR of large multilamellar vesicles as a function of temperature. Liposomes were prepared and treated as in Fig. 2, and incubated (closed symbols) or not (open symbols) at 37°C for 1 hour with azithromycin at drug:phospholipid molar ratio of 0.2 at pH 5.4 (circles), 6.0 (triangles) and 7.0 (squares). $\Delta\sigma$ values are means of three determinations.

Figure 4: Atomic force microscopy (AFM) topographic images showing the influence of azithromycin on the nanoscale organization of mixed DPPC/cholesterol Langmuir-Blodgett (LB) monolayers supported on mica: A, DPPC/cholesterol (2:1 molar ratio) monolayer; B, C, D, DPPC/cholesterol/azithromycin (4:2:1.2 molar ratio) monolayers. The image size is 5 µm x 5 µm for A, B, C and 4 µm x 4 µm for D. The *z*-range is 15 nm (A) and 5 nm (B, C, D). In Fig. 4 D, a 2 µm x 2 µm image was first recorded under high load followed by recording a 4 µm x 4 µm image

of the same area under normal load. All LB films were prepared on a water subphase adjusted at pH 7.1 with 10 mM Tris buffer.

Figure 5: Effect of azithromycin on the insertion of (A) *N*-(lissamine rhodamine B sulfonyl)diacylphosphatidylethanolamine (*N*-Rh-PE), (B) 6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosyl phosphocholine (C_6-NBD-SM), and (C) 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) in J774 mouse macrophages. Cells were either untreated (open squares) or pre-treated with 100 mg/L azithromycin for 3 h (filled squares) prior to labeling at 4°C with increasing concentrations of each lipid tracers. The insertion of *N*-Rh-PE was measured after addition of 0.5 % SDS, an anionic surfactant, to disrupt J774 mouse macrophages and therefore to obtain a measure of the total content of *N*-Rh-PE inserted in the plasma membrane. Values are means ± SD of 3 dishes. Experiments were reproduced 4, 4 and 3 times in A, B and C respectively.

Figure 6: Effect of azithromycin on the insertion of (A) *N*-Rh-PE, (B) C_6-NBD-SM and (C) TMA-DPH in the plasma membrane of J774 cells in function of the extracellular concentration of methyl-β-cyclodextrin. Cells were either untreated (open squares) or treated with 100 mg/L azithromycin for 3h at 37°C (closed squares), then incubated for 30 min with the indicated concentrations of methyl-β-cyclodextrin in FCS-and azithromycin-free medium and finally incubated at 4°C with 5 µM *N*-Rh-PE (A), 4 µM C_6-NBD-SM (B) or 2 µM TMA-DPH (C). Results are mean ± SD of 3 dishes. Experiments were reproduced twice in each panel.

Figure 7: Effect of azithromycin on TMA-DPH fluorescence anisotropy (r) in the plasma membrane of J774 macrophages. After 3 hours of preincubation with the indicated azithromycin concentration, cells were incubated for 30 sec at 37°C with 5 µM TMA-DPH and washed once with 1% trypsin in PBS at room temperature. Cells were recovered in PBS and assayed at room temperature for fluorescence anisotropy. Values are means ± SD of 3 dishes. Experiments were reproduced 3 times.

Figure 8: Confocal microscopy of J774 cell surface by labeling with red fluorescent concanavalin A at 4°C. Cells were either untreated (A) or preexposed to 100 mg/L azithromycin for 3 h at 37°C (B). Cells were then cooled, labeled with concanavalin A TRITC (red) at 4°C, fixed and finally examined by confocal microscopy. Bar is 10 µM.

Figure 9: Effect of azithromycin on TMA-DPH fluorescence anisotropy (r) in J774 upon internalization. Cells were either untreated (open squares) or pre-treated with azithromycin (100 mg/L for 3 h filled squares) prior to incubation with 5 µM TMA-DPH for 3 min at 37 °C. Cells were then washed 7 times at 4°C with PBS plus 5% (w/v) BSA and 4 times with PBS. They were thereafter incubated for the indicated times in TMA-DPH-free medium and gently recovered. Fluorescence was measured with a Perkin-Elmer LS-50 under conditions of negligible scattering. Experiments were reproduced 3 times.

Figure 1

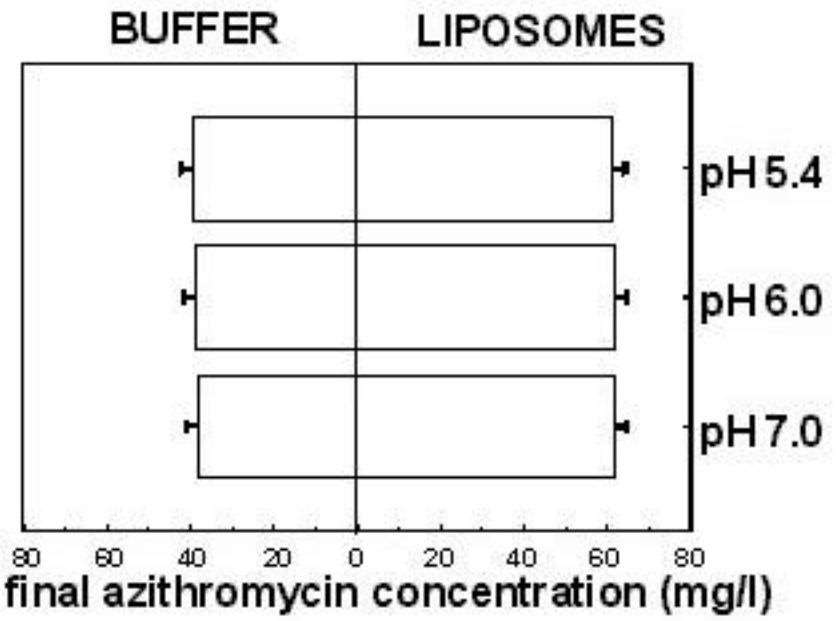

BUFFER
LIPOSOMES
pH 5.4
pH 6.0
pH 7.0
80
60
40
20
0
20
40
60
80
final azithromycin concentration (mg/l)

Figure 2

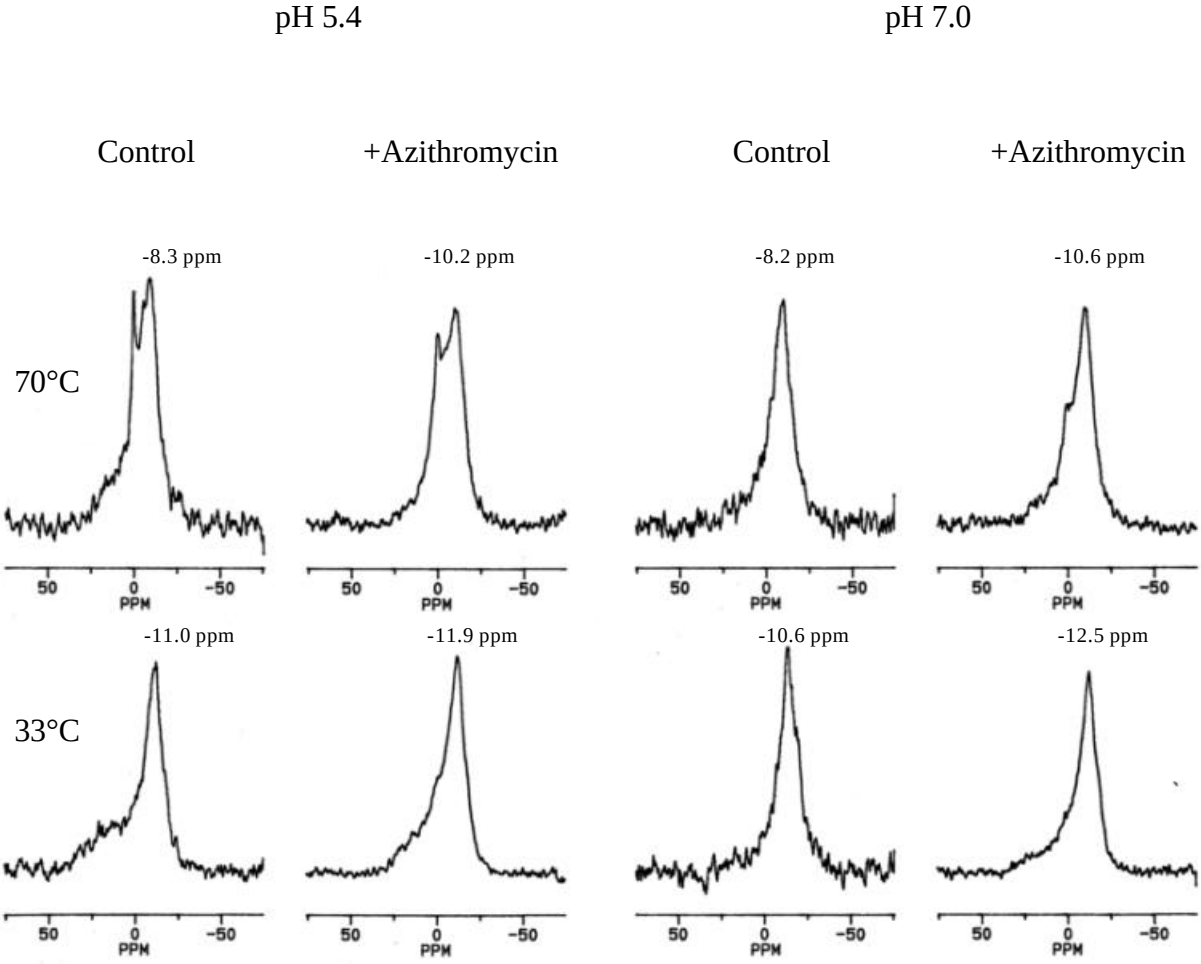

pH 5.4
pH 7.0
Control
+Azithromycin
Control
+Azithromycin
-8.3 ppm
-10.2 ppm
-8.2 ppm
-10.6 ppm
70°C
-11.0 ppm
-11.9 ppm
-10.6 ppm
-12.5 ppm
33°C
50
0
-50
PPM

Figure 3

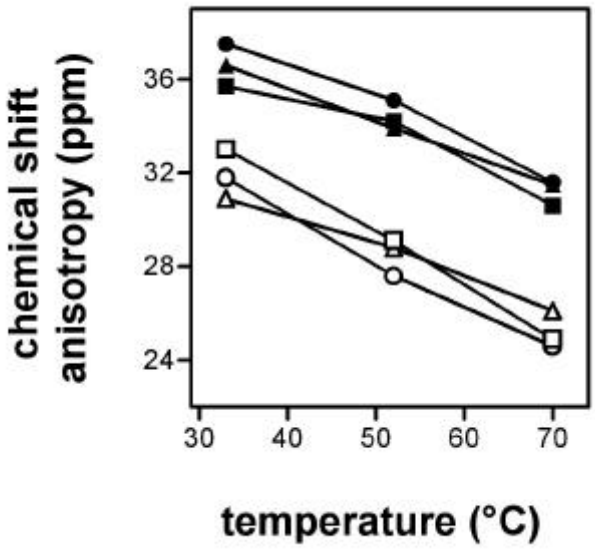

chemical shift
anisotropy (ppm)
36
32
28
24
30
40
50
60
70
temperature (°C)

Figure 4

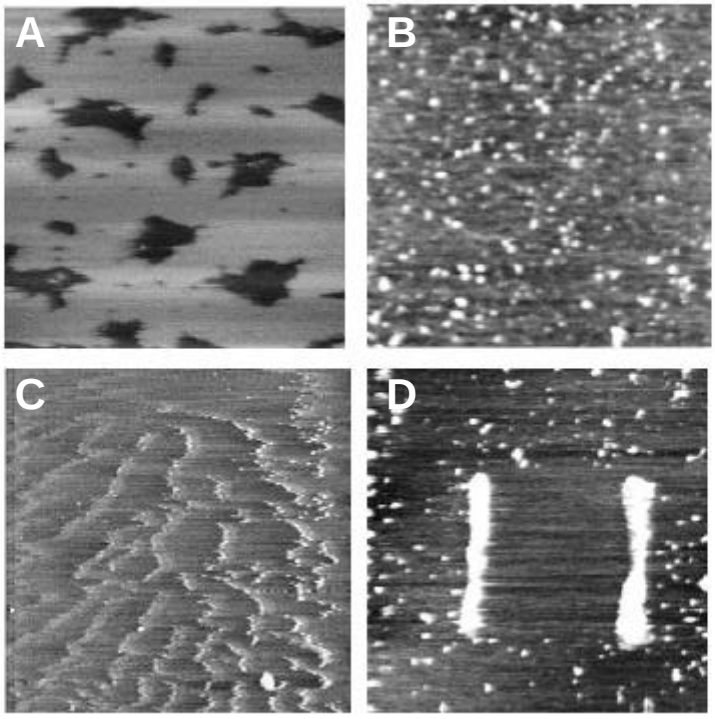


Figure 5

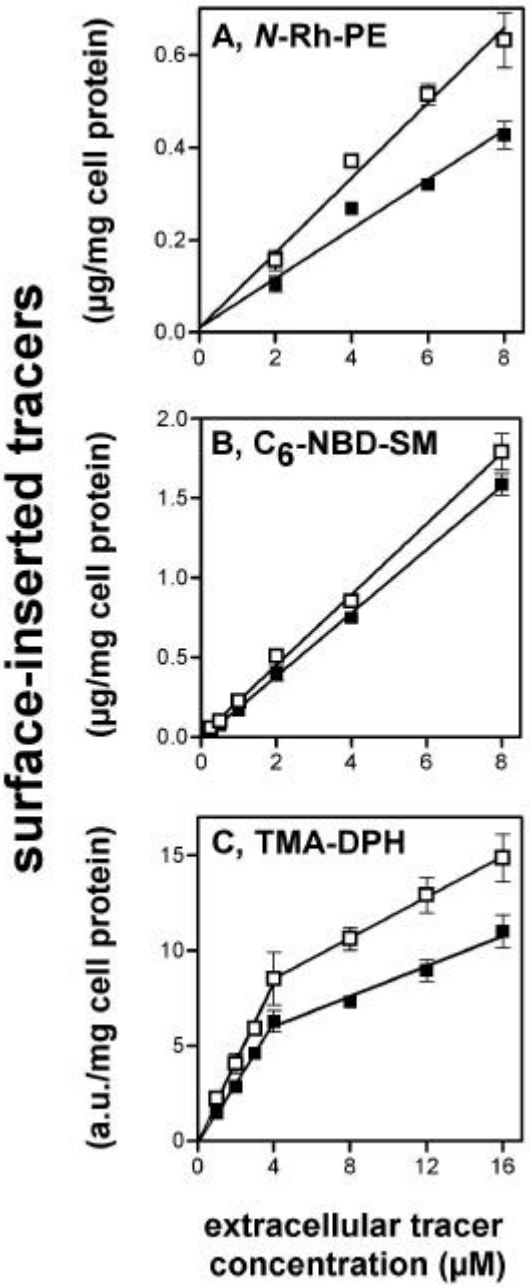

surface-inserted tracers
A, N-Rh-PE
(µg/mg cell protein)
0.0
0.2
0.4
0.6
0
2
4
6
8
B, C6-NBD-SM
(µg/mg cell protein)
0.0
0.5
1.0
1.5
2.0
0
2
4
6
8
C, TMA-DPH
(a.u./mg cell protein)
0
5
10
15
0
4
8
12
16
extracellular tracer concentration (µM)

Figure 6

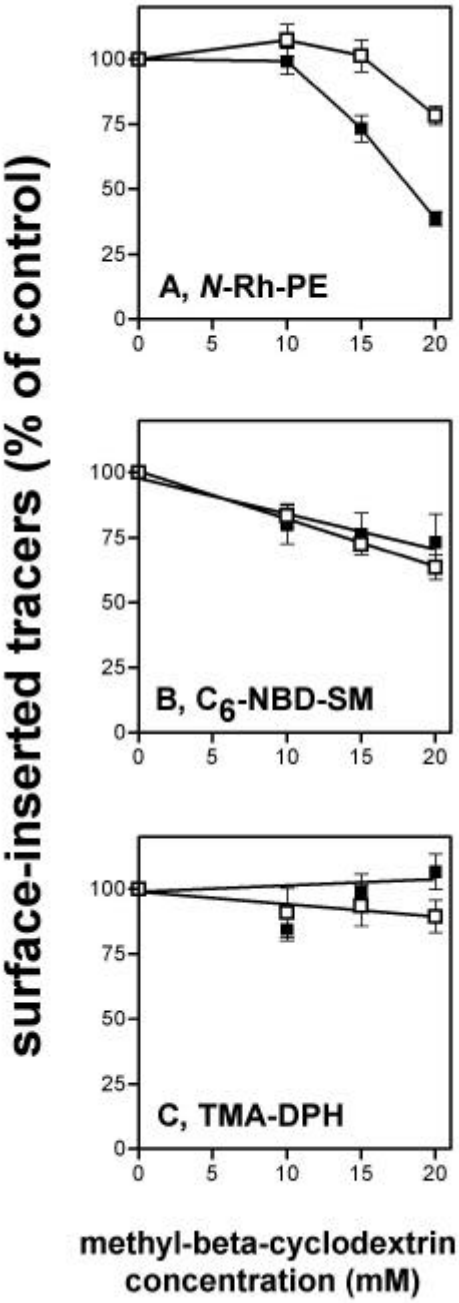

surface-inserted tracers (% of control)
A, N-Rh-PE
B, C6-NBD-SM
C, TMA-DPH
methyl-beta-cyclodextrin concentration (mM)

Figure 7

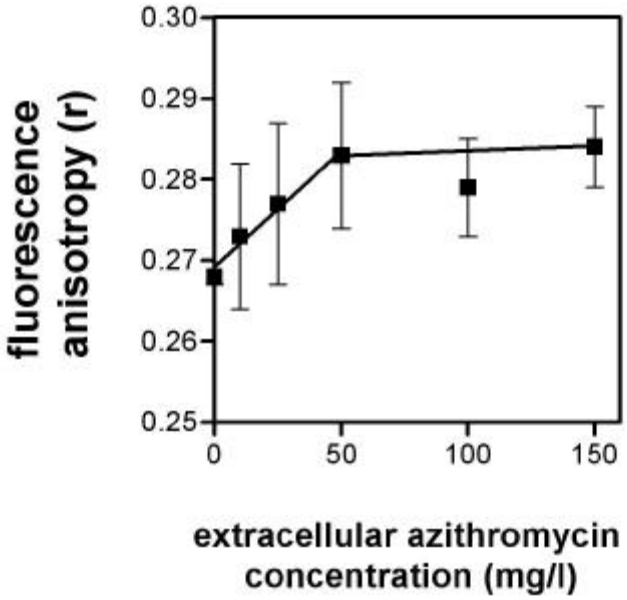
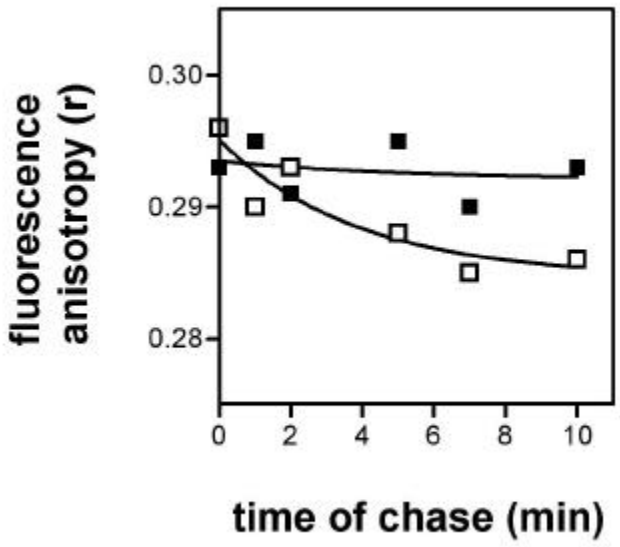

fluorescence anisotropy (r)
0.30
0.29
0.28
0.27
0.26
0.25
0
50
100
150
extracellular azithromycin concentration (mg/l)

Figure 9.


fluorescence anisotropy (r)
0.30
0.29
0.28
0
2
4
6
8
10
time of chase (min)

CHAPTER 4. GENERAL DISCUSSION

1. Azithromycin does not equally affect all endocytic pathways

The study made on J774 mouse macrophages discloses several striking findings. Indeed, azithromycin: (i) severely inhibits fluid-phase endocytosis; (ii) inhibits to a comparable extent bulk-membrane endocytosis; (iii) delays sequestration of Tf/receptor and PAP/receptor into clathrin-coated pits at the cell surface, but does not inhibit the subsequent rate of internalization; (iv) delays recycling of Tf without affecting its subsequent rate of efflux; (v) causes a major vacuolation of the late endocytic apparatus and severely inhibits its accessibility by fluid-phase endocytic tracers and immune complexes internalized by the Fcγ-receptors; and (vi) does not slow down phagocytosis nor impede access of phagosomes to swollen late endosomes/lysosomes.

The strong effect of azithromycin on fluid-phase endocytosis of HRP is also observed in rat foetal fibroblasts, by biochemical and morphological studies. However, in this cell type, no effect has been observed on receptor-mediated endocytosis of Tf, neither on cell-surface binding, nor on ligand internalization. Three hypotheses could explain this difference: (i) the rate of internalization of Tf in the two cell types, which is approx. 4 times slower in rat foetal fibroblasts than in J774 macrophages; (ii) the size of internal pools for TfRs, which represent 60-80 % of the cellular TfR content in J774 macrophages and which could be less important in fibroblasts; and (iii) the properties of endosomes, i.e. the shape, pH and membrane properties of these organelles, which could be different in both cell types.

2. Azithromycin could perturb three distinct steps of the endocytic pathways

In addition to the observation that azithromycin does not equally affect all endocytic pathways, we also distinguish three steps of the endocytic pathways which are affected by azithromycin.

We propose that the first step affected by azithromycin could be at the plasma membrane. Indeed, we have shown that azithromycin: (i) delays sequestration of Tf/receptor and PAP/receptor complexes into clathrin-coated pits at the cell surface; (ii) does not inhibit their subsequent rate of internalization while it affects fluid-phase endocytosis of HRP and LY; (iii) perturbs pinocytosis but not phagocytosis. Since biogenesis of phagolysosomes proceeds through sequential interactions with the endocytic apparatus, and since fluid-phase and receptor-mediated endocytosis are connected at the level of early endosomes, this implies that the effect of azithromycin on the endocytic traffic would take place on the very early steps of endocytosis, before early endosomes. We therefore suggest that azithromycin could perturb endocytosis by impairing lateral mobility of receptors and by preferentially affecting the formation of primary non-clathrin-coated pits.

The second step affected by azithromycin could be at sorting and/or recycling endosomes. Indeed, we have shown that azithromycin delays the recycling of TfRs. This could occur by impairing their lateral mobility at the endosomal membrane, thereby delaying orientation into the recycling pathway. Major intracellular sequestration of TfRs has been observed in J774 macrophages and accounts for the corresponding decrease of TfR-mediated endocytosis. The relocation of receptors was not observed with FcãRs, explaining why inhibition on their endocytosis is marginal in comparison with that of Tf. Our results are consistent with the observation that internal pools for TfRs in J774 mouse macrophages represent 60-80 % of the cellular TfR content (Buys and Kaplan, 1987), whereas total intracytoplasmic membrane area of the same cells containing FcRs is probably not more than 20 % of the plasma membrane area (Mellman et al, 1984).

The third step perturbed by azithromycin could be at the level of fusion between endosomes and lysosomes, but not between phagosomes and lysosomes. We have shown that azithromycin causes a major vacuolation of the late endocytic apparatus and severely inhibits its accessibility by HRP and PAP, but does not impair access of phagosomes to swollen late endosomes/lysosomes, since the latter are fully accessed by latex beads.

3. The effect of azithromycin on fluid-phase endocytosis is rapid, reversible and directly related to the cellular drug content

Because a strong effect of azithromycin was observed on fluid-phase endocytosis, we analyzed this process in non-phagocytic cells (fibroblasts) as well as in phagocytic cells (macrophages). We observed that the effect of azithromycin: (i) is rapid; (ii) increases linearly with the extracellular drug concentration in macrophages, but levels off at 25 mg/l in fibroblasts; (iii) is not related to the extracellular but well to the intracellular drug content, since the inhibition is abolished if both cells are pretreated with azithromycin in the presence of 20 μM monensin; (iv) does not require the continued presence of the drug, lasts for at least 4 h after drug removal; (v) is almost completely reversible after 24 h of chase in both cell types, after a 3-h pretreatment with the drug; and (vi) is not correlated with the phospholipidosis in fibroblasts, nor in macrophages since it does not develop in the latter.

4. Impairment of endocytosis by azithromycin could more likely be attributed to its capacity to interact with membranes than by a general toxic effect, phospholipidosis, acidotropic swelling and lysosomal pH increase

We have shown that treatment of rat foetal fibroblasts and J774 macrophages by azithromycin for 3 h causes an intense vacuolation of phagosomes and late endosomes/lysosomes. The presence of these vacuoles has been revealed by electron microscopy, and their nature has been further examined by confocal microscopy using lucifer yellow (a fluorescent fluid-phase tracer) and lysoTracker green (a fluorophore which concentrates in acidified compartments) in fibroblasts and macrophages respectively. Both substances label azithromycin-induced vacuoles, indicating that these structures belong to the late endosome/lysosome compartments. We have also observed that the fluorescence intensity is decreased when the vacuole size is increased. For this reason, we have tested, in macrophages, the hypothesis that azithromycin could, like other weak bases, perturb lysosomal pH, by measuring cellular accumulation of lysoTracker green in a biochemical assay. We have observed a two-fold decrease of the lysoTracker accumulation in azithromycin-treated macrophages, suggesting that, at least in macrophages, azithromycin is able

to impair endosomal/lysosomal pH. Another important observation was that a 3 days treatment of fibroblasts with azithromycin resulted in the accumulation of very large vesicles filled with phospholipids and cholesterol.

Based on these observations, how can we explain the mechanism of azithromycin action? First, it is important to note that impairment of endocytosis by azithromycin could not be attributed to a general toxic effect neither on rat foetal fibroblasts nor on J774 mouse macrophages, since no major change in ATP levels, plasma membrane integrity, and protein and DNA syntheses could be observed during the period of study. Ultrastructure of organelles also appeared normal, except for occasional Golgi swelling and major alteration of endosomal and lysosomal compartments (see above).

Second, azithromycin effect could not be attributed to the accumulation of phospholipids in lysosomes, since the inhibition of endocytosis was not correlated with the development of phospholipidosis in fibroblasts, and since no phospholipidosis occured in macrophages (either because the phospholipid turn-over is more rapid in J774, or because lysosomal activity is more intense, or because J774 macrophages would have died before).

Third, the impairment of fluid-phase uptake, of Tf recycling and of fusion with lysosomes could be related to the intense vacuolation caused by the drug. These vacuoles must be considered as being part of a dynamic late endosomal/lysosomal compartment, since they were stained with the lysoTracker. These swelled late endosomes/lysosomes could become unable to fuse with incoming pinocytic vesicles. However, this hypothesis could not explain two properties of azithromycin, i.e. the delayed sequestration of Tf and PAP into coated pits at the cell surface, and the absence of effect on phagocytosis and on the accessibility of latex beads to lysosomes.

A fourth hypothesis is that azithromycin accumulation in endosomes causes an alteration of endosomal acidification which could be responsible for the reduced fluid-phase endocytosis, the impairment of the capacity of endosomes to deliver their load to lysosomes, and the sequestration of TfRs. These compartments are indeed less acidic in comparison to controls. These effects could reflect an inhibition of the recruitment or function of the machinery catalysing the transfer of material to lysosomes. In this context, a subset of COPs has been shown to associate with endosomes in a pH-dependent manner and to mediate trafficking between early and late endosomes. However, perturbation of acidification does not directly account for three properties of

azithromycin, including: (i) the delayed sequestration of Tf and PAP into coated pits at the cell surface, without affecting the rate of internalization; (ii) the delay of Tf recycling without impairment of subsequent rate of efflux, contrasting with the effects of monensin or bafilomycin A1, both drugs able to perturb lysosomal pH; and (iii) the absence of effect on phagocytosis and on the accessibility of latex beads to lysosomes.

Another possibility is that azithromycin interferes with microtubules and/or microfilaments, perturbing therefore membrane transport during endocytosis. However, experiments made in both cell types with nocodazole, a microtubule inhibitor, does not support this hypothesis, since both drugs show only additive effects on the inhibition of fluid-phase endocytosis of HRP.

Thus, from all these observations, the most plausible hypothesis which could explain: (i) the impairment of fluid-phase endocytosis, (ii) the reduced lateral mobility at the plasma membrane, (iii) the impairment of Tf recycling, (iv) the reduced transfer of fluid-phase tracers to lysosomes, and (v) the absence of effect on phagocytosis, would be direct interaction of azithromycin with membranes. Indeed, a part of cell-associated azithromycin is not sedimentable and therefore presumably found in the cytosol (Carlier et al, 1994) and could have access to the inner leaflet of the pericellular membrane and the cytosolic leaflet of endosomal membranes. Two arguments allow us to suggest that azithromycin could perturb endocytosis by interacting with the inner leaflet of the plasma membrane rather than with the outer leaflet: (i) the inhibition of HRP accumulation is abolished if both fibroblasts and macrophages are pretreated with azithromycin in the presence of 20 μM monensin; and (ii) azithromycin preferentially interacts with acidic phospholipids which are mostly located in the inner leaflet of the plasma membrane. Likewise, acidic phospholipids are also mostly located in the cytosolic leaflet of the endocytic compartments.

5. Comparison of rat foetal fibroblasts and J774 mouse macrophages for characterization of azithromycin properties and perturbation of endocytosis

In this section, we have compared the behavior of both J774 mouse macrophages and rat foetal fibroblasts in control conditions but also after cell pretreatment with azithromycin. Different comparisons were made: (i) azithromycin accumulation; (ii) vacuolation induced; (iii)

phospholipidosis induced; (iv) cellular viability; (v) fluid-phase endocytosis in control and pretreated cells; and (iv) receptor-mediated endocytosis in control and pretreated cells.

Parameter	**Rat foetal fibroblasts**		**J774 macrophages**
	< 24 h-pretreatment (3 h-pretreatment unless stated otherwise)	**24 h-pretreatment** (72 h-pretreatment unless stated otherwise)	**< 24 h-pretreatment** (3 h-pretreatment unless stated otherwise)
Characterization of AZ uptake and release in function of			
extracellular AZ concentration	partial saturation from 25 mg/l	partial saturation from 25 mg/l	linearly increases with the extracellular concentration
time of preincubation with AZ	maximal at 3 h	same level of accu-mulation until < 72 h increases at 72 h	maximal at 1 h
time of AZ release	half-life of 3.6 h	50 % release after 48 h of chase	half-life of 3 h
incubation with 100 mg/l AZ	for 3 h : 40 –50 µg / mg cell protein	for 72 h : 70 – 80 µg / mg cell protein	for 3 h : 60 – 80 µg / mg cell protein
incubation with AZ + monensin	abrogates AZ accumulation by > 90 %	ND	abrogates AZ accumulation by > 90 %
Vacuoles and overloaded-vesicles induced by AZ			
vacuole size	up to 3 µm	up to 5 µm	up to 10 µm
Phospholipidosis induced by AZ			
phospholipid content (% of control)	up to 115 %	up to 160 %	no development
Effect of AZ on cellular viability			
LDH release	no effect	increase	no effect
protein synthesis	no effect	no effect	no effect
DNA synthesis	no effect	no effect	no effect
ATP content	no effect	up to 50 % decrease	no effect

Fluid-phase endocytosis in control cells			
clearance after 5 min of uptake	2 nl / min / mg cell prot	3.5 nl / min / mg cell prot	11 nl / min / mg cell prot
regurgitation after 15 min load	ND	40 % release	20 % release
Effect of AZ on fluid-phase endocytosis			
internalization	ND	maximal after 2 min	maximal after 2 min
regurgitation	ND	no effect	slight increase
fluid transfer to vacuolated structures caused by AZ	HRP has no access to vacuoles (EM) LY has a slight access to vacuoles (FM)	HRP has no access to phospholipid and cholesterol overloaded vesicles (EM)	HRP has no access to vacuoles (EM and FM)
typical lysosomes	reduced number of HRP-containing lysosomes (EM)	reduced number of HRP-containing lysosomes (EM)	reduced number of HRP-containing lysosomes (EM)
Characterization of the inhibition of HRP accumulation by AZ in function of			
extracellular AZ concentration	levels off at 25 mg/l	levels off at 50 mg/l	linearly increases with extracellular concentration
time of preincubation with AZ	maximal after 3 h	same level of inhibition until < 72 h increased effect at 72 h	maximal after 1 h
time of AZ release	**half-life of the inhibition of HRP accumulation of 11.8 h**	50 % decrease after 48 h of chase	half-life of 4 h after 3 h of persisted effect
incubation with AZ + monensin	abrogates inhibition	ND	abrogates inhibition
correlation with	AZ cellular content not phospholipidosis		AZ cellular content
Receptor-mediated endocytosis in control cells			
cell surface-binding	ND	400 fmol / mg cell protein	1200 fmol/mg cell protein
internalization	ND	8 % internalized per min	> 30 % internalized per min
recycling	ND	ND	half-life of 18 min
Effect of AZ on receptor-mediated endocytosis			

cell surface-binding	ND	no effect	50-80 % reduction
internalization	ND	no effect	1-min delay
recycling	ND	ND	half-life of 12 min after a 10-min delay

Table 9. Comparison of rat foetal fibroblasts and J774 mouse macrophages for characterization of azithromycin properties and perturbation of endocytosis. ND, not determined.

6. Azithromycin may perturb endocytosis by membrane interaction and perturbation of membrane properties

Based on this hypothesis, we have therefore explored, in the third part of this work, the capacity of azithromycin to interact with membranes and to perturb some membrane properties, by using J774 mouse macrophages as well as model membranes such as liposomes and Langmuir-Blodgett monolayers.

First, we have shown that azithromycin was able to bind to and interact with negatively-charged phospholipid bilayers at pH 6.0 and 7.0, close to those of endosomes and plasma membrane. Moreover, we have confirmed the observation of Montenez et al (1996) that azithromycin interacts not only with phosphate groups but also with the lipophilic zones of the bilayer.

Second, because the plasma membrane surface asymmetry and the formation of cholesterol-based membrane microdomains are regarded as driving forces for vesicle formation during endocytosis in living cells (Farge, 1995; Farge et al, 1999; for a review, see Huttner and Zimmerberg, 2001), we examined the effect of azithromycin on membrane organization. First, by using atomic force microscopy, we demonstrated that addition of azithromycin to the DPPC/cholesterol mixture dramatically alters the film properties, suggesting a membrane destabilizing effect of azithromycin. Second, we showed that azithromycin was able to decrease the insertion in the plasma membrane of three membrane tracers, C_6-NBD-SM by 15 %, TMA-DPH by 25 %, and *N*-Rh-PE by 40 %. In the presence of methyl-β-cyclodextrin, the effect of azithromycin on *N*-Rh-PE incorporation was enhanced, suggesting that both drugs acted

synergistically. The interpretation of the effect of methyl-β-cyclodextrin is complicated, since this compound which induces a depletion of cholesterol, perturbs lateral organization of the plasma membrane but also its fluidity.

Membrane fluidity influences endocytic and phagocytic activities (Mahoney et al, 1977; Calder et al, 1990), and clathrin-dependent and clathrin-independent endocytosis can be differentiated by means of membrane fluidity measurements (Liaubet et al, 1994; Coupin and Kuhry, 1999). We therefore explored membrane fluidity, and suggested a decrease of membrane fluidity induced by the interaction of azithromycin with lipid membranes, as measured by polarization fluorescence techniques, and evidenced by the absence of concanavalin A-induced patching of surface glycoproteins. Further experiments are in progress to confirm this observation.

In conclusion, we showed that azithromycin is able to interact with lipids, to destablize membrane and to modify its biophysical properties. Since these membrane properties are involved in the process of vesicle formation *in vivo*, we suggest that azithromycin would perturb endocytosis by this way. Among the major driving forces thought to be involved in the vesicle formation are the polymerization of proteins such as clathrin onto the cytoplasmic phospholipid leaflet, cortical actin remodelling, and the generation of phospholipid number asymmetry between the two leaflets of the plasma membrane (Farge and Devaux, 1992; Devaux and Zachowski, 1994; Farge, 1995; Zha et al, 1998; Huttner and Zimmerberg, 2001). One might therefore suggest that azithromycin selectively impairs clathrin-independent endocytosis and does not perturb receptor-mediated endocytosis or phagocytosis, because CCVs and phagosomes are covered by clathrin lattices and actin respectively, both of which would impose a local constraint on membrane fluidity. Perturbation of membrane fluidity could also explain the delay of receptor mobility at the level of plasma membrane as well as in endosomes. Perturbation of fusion between endosomes and lysosomes could also be explained by an impairment of membrane fluidity and/or organization. Since actin is sometimes involved in the fusion of phagosomal and endosomal compartments (Guerin and de Chastellier, 2000), we could explain why azithromycin does not perturb this heterotypic fusion, once again because a constraint could be imposed by the actin cytoskeleton.

7. Is the perturbing effect of azithromycin on membrane fluidity and organization direct or indirect ?

Although it is clear from these studies that azithromycin is able to interact with membranes and to modify their properties, we do not currently know by which mechanism this perturbation occurs.

With regard to lipids, membrane fluidity is influenced by fatty acyl length and insaturation, by cholesterol content, and by membrane organization in microdomains, but not by phospholipid head group modifications (for reviews, see Spector and Yorek, 1985; Mukherjee and Maxfield, 2000). It is possible that azithromycin would perturb the activity of enzymes involved in the very early steps of endocytic trafficking, impairing thereby membrane lipid composition and therefore its fluidity. Among these are PI 3-kinases, PLD and the neutral sphingomyelinase (see section 7.2.2.).

PI 3-kinases are involved in fluid-phase endocytosis, internalization of Tf, clathrin-mediated endocytosis, and phagocytosis of large latex beads (Clague et al, 1995; Li et al, 1995; Cox et al, 1999). However, it is unlikely that azithromycin would perturb the activity of this enzyme, since we have shown that wortmannin does not impair fluid-phase endocytosis of HRP neither in fibroblasts nor in macrophages (data not shown).

PLD plays a key role in AP-2 (a component of clathrin-coated pits) recruitment on plasma membrane and endosomes (West et al, 1997). It is therefore possible that azithromycin perturbs the sequestration of receptors into clathrin-coated pits by impairing PLD activity.

Another possibility is that azithromycin could perturb clathrin-independent endocytic pathways by the inhibition of the neutral sphingomyelinase. Indeed, sphingomyelinase induces the formation of numerous vesicles that pinch off from the plasma membrane, and which are able to deliver fluid-phase markers to late endosomes/lysosomes and return recycling receptors back to the plasma membrane (Zha et al, 1998). In addition, we have shown in our model of rat foetal fibroblasts that azithromycin is able to strongly inhibit the activity of lysosomal sphingomyelinase. In this context, we propose to study the effect of azithromycin on the activity of neutral

sphingomyelinase, which could be involved in vesicle formation at the plasma membrane (see section 7.2.2.).

8. Is azithromycin an usefull agent for controlled perturbations of endocytic trafficking?

It is clear that azithromycin, like other pharmacological agents, pertubs many, but not only one, steps of endocytosis. However, all these steps were not equally affected by the same concentration of azithromycin. Indeed, inhibition of fluid-phase pinocytosis, accessibility of Tf surface receptors to digestion, delay of receptor-mediated endocytosis show distinctive concentration dependence. Indeed, different IC50 were found for fluid-phase pinocytosis (<100 mg/l), accessibility of cell surface TfR to pronase-digestion (25 mg/l), and receptor-mediated endocytosis (>100 mg/l).

As described in the introduction, PEG-chols are also able to selectively inhibit clathrin-independent endocytosis at low concentrations (Ishiwata et al, 1997; Baba et al, 2001). Since agents perturbing clathrin-independent pathway are clearly lacking (see Dautry-Varsat, 2001), azithromycin could be usefull because it preferentially inhibits this internalization route. Indeed, some receptors such as the interleukin-2 receptor, and toxins are thought to enter the cell by a still obscure clathrin-independent pathway. However, this route is less characterized and still less is the cell signalling (see Lamaze et al, 2001).

Azithromycin could also be interesting for studying the recycling pathway (see Sönnichsen et al, 2000) and the fusogenicity between phagosomes and lysosomes (Jahraus et al, 1998). Indeed, we have shown that, in macrophages, the drug delays recycling of Tf without affecting its subsequent rate of efflux. It also causes a major vacuolation of the late endocytic apparatus and severely inhibits accessibility of these structures by fluid-phase endocytic tracers and immune complexes internalized by the Fcγ-receptors (PAP immune complexes), but not their accessibility to latex beads which are internalized by phagocytosis.

CHAPTER 5. PERSPECTIVES

Our work could be oriented into two directions. First, at short term, it would be interesting to continue the evaluation of azithromycin effect on different endocytic modes/pathways and the mechanism of azithromycin action. A second approach, at long term, is the use of azithromycin as an agent able to perturb endocytosis to further understand some pathways and steps of endocytosis.

1. Short-term perspectives

1.1. Effect of azithromycin on different endocytic modes/pathways

The effect of azithromycin on the rate of membrane internalization was evaluated, in our work, by using *N*-Rh-PE, a tracer which forms microaggregates in the plane of the plasma membrane, resulting in fluorescence self-quenching, which is relieved by endocytosis and fusion with pre-existing endosomes due to dilution with unlabelled membrane. In the future, it could be interesting to confirm or infirm the results obtained with *N*-Rh-PE by using C_6-NBD-SM, a fluorescent lipid probe which allows for kinetic studies of membrane internalization and recycling (Hao and Maxfield, 2000). After nonselective internalization, this tracer exits sorting endosomes, enters the ERC and then returns to the plasma membrane. Because these processes are rapid, we will evaluate the effect of azithromycin on the rate of entry and recycling of C_6-NBD-SM, by using very short times of pulse and chase, as described in Hao and Maxfield (2000). Another approach could be the use of the plant toxin ricin that binds both glycoproteins and glycolipids with terminal galactose, labels thereby the cell surface and has proven valuable as a bulk membrane tracer in studies of endocytosis (Sandvig and van Deurs, 1999; see Dautry-Varsat, 2001). To evaluate the effect of azithromycin on endocytosis and recycling of the toxin, we would use ^{125}I-ricin.

Preliminary results indicate that azithromycin inhibits internalization of the B subunit of cholera toxin to the same extent than fluid-phase endocytosis of HRP and LY. Cholera toxin binds to a specific glycosphingolipid, GM1, which has been shown to be localized in caveolae by ultrastructural studies, and therefore entry cells by a clathrin-independent process (see Mukherjee et al, 1997). It could therefore be interesting to examine in more details this effect, link it with the other perturbations observed, and with the extracellular concentration of azithromycin.

1.2. Mechanism of azithromycin action

Among the hypothesis to explain impairment of endocytosis by azithromycin are the intense vacuolation caused by the drug, an alteration of endosomal acidification, and an interaction between azithromycin and membranes. Although the latter is the most plausible hypothesis, we cannot eliminate the two others. To go further, we would therefore: (i) identify the swelled structures and the TfR-containing structures caused by the drug; (ii) evaluate the ability of azithromycin to perturb lateral mobility of receptors and sequestration into coated pits; and (iii) evaluate the effect of azithromycin on membrane properties involved in endocytosis.

1.2.1. Identification of the swelled structures and of the TfR-containing structures caused by azithromycin, and accessibility of these structures

The identification of swelled structures could be done by colocalizing, in confocal microscopy, a fixable lysoTracker with (i) rab5, (ii) rab7 and LBPA and (iii) Lamps. Rab5 is present on clathrin coated vesicles and early endosomes. Rab7 is present on late endosomes and LBPA is a rare negatively charged lipid which concentrates in the inner involutions of late endosomes. Lamps are present in late endosomes and lysosomes. Using antibodies against these molecules and an antibody against transferrin-receptors, we would be able to identify the vacuolated structures.

Vacuolation might explain intracellular sequestration of TfRs, that are found in dilated structures as shown by confocal microscopy. Vacuolation by itself does not provide a direct explanation for the inhibition of fluid phase endocytosis and the delay in TfRs recycling, but could result in the sequestration of rate-limiting factor(s) for upstream endocytic steps. To better understand the mechanism by which azithromycin causes intense intracellular accumulation of TfRs, we have first to identify these compartments. This could be done by colocalizing, in confocal microscopy, TfRs with (i) rab5, (ii) rab7 and LBPA and (iii) Lamps.

Because experiments with rat foetal fibroblasts suggest that LY, but not HRP, has a slight access to swollen endosomes/lysosomes and that HRP and PAP immune complexes have no access to these structures in macrophages, we propose to deepen these observations by using bovine serum albumin-gold conjugates as a tracer of fluid-phase endocytosis (Bright et al, 1997). Because

this is a particulate tracer, it would not be soluble in these compartments, and this would allow us to infirm or confirm that fluid-phase endocytic tracers have no access to the swollen acidic compartments.

1.2.2. Effect of azithromycin on receptor motility in the plasma membrane and sequestration into coated pits

We have shown that clustered concanavalin A receptors are not observed in the plasma membrane of J774 macrophages treated with azithromycin, suggesting a reduced membrane fluidity which inhibit the capacity of glycoproteins to move in the lateral plane of the plasma membrane and therefore to aggregate. First, we have to confirm these results and to be sure that azithromycin would not induce a redistribution of surface glycoproteins inside the cells, since it was the case for TfRs. This could be done by biochemical measurement of fluorescent concanavalin A bound at the cell surface in cells treated or not by azithromycin.

We have also shown that azithromycin delays sequestration in clathrin-coated pit structures of receptor-bound ligands, as was most clearly evidenced for PAP/Fcγ-receptor complexes (~8 minutes) and causes a major delay of transferrin-receptors recycling (~10 minutes). Because these results suggest a perturbation of receptor mobility at different levels in the endocytic pathway, we would therefore evaluate the effect of azithromycin on receptor mobility and clustering into clathrin-coated pits, by using fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) techniques. First, the mobility of FcγR (using fluorescent receptor antibodies) and concanavalin A receptors (using fluorescent concanavalin A) in the plasma membrane of J774 mouse macrophages would be examined by FRAP. After labelling, we will examine a small region of the surface by fluorescence microscopy, photobleach this region, and finally monitor the recovery of fluorescence intensity. If the labelled component is mobile, bleached molecules will leave and unbleached molecules enter the illuminated region, which results in an increase in the fluorescence intensity. This would allow to evaluate the effect of azithromycin on the diffusion capacity of both receptors and if several receptors sites can be distinguished in cell membranes. Second, we would use FRET to evaluate on one hand the assembly and mutual proximity of TfR and FcγR using fluorescent conjugated monoclonal antibodies, and on the other hand the sequestration of FcγR into coated pits, using green fluorescent protein-clathrin light chains and FcγR antibodies.

1.2.3. Effect of azithromycin on membrane properties involved in endocytosis

First, we would continue to evaluate the effect of azithromycin on membrane domain formation by atomic force microscopy. It could be interesting to first evaluate the effect of azithromycin on the organization of Langmuir-Blodgett monolayers made of 1,2-dioleoyl-glycero-3-phosphocholine (DOPC): cholesterol (instead of DPPC: cholesterol). DOPC is a fluid glycerophospholipid, whereas DPPC is a solid lipid (Rinia et al, 2001). Second, we would add azithromycin after deposing the monolayer at the air-water interface (instead of mix azithromycin with lipids). Another interesting perspective is the use of lipid bilayers (instead of monolayers). Bilayers of different lipid compositions would be prepared by the Langmuir-Blodgett method. Such bilayers, which are asymmetric, can function as a model for biological membranes (Rinia et al, 1999). Concerning the atomic force microscopy itself, it could be useful to use mica modified with 3-aminopropyl-triethoxy-silane (APTES) (instead of mica). When mica is modified with APTES, the amino groups give the surface a net positive charge and a more polar surface than pure mica (Leonenko et al, 2000). This system might be better than mica for studying the effect of azithromycin on the interaction of a lipid mixture with the surface. Finally, more recent dynamic imaging modes now offer the possibility of probing nanomechanical properties. Among these, the tapping mode atomic force microscopy has proved useful to elucidate variations in material properties such as adhesion, friction and microelasticity. It is possible, with this system (instead of the widely used static contact mode), to directly visualize phase-separated lipid bilayers with different surface charge characteristics (Deleu et al, 2001).

Membrane fluidity, measured by TMA-DPH fluorescence anisotropy, in the plasma membrane and during membrane internalization is probably decreased in presence of azithromycin. We would first relate these results with those obtained with methyl-â-cyclodextrin. First, we would verify that this agent effectively extracts cholesterol. Second, we would evaluate in these conditions the binding or the insertion of azithromycin into membranes.

Based on the facts that: (i) membrane fluidity (or microviscosity) is only a small part of membrane tension, that is the result of the combination of the membrane cytoskeleton adhesion force and the in-plane membrane tension; (ii) membrane tension is an important property in regulating cell functions such as endocytosis; and (iii) alteration of membrane tension and curvature could be obtained by differences between outer and inner leaflet area, that in turn could be the result of the addition of amphipathic drugs that preferentially partition into either the outer

or the inner leaflet of the phospholipidic bilayer, selectively increasing that leaflet's surface area (Oghalai et al, 2000), we propose to evaluate the effect of azithromycin on membrane tension. It is possible to measure membrane tension of biological membranes from the force that is exerted on membrane tethers. Tethers can be formed by pulling on membrane-attached beads with lazer tweezers and the displacement of the beads in the lazer tweezers gives a rapid readout of the force on the beads. This can be viewed by a video enhanced differential interference contrast (DIC) microscope equiped with lazer optical tweezers. Since both membrane tension and membrane cytoskeleton adhesion contribute to the tether force, it could be interesting to separate plasma membrane from the cytoskeleton, which occurs in membrane blebs. Membrane blebs are spherical membrane extensions that can be seen at the periphery of eukaryotic cells during mitosis for example or on cells injured by physical and chemical stress. When the membrane tension term is substracted from the apparent membrane tension over the cytoskeleton, the membrane cytoskeleton adhesion term can be estimated (Dai and Sheetz, 1999). All these measurements could be done in the absence or the presence of azithromycin.

Because the reduced incorporation of *N*-Rh-PE in the plasma membrane by azithromycin suggests either a decrease of insertion or a decrease of membrane availability, we have to test the latter. The experiment should be repeated by preincubating J774 macrophages with azithromycin at 4°C, or during a time shorter than 3 h (15 min for example) to avoid membrane sequestration in lysosomes.

Another membrane property which is involved in endocytosis is membrane asymetry, a property which is also related to membrane tension. Indeed, a difference in surface tension between the inner and the outer monolayers of the plasma membrane exists and has been proposed to be a driving force of endocytic vesiculation. This difference between both leaflets could be mechanically generated by the phospholipid number asymetry that could be obtained by the addition of exogenous PS which is translocated by a flippase activity to the inner leaflet of the plasma membrane (Rauch and Farge, 2000). We would evaluate the addition of PS and azithromycin on endocytosis of Tf, HRP and C_6-NBD-SM.

Fourth, we would evaluate the effect of azithromycin on the activity of some enzymes involved in the very early steps of endocytic trafficking, and which are important for membrane lipid composition. Among these enzymes are PLA_2, PLD and sphingomyelinase. PLA_2 is involved in vesicle fusion along the endocytic pathway (Mayorga et al, 1993) and in immunoglobulin G-

mediated phagocytosis (Lennartz et al, 1997). Likewise, PLD has been implicated in the recruitment of the AP-2 adaptor onto endosomes and the plasma membrane (West et al, 1997). Finally, addition of exogeneous sphingomyelinase has been shown to induce formation of numerous vesicles that pinch off from the plasma membrane in macrophages and fibroblasts (Zha et al, 1998). Sphingomyelinase is also involved in immunoglobulin G-dependent phagocytosis (Hinkovska-Galcheva et al, 1998). We will evaluate the implication of these enzymes in endocytosis of Tf, HRP and C_6-NBD-SM. For this purpose, we will investigate to what extent externaly added PLA_2 and PLD and sphingomyelinase could affect endocytosis of the different tracers. In parallel, we will use inhibitors of these enzymes, bromoenol lactone, a PLA_2 inhibitor (Mayorga et al, 1993), neomycin, a PLD inhibitor (West et al, 1997), and scyphostatin, an inhibitor of neutral sphingomyelinase (Bernardo et al, 2000), to investigate the potential role of such enzymes in endocytosis.

2. Long-term perspectives

Whereas it is clear that rigourous studies with pharmacological agents require several controls, it is suggested that azithromycin, or related agents, could be useful for kinetics studies addressing the contribution of clathrin-independent pinocytosis (Dautry-Varsat, 2001), sorting steps in the recycling pathway (Sönnichsen et al., 2000), fusogenicity of phagosomes (Jahraus et al., 1998).

Since azithromycin is able to selectively impair clathrin-independent internalization, this drug could be useful to evidence the implication of clathrin-independent endocytosis for the internalization of receptors, such as the interleukin-2 receptor. Furthermore, azithromycin could be useful to identify the internalization mode of β2-adrenergic receptors that are internalized via coated pits in some cell types, but may enter in a different manner in other cell type.

We have shown that the drug delays recycling of Tf without affecting its subsequent rate of efflux. Azithromycin could therefore help to better understand sorting steps in endocytic pathway.

Azithromycin also causes a major vacuolation of the late endocytic apparatus and severely inhibits accessibility of these structures by fluid-phase endocytic tracers and immune complexes internalized by the Fcγ-receptors, but not their accessibility to latex beads which are internalized by phagocytosis. So, using azithromycin, it is possible to block fusion between endosomes and

lysosomes and keep intact fusion between phagosomes and lysosomes, and therefore study the machinery involved in the latter.

CHAPTER 6. REFERENCES

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