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

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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 HRP-labeled 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 result in 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 cellular concentrations. We suggest that dicationic macrolides inhibit fluid-phase pinocytosis by impairing the formation of pinocytic vacuoles and endosomes.

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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, 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, 1999), and induces a mixed lysosomal storage disorder involving phospholipids (Van Bambeke et al., 1996, 1998) and cholesterol (Montenez, 1996).

In this study, we have examined whether azithromycin and erythromycylamine (another dicationic macrolide described by Massey et al. (1970)) could affect earlier steps of the endocytic

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process. 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 level. 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, 1997).

We found that azithromycin and erythromycylamine 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 collection of cells

Primary cultures of rat fibroblasts were obtained by trypsinization of eviscerated 18 days-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 s 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 determined 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 cellular 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 was 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 s each with 155 mM NaCl; two washes of 5 and 1 min each with complete culture medium; 5 washes of 30 s 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 s 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 (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. All values 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 cellular protein content.

Peroxidase cytochemistry and microscopy

We used the method described in detail by 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 0.1 M

cacodylate buffer, pH 7.4; (*2*) after post-fixation, the samples were stained "en bloc" for 2 h in the dark with 0.5% uranyl acetate. Semi-thin (approximately 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× 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 coverglasses (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 800–1300 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 non-specific binding. Cells were washed 3 times with PBS-Ca^{2+}–Mg^{2+} and reincubated at 37 °C 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-sensitive) 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-^3H]leucine, [methyl-^3H]thymidine, [^{14}C]urea and [U-^{14}C]sucrose were obtained from Amersham-Pharmacia (Piscataway, 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 concentrations (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 × mg of cell protein^{-1} × 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 × mg of cell protein^{-1} × 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, protein and DNA syntheses and cell volume and moderately decreases the 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 I shows the main criteria investigated in this aim in fibroblasts incubated with 50 mg/l azithromycin (66 μM; the concentration routinely used in subsequent experiments) for up to the maximum 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 incubation, 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).

Tab. I. Influence of 50 mg/l azithromycin on plasma membrane integrity, protein and DNA 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 *
[3H]Leucine incorporation [b]	97.2 ± 0.8 NS	116.1 ± 7.9 NS	111.7 ± 6.8 NS	118.1 ± 10.4 NS
[3H]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; * significantly different from the matched control (* $p < 0.05$; ** $p < 0.01$); NS not significant from the matched control.

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 for 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 approximately 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 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

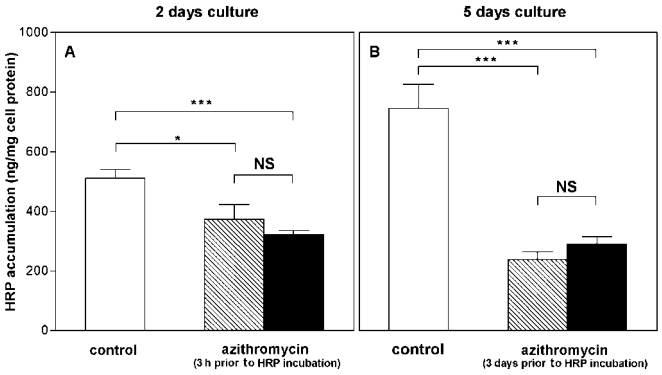

Fig. 1. Effect of azithromycin on the accumulation of HRP in rat foetal fibroblasts cultured for 2 days (**A**) or 5 days (**B**). Cells were either untreated (controls; *open bars*) or incubated with 50 mg/l azithromycin for the last 3 h (**A**) or 3 days of culture (**B**) prior to a 2-h HRP challlenge. 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 ± SD of 3 dishes. Statistical analysis (Student's t test): NS, not 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.

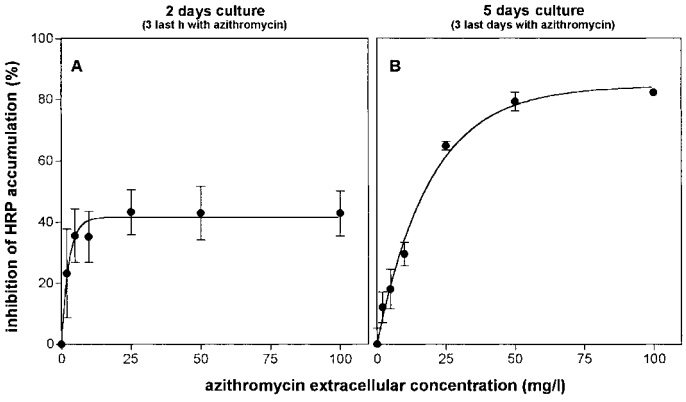

Fig. 2. Effect of azithromycin concentration on the inhibition of HRP accumulation in rat foetal fibroblasts cultured for 2 days (**A**) or 5 days (**B**). Cells were treated with azithromycin for the last 3 h (**A**) or 3 days of culture (**B**) prior to a 2-h HRP challenge. Values are means ± SD of 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).

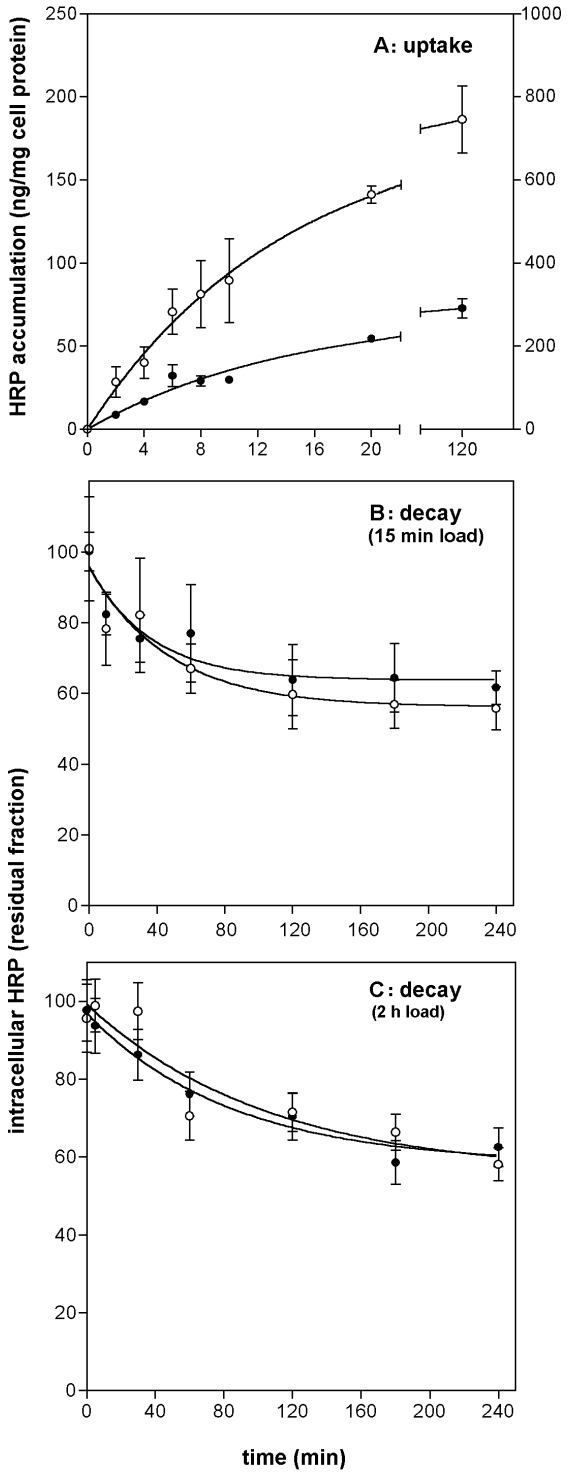

Fig. 3. Effect of azithromycin on the kinetics of HRP uptake (**A**) and decay (**B, C**) in rat foetal fibroblasts cultured for 5 days. Cells were either untreated (control; *open circles*) or exposed to 50 mg/l azithromycin for the last 3 days of culture (*filled circles*) prior to HRP challenge. Values are means ± SD of 3 dishes. (**A**) 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. (**B, C**) 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.

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)) (data not shown).

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 Figures 4 (light microscopy; fibroblasts treated for 3 days with azithromycin and exposed for 5 min to HRP, Fig. 4B) and 5 (electron microscopy; cells treated for 3 h or 3 days with azithromycin and incubated for 5 min or 2 h with HRP, Figs. 5C–F). 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

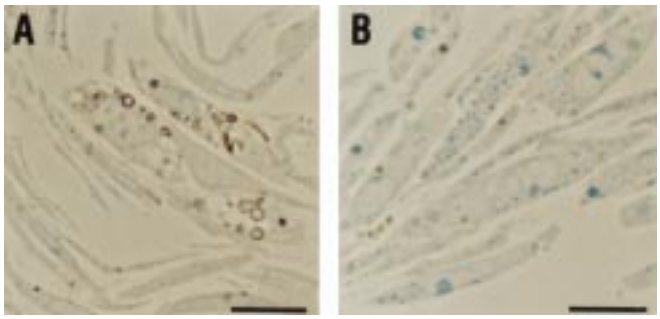

Fig. 4. Light microscopic analysis after peroxidase cytochemistry (semi-thin plastic sections). Control cells (**A**) and cells treated with 50 mg/l azithromycin for 3 days (**B**) were exposed to HRP for 5 min prior to fixation, processed for HRP cytochemistry (brownish staining) and slightly counterstained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

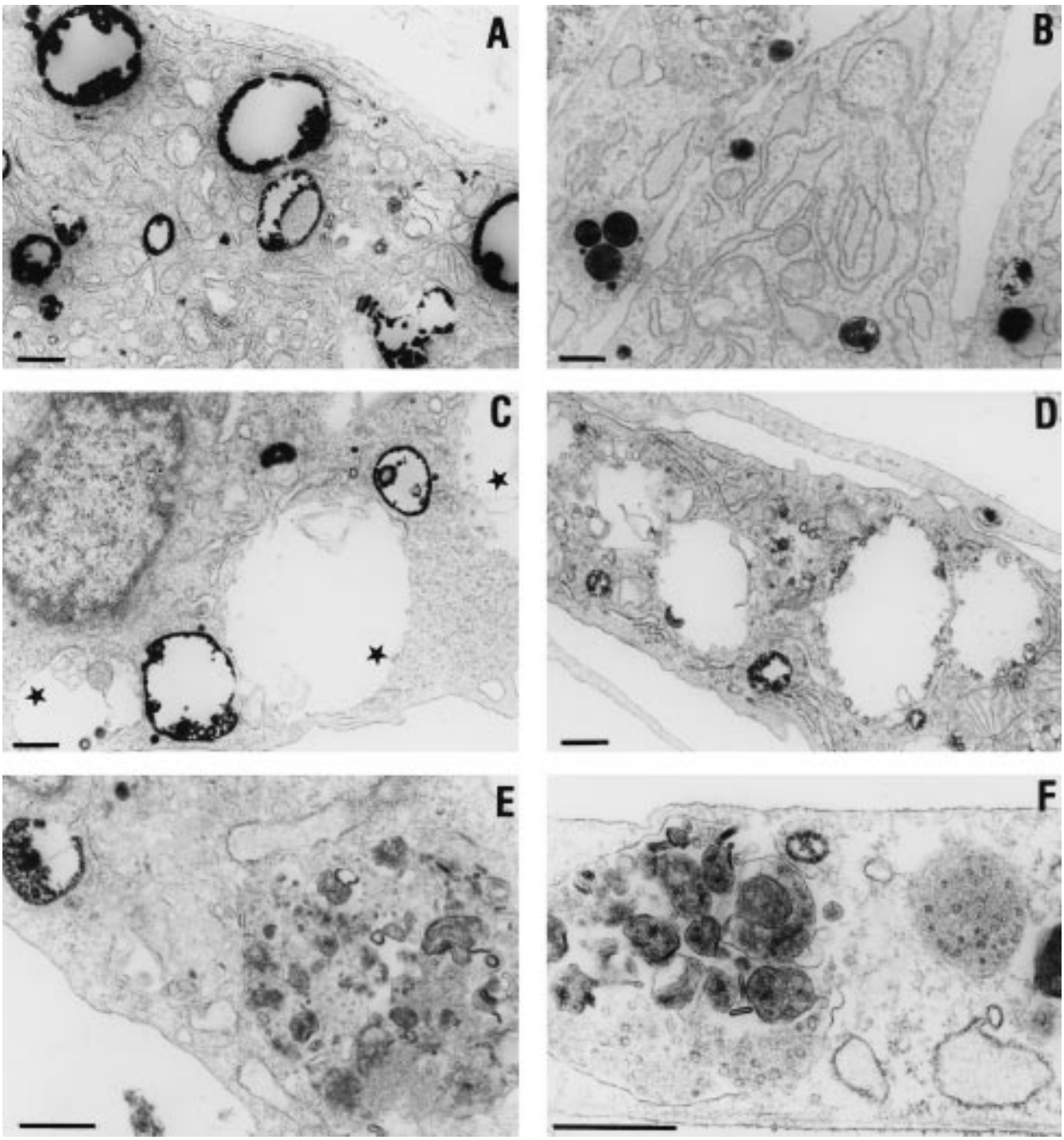

Fig. 5. Ultrastructural peroxidase cytochemistry. Control and azithromycin-treated cells were challenged with HRP for 5 min (**A, C, E**) or 2 h (**B, D, F**). *Upper panels*: control cells. At 5 min (**A**), typical pinocytic vesicles and early endosomes are stained by the reaction product at the luminal side of their limiting membrane. At 2 h (**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 for 5 min (**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 (**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 (**E**) as well as at 2 h (**F**). Bars are 0.5 μm.

(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 with 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 products (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 vesicles 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 refractive 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 less intensely labeled (compare Figs. 6D 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).

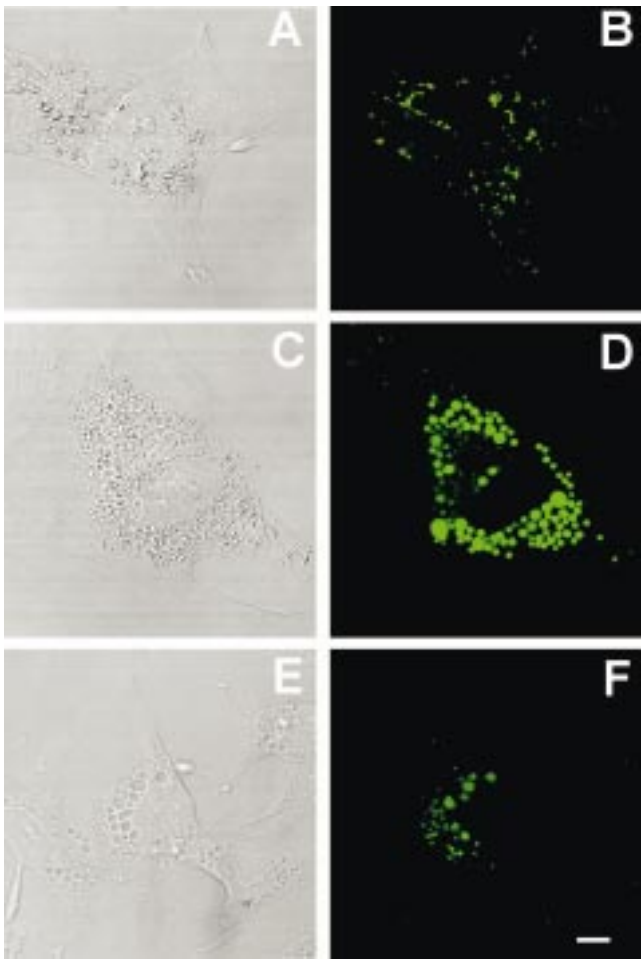

Fig. 6. Phase-contrast (**A, C, E**) and confocal fluorescence microscopy (**B, D, F**) of cells exposed to lucifer yellow (1 g/l). (**A, B**) Control cells; (**C–F**) cells treated with azithromycin (50 mg/l). (**A, B**) Cells incubated with lucifer yellow for 14 h; (**C, D**) cells incubated with lucifer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

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

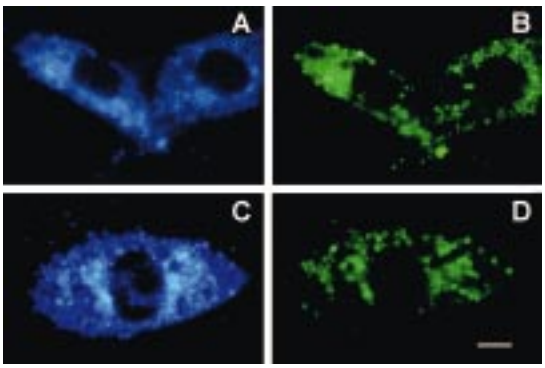

Fig. 7. Fluorescence microscopy of cells treated with 50 mg/l azithromycin for 3 days, incubated overnight with FITC-BSA, and reacted with filipin (50 mg/l) after fixation. (**A, C**) Fluorescence of filipin; (**B, D**) fluorescence of FITC-BSA. Bar is 10 μm.

azithromycin for only 3 h, both to minimize the risk of 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 cellular accumulation of azithromycin. Monensin is also known to stimulate HRP regurgitation in fibroblasts without modifying its influx (Cupers et al., 1997). Table II shows the results of an experiment in which HRP uptake was measured in fibroblasts treated for 3 h with either azithromycin or monensin alone or in 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 cellular accumulation of HRP 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 endocytosed 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 20 mg/l (see (Verrey et al., 1995)). The effect of the combination of nocodazole and azithromycin 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 II 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 the 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).

Figure 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 cellular drug levels, but were independent of the variations in phospholipid contents. Interestingly, the cellular phospholipid

Tab. II. Inhibition of HRP accumulation by azithromycin, monensin and nocodazole.

Treatment	HRP challenge [1]	Inhibition of HRP accumulation (%) [+]	Azithromycin accumulation (μg/mg cell 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). [+] 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 the extracellular medium during HRP challenge.

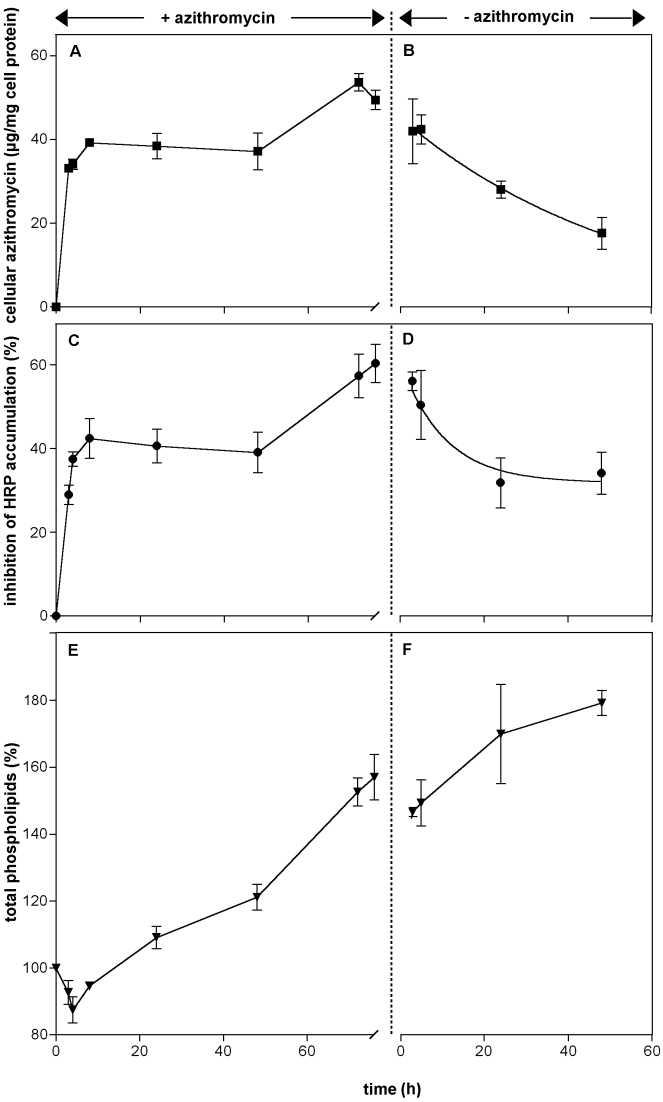

Fig. 8. Comparison of the kinetics of cellular accumulation and retention of azithromycin, upon pulse and chase (**A, B**), of inhibition of HRP accumulation (**C, D**; 2 h challenge) and of total cell phospholipid content (**E, F**). After 2 days of culture, cells were treated with 50 mg/l azithromycin 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. In the *left panels* (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 onwards ($p < 0.01$). In the *right panels* (azithromycin chase), values of cellular drug content and 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.

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 cellular 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 cellular phospholipid content, by manipulating the drug concentrations and times of exposure, together with variable withdrawal periods. The results of all these experiments are combined in Fig. 9. Inhibition of HRP uptake closely correlated with cellular azithromycin 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 I), 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 cellular concentration, 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 III shows that the inhibition of HRP uptake is directly related to the actual molar content of either drug in the cell (~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

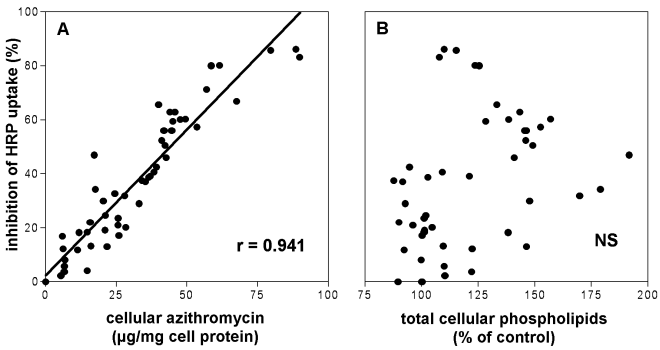

Fig. 9. Correlation between the inhibition of HRP uptake versus the cellular azithromycin content (**A**) or the cellular total phospholipid content (**B**). Compilation of a series of independent experiments in which the extracellular concentrations of azithromycin were varied from 0 to 100 mg/l and the incubation times from 0 to 3 days (n = 51).

Tab. III. Inhibition of HRP accumulation by azithromycin and erythromycylamine.

Drug	Extracellular concentration (mg/l) [μM]	Drug accumulation (μg/mg cell protein) [nmol/mg cell protein]	Inhibition of HRP accumulation	
			%	% per nmol of accumulated drug/ mg cell protein
Azithromycin	50 [66]	61.6 ± 4.8 [82.2 ± 6.4]	80.0 ± 3.1 ***	1.0
Erythromycylamine	250 [340]	48.0 ± 5.7 $^{+}$ [65.3 ± 7.7 $^{+}$]	51.3 ± 6.7 ***, $^{++}$	0.8

Values are means ± SD of 3 dishes. * significantly different from controls (*** $p < 0.001$); $^{+}$ significantly different from 50 mg/l azithromycin ($^{+}$ $p < 0.05$; $^{++}$ $p < 0.01$).

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 approximately 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 approximately 7 h; Steinman et al., 1974, 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 had accessed 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 to be a part of 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 impair 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 (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., 1987, 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 to 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; 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 macrolide 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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