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The Macrolide Antibiotic Azithromycin Interacts with Lipids and Affects Membrane Organization and Fluidity: Studies on Langmuir-Blodgett Monolayers, Liposomes and J774 Macrophages

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Abstract. The macrolide antibiotic azithromycin was shown to markedly inhibit endocytosis. Here we investigate the interaction of azithromycin with biomembranes and its effects on membrane biophysics in relation to endocytosis. Equilibrium dialysis and ^{31}P NMR revealed that azithromycin binds to lipidic model membranes and decreases the mobility of phospholipid phosphate heads. In contrast, azithromycin had no effect deeper in the bilayer, based on fluorescence polarization of TMA-DPH and DPH, compounds that, respectively, explore the interfacial and hydrophobic domains of bilayers, and it did not induce membrane fusion, a key event of vesicular trafficking. Atomic force microscopy showed that azithromycin perturbed lateral phase separation in Langmuir-Blodgett monolayers, indicating a perturbation of membrane organization in lateral domains. The consequence of azithromycin/phospholipid interaction on membrane endocytosis was next evaluated in J774 macrophages by using three tracers with different insertion preferences inside the biological membranes and intracellular trafficking: C_6-NBD-SM, TMA-DPH and *N*-Rh-PE. Azithromycin differentially altered their insertion into the plasma membrane, slowed down membrane trafficking towards lysosomes, as evaluated by the rate of *N*-Rh-PE self-quenching relief, but did not affect bulk membrane internalization of C_6-NBD-SM and TMA-DPH. Azithromycin also decreased plasma membrane fluidity, as shown by TMA-DPH fluorescence polarization and confocal microscopy after labeling by fluorescent concanavalin A. We conclude that azithromycin directly interacts with phospholipids, modifies biophysical properties of membrane and affects membrane dynamics in living cells. This antibiotic may therefore help to elucidate the physico-chemical properties underlying endocytosis.

Key words: Azithromycin — Lipids — AFM — Endocytosis — Macrophages — Membrane

Introduction

Constitutive membrane trafficking involves the movement of membrane lipids and associated proteins among and between intracellular organelles and the plasma membrane. This transport mostly involves vesicular trafficking between donor and target membranes. The generation of transport vesicles through budding processes and the fusion of vesicles with acceptor membranes clearly must involve a major reorganization in membrane structure and, accordingly, it is likely that changes of lipid components or alterations in membrane lipidic organization could

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Abbreviations: C_6-NBD-SM, 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosyl phosphocholine; N-*Rh*-PE, *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; R18, octadecylrhodamine B; AFM, atomic force microscopy; MLV, multilamellar vesicles; LUV, large unilamellar vesicles, SUV, small unilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine.

significantly affect these processes. With respect to endocytosis, several steps in vesicular transport including (i) the definition of the location where vesicles will form; (ii) the rearrangement of the lipid bilayer that must occur during vesicle fission and fusion; and (iii) the coordination of the various steps of vesicle formation to ensure membrane homeostasis and to respond or adopt to cellular metabolism, could be facilitated by lipids and/or by enzymes that modify membrane lipids (Roth & Sternweis, 1997). In turn, drugs that interact with lipid membrane might affect endocytosis and/or the function of membrane-associated proteins or membrane trafficking.

Many drugs with different chemical structures and pharmacological effects are known to bind to membrane lipids and to alter physical properties. Typical examples include local anesthetics (Bazzoni & Rasia, 2001; Raucher & Sheetz, 2001), non steroidal anti-inflammatory agents (Grage et al., 2000), calcium channel-blocking drugs (Mason et al., 1999), anti-psychotic drugs (Agasosler et at., 2001; Jutila et al., 2001), as well as anti-cancer (Saint-Laurent et al., 2001), anti-parasital (Go & Feng, 2001), anti-fungal (Hing, Schaefer & Kobayashi, 2000; Mingeot-Leclercq et al., 2001; Milhaud et al., 2002), anti-bacterial (Mingeot-Leclercq & Tulkens, 1999) and anti-viral agents (Asawakarn, Cladera & O'shea, 2001). Unfortunately, only few studies have been devoted to relate the effects of these drugs on membrane properties underlying endocytosis (Mukherjee, Ghosh & Maxfield, 1997; Marsh, 2001).

Our recent work (Tyteca et al., 2001; 2002) has shown that azithromycin, a dicationic macrolide antibiotic (Djokic, Kobrehel & Lazarevski, 1987; Bright et al., 1988), markedly inhibits fluid-phase endocytosis and induces a delay between ligand binding to their receptors and internalization into clathrin-coated pits, without affecting the subsequent rate of endocytosis. Remarkably, phagocytosis was not affected. This suggested that azithromycin could act by perturbing lateral mobility of receptors and/or the budding of endocytic pits into endocytic vesicles, and prompted us to investigate whether azithromycin interacts directly with phospholipids and alters the biophysical properties of biological membranes, so as to impair endocytosis.

Using both acellular models and cultured cells, we report here a detailed molecular analysis of azithromycin interaction with membrane phospholipids. First, we studied the binding of azithromycin to lipids using liposomes as models of membranes and discriminated the contribution of phosphate groups, interfacial and hydrocarbon domains of phospholipids to this interaction in relation with a potential effect of azithromycin on membrane fusion. Second, by using Langmuir-Blodgett monolayers, we investigated the effect of azithromycin/phospholipid interaction on membrane organization. Third, using J774 macrophages as cellular model, we examined the influence of azithromycin on plasma membrane insertion of three membrane tracers that explore distinct transversal or lateral domains and undergo different intracellular trafficking, namely C_6-NBD-SM [6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosyl phosphocholine], TMA-DPH [trimethylammonium-diphenylhexatriene] and *N*-Rh-PE [*N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine]. The effects of azithromycin/phospholipid interaction on plasma membrane fluidity and intracellular trafficking of these tracers were also investigated and observations will be discussed in relation to the effect of azithromycin on endocytosis.

Materials and Methods

Liposome Preparation

Liposomes were made of a mixture of cholesterol, phosphatidylcholine, sphingomyelin and phosphatidylinositol (5.5:4:4:3, molar ratio), either alone or combined with phosphatidylethanolamine (5.5:4:1.7:3:2.3, molar ratio). When phosphatidylethanolamine was included, the sphingomyelin content was decreased so as to maintain a constant phospholipid to cholesterol molar ratio at 2:1. Lipids were dissolved in chloroform:methanol (2:1, v:v) in a round-bottomed flask. The solvent was evaporated under vacuum (Rotavapor R Buchi RE-111, Buchi, Flawil, Switzerland) to obtain a thin film of lipids that was dried overnight in a vacuum dessicator. The dry lipid films were hydrated for 1 h under nitrogen at 37°C in 40 mM Tris maleate buffer, pH 5.4, 6.0, or 7.0. According to the type of experiment performed, this suspension was either sonicated to yield small unilamellar vesicles (SUV; Montenez et al., 1999), or submitted to five cycles of freezing/thawing to obtain multilamellar vesicles (MLV). These were further extruded to produce large unilamellar vesicles (LUV; Van Bambeke et al., 1993). The actual phospholipid content of each preparation was determined by phosphorus assay (Bartlett, 1959) and the concentration of liposomes was adjusted for each type of experiment.

Equilibrium Dialysis

Binding of azithromycin to SUV (15.7 mM in lipids) was investigated by equilibrium dialysis using a Dianorm apparatus (Van Bambeke et al., 1996; Montenez et al., 1999), except that the molecular weight cut-off was ~10 kDa and that dialysis was performed overnight at 4°C under constant rotation at 8 rpm.

The initial drug concentration was set at 100 mg/L (132 μM). The method was validated to ensure binding equilibrium and full drug recovery at the three pH values used. Azithromycin was assayed in the chamber without liposomes (free drug concentration [D_{free}]) and the total concentration of free and bound drug in the chamber containing liposomes ($D_{free} + D_{bound}$) was calculated as the initial concentration ($D_{initial}$) minus the concentration of free drug ($D_{initial} - D_{free}$; Van Bambeke et al., 1996).

^{31}P Nuclear Magnetic Resonance (NMR) Studies

The effect of azithromycin on membrane lipid mobility and organization was studied on MLV. The experimental chemical shift

anisotropy depends on the motions of the phosphodiester moiety. Rapid motion with limited amplitude along the molecular axis of the phospholipid normal to the membrane produces a supplementary averaging of the anisotropy tensor. The effective tensor still has axial symmetry but the chemical shift anisotropy is reduced. If motional freedom increases, or, in other words, if the amplitude and/or rate of the precession motion becomes more important, $\Delta\sigma$ decreases. This is generally observed when the liposomes are heated. The effective chemical shift anisotropy ($\Delta\sigma$) 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, the $\Delta\sigma$ value was deduced 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 [$\sigma_i = 1/3\ (2\ \sigma_{\perp} + \sigma_{||})$], and since the anisotropic part of this tensor is defined as $\Delta\sigma = \sigma_{||} - \sigma_{\perp}$, it follows that $\Delta\sigma = 3\ (\sigma_i - \sigma_{\perp})$ (Seelig, 1978).

Control samples of 3 mL of liposomes (37.5 mM in lipids) were prepared from concentrated MLV suspension (100 mM) by addition of appropriate buffer and 500 µL of D_2O for locking on the deuterium signal. Treated samples were obtained by adding concentrated drug solutions to reach final concentrations up to 5 mM azithromycin and 10 mM glucosamine (the latter was used as negative control; Mingeot-Leclercq et al., 1989).

Proton-decoupled ^{31}P NMR spectra were acquired on a Bruker AC 250 spectrometer (Bruker, Wissembourg, France). A 10-mm broad-band 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, 4 K data points, and 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 52°C and 70°C with 15 min of equilibration before data acquisition at the next temperature.

DPH and TMA-DPH Fluorescence Polarization Studies in Liposomes

Fluorescence polarization studies were performed on LUV at a 0.314 mM final lipid concentration. Incorporation of two fluorescent markers with distinct level of insertion in the membrane, DPH and TMA-DPH (*see* Kitagawa et al., 1991; Kaiser & London, 1998) was achieved at a molar ratio to lipids 1:250, by vigorous mixing followed by preincubation at 37°C for 1 h. Labeled LUV were mixed with azithromycin (132 µM final) or benzyl alcohol (as positive control; 30 mM final [Friedlander et al., 1987]), incubated at 37°C for 30 min, brought to 45°C in 15 min, and stabilized at that temperature during 5 min before the measurements (Montenez et al., 1996). The fluorescence emitted in the planes parallel (I_{par}) and perpendicular (I_{per}) to that of the polarized excitation light was then measured while samples were cooled down to 10°C at a rate of 50°C/h. Results were expressed as polarization values ($P = [I_{par} - I_{per}]/[I_{par} + I_{per}]$). Fluorescence was measured on an LS-50 Perkin-Elmer spectrofluorimeter (Perkin-Elmer, Beaconsfield, UK), equipped with a special adaptor for polarization measurements, and operating with λ_{exc} at 365 ± 5 nm and λ_{em} at 427 ± 3 nm for DPH or λ_{exc} 360 ± 5 nm and λ_{em} 435 ± 4 nm for TMA-DPH. The sample was kept under gentle stirring throughout the experiment and its temperature was continuously monitored by a sensor placed into the measuring unit coupled with a DC5 programmable circulator bath (Haake, Karlsruhe, Germany).

Fluorescence Dequenching of Octadecylrhodamine B (R_{18}).

These studies were performed exactly as described (Van Bambeke et al., 1995), using a mixture of labeled and unlabeled LUV (5 µM final lipid concentration), and based on recording the increase of fluorescence upon R18 dequenching by dilution (Hoekstra et al., 1984). Labeled and unlabeled liposomes were mixed at a 1:4 ratio and the fluorescence of the preparation was followed upon addition of increasing azithromycin concentrations (0.65 µM to 800 µM) at room temperature for 20 min, on a Perkin-Elmer LS-30 spectrofluorimeter using λ_{exc} at 560 nm and λ_{em} at 590 nm. Melittin was used as a positive control (Morgan et al., 1983; Van Bambeke et al., 1995).

Langmuir-Blodgett (LB) Monolayer Preparation

LB monolayers were prepared from 1 mM of dipalmitoylphosphatidylcholine (DPPC): cholesterol (2:1) at 25°C with an automated system (LFW2 3″5, Lauda, Königshofen, Germany) in the presence or absence of azithromycin, initially dissolved at 1 mM in chloroform:methanol (2:1). A mixture of DPPC:cholesterol:azithromycin (4:2:1.2 molar ratio) was spread on 10 mM Tris aqueous phase adjusted at pH 7.1 with HCl. After evaporation of the solvent for 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 at 90° 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) Studies

AFM imaging was performed at room temperature using an 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, USA) 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 stated otherwise, the applied force was maintained below 1 nN.

Cell Culture and Incubation with Azithromycin

J774 mouse macrophages, a cell line derived from a mouse reticulosarcoma (Snyderman et al., 1977), were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). For each series of experiments, cells were seeded at 5.5×10^4 cells/cm^2 and grown until confluency (2 days). Cells were then incubated for 3 h with the indicated concentrations of azithromycin before addition of membrane tracers (*N*-Rh-PE, C_6-NBD-SM, and TMA-DPH) or concanavalin A (*see* below).

Insertion of *N*-Rh-PE, C_6-NBD-SM and TMA-DPH in the Plasma Membrane of J774

Cells were cooled down at 4°C, then incubated with *N*-Rh-PE or C_6-NBD-SM in FCS-free medium for 30 min, or with TMA-DPH in phosphate-buffered saline (PBS) for 2 min.

To measure *N*-Rh-PE plasma membrane insertion, cells were washed at room temperature twice with PBS for 30 s each and once rapidly with 1% trypsin in PBS, then recovered intact in PBS by gentle removing from the dish to yield a homogeneous suspension. Sodium dodecylsulphate (SDS) was then added to a final concentration of 0.5% (w/v). These conditions allow to assess by fluo-

rescence both *N*-Rh-PE content of the plasma membrane and self quenching (*see* below) (Perkin Elmer LS-30 spectrofluorimeter; λ_{exc} 560 nm and λ_{em} 590 nm). Content was determined by reference to a standard curve generated from known amounts of *N*-Rh-PE in 0.5% (w/v) SDS.

To determine C_6-NBD-SM plasma membrane insertion, cells were rapidly washed at 4°C five times in PBS supplemented with 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$ (PBS-Ca^{2+}-Mg^{2+}), then lysed in 0.05% (v/v) Triton X-100. Cell-associated C_6-NBD-SM was similarly measured by fluorimetry (λ_{exc} 465; λ_{em} 530 nm) and content determined by reference to a standard curve generated from known amounts of C_6-NBD-SM in 0.05% (v/v) Triton X-100.

To examine TMA-DPH plasma membrane insertion, cells were washed and recovered as described for *N*-Rh-PE. Cell- associated TMA-DPH was assessed on intact cells in PBS by fluorimetry (λ_{exc} 362; λ_{em} 435 nm).

Cellular Accumulation of *N*-Rh-PE and Internalization of C_6-NBD-SM and TMA-DPH in J774 Macrophages

N-Rh-PE cellular accumulation (*cf.* definition of accumulation *vs.* internalization, *see* below) was measured by self-quenching relief upon dilution with unlabeled intracellular membrane. *N*-Rh-PE is indeed a non-exchangeable lipid analog, by opposition to C_6-NBD-SM and TMA-DPH, and can therefore not be removed from the plasma membrane after insertion. N-Rh-PE-labeled cells were reincubated in FCS- and tracer-free medium at 37°C for up to 30 min, washed with PBS twice for 30 s each, and recovered as described above. Self-quenching was immediately determined by measuring fluorescence before (*F1*) and after (*F2*) addition of 0.5% SDS, and calculated as $[(F2-F1)/F2]\cdot 100\%$.

To monitor C_6-NBD-SM internalization, labeled cells were incubated in fresh FCS- and tracer-free medium at 37°C for up to 30 min, followed by extensive washing at 4°C (back-exchange): three washes with PBS-Ca^{2+}-Mg^{2+} for 30 s each, two washes with PBS-Ca^{2+}-Mg^{2+} supplemented by 5% (w/v) bovine serum albumin (BSA) for 5 min and 1 min and finally five washes with PBS-Ca^{2+}-Mg^{2+} for 30 s each. This procedure removed 96 to 97% of the surface label.

To assay TMA-DPH internalization, labeled cells were incubated into FCS- and tracer-free medium at 37°C for up to 30 min, followed by extensive washing (back-exchange) at 4°C: seven washes of 30 s each with PBS plus 5% (w/v) BSA, followed by four washes of 10 s with PBS. This procedure also removed 96 to 97% of the surface label.

TMA-DPH Fluorescence Polarization Measurements of the J774 Plasma Membrane

To measure plasma membrane fluidity, cells were incubated at 37°C with 5 μM TMA-DPH for 30 s, then washed once with 1% trypsin in PBS at room temperature. After 30 s, cells were recovered to give a homogeneous suspension as above. Cells were immediately assayed at room temperature for fluorescence polarization (Perkin Elmer LS-50B spectrofluorimeter; λ_{exc}, 360 nm and λ_{em}, 435 nm). Each sample was measured six times for 15 s.

Labeling of the J774 Macrophages Cell Surface by Concanavalin A

Confocal microscopy studies were performed on J774 macrophages seeded at lower density (1.3×10^4 cells per cm^2) to facilitate the analysis of well-defined cells. After washing, cells were cooled down at 4°C in PBS-Ca^{2+}-Mg^{2+} and incubated with concanavalin A-tetramethylrhodamine isothiocyanate (20 μg/ml) in PBS-Ca^{2+}-Mg^{2+} supplemented with 1% 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, washed and examined with an MRC1024 confocal scanning equipment (Bio-Rad, Richmond, CA) mounted on a Zeiss Axiovert confocal microscope (Zeiss, Oberkochen, Germany), using λ_{exc} at 555 and λ_{em} at 580 nm.

Azithromycin and Protein Assays

Azithromycin was assayed by a microbiological assay using a disc-plate technique, as described in Montenez et al. (1999). Proteins were assayed by the Lowry procedure (Lowry et al., 1951) using BSA as a standard. Values of all cell constituents were normalized by reference to the cell 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). Azithromycin was dissolved in 0.1 N HCl and thereafter diluted to the desired final concentrations. Glucosamine, sphingomyelin, cholesterol, melittin, benzyl alcohol, and concanavalin A-tetramethylrhodamine isothiocyanate (TRITC) were from Sigma-Aldrich (St Louis, MO). C_6-NBD-SM, TMA-DPH, DPH and R18 were from Molecular Probes (Eugene, OR). *N*-Rh-PE was obtained from Avanti polar lipids (Alabaster, AL). Egg phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and DPPC were from Lipid Products (Redhill, UK). All culture sera and media were supplied by Life Technologies (Paisley, UK). Other reagents were from Merck (Darmstadt, Germany).

Statistical Analyses

Statistical comparisons of experimental values and slopes 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). Unless specified otherwise, differences were considered significant at $P < 0.05$.

Results

Azithromycin Interacts with Lipidic Model Membranes

Binding of azithromycin to lipids was measured by equilibrium dialysis at pH 5.4, 6.0, and 7.0. At an initial drug concentration of 100 mg/L (132 μM) 23.4% ± 5.3 of azithromycin bound to small unilamellar vesicles (SUV), at pH 7.0 (mean of 3 independent experiments with 3 measurements each). Binding varied with drug concentration (13.0% for 50 mg/L azithromycin) but was independent of the pH investigated (Table 1). Similar results were obtained with large unilamellar vesicles (LUV) and when phosphatidylethanolamine was added to liposomes to better mimic the lipid composition of cell membranes (*data not shown*).

Table 1. Influence of pH on the binding of azithromycin to lipids and on the effect of azithromycin on effective chemical shift anisotropy ($\Delta\sigma$) of ^{31}P NMR spectra and fluorescence polarization values (P) of TMA-DPH and DPH

pH	% of Binding[1] (equilibrium dialysis)	$\Delta\sigma$[1,3] (ppm)			P[2,3]			
					TMA-DPH		DPH	
		33°C	52°C	70°C	15°C	37°C	15°C	37°C
5.4	21.7 ± 6.5	35.8 ± 1. 2 *vs.*[4]	34.3 ± 1.1 *vs.*	29.03 ± 1.07 *vs.*	0.390 ± 0.015 *vs.*	0.351 ± 0.024 *vs.*	0.397 ± 0.014 *vs.*	0.348 ± 0.012 *vs.*
		33.0 ± 1.1	29.2 ± 1.0	24.98 ± 1.00	0.385 ± 0.006	0.369 ± 0.012	0.389 ± 0.017	0.332 ± 0.008
6.0	23.1 ± 6.0	36.3 ± 1.1 *vs.*	33.7 ± 1.1 *vs.*	31.24 ± 1.11 *vs.*	0.382 ± 0.013 *vs.*	0.350 ± 0.013 *vs.*	0.398 ± 0.008 *vs.*	0.305 ± 0.032 *vs.*
		30.7 ± 1.2	28.8 ± 1.1	26.24 ± 1.07	0.391 ± 0.007	0.366 ± 0.009	0.399 ± 0.004	0.318 ± 0.017
7.0	23.4 ± 5.3	37.6 ± 1.2 *vs.*	35.0 ± 1.2 *vs.*	31.38 ± 1.09 *vs.*	0.384 ± 0.015 *vs.*	0.362 ± 0.012 *vs.*	0.378 ± 0.007 *vs.*	0.334 ± 0.004 *vs.*
		31.8 ± 1.1	27.7 ± 1.2	24.51 ± 1.05	0.384 ± 0.008	0.375 ± 0.004	0.374 ± 0.018	0.305 ± 0.056

[1] Mean ± SD of three independent experiments.
[2] Mean ± SD of three dishes from one experiment.
[3] Values of effective chemical shift anisotropy and fluorescence polarization are compared to control values.
[4] Data are presented as azithromycin *vs.* control.

Azithromycin Reduces the Mobility of Phosphate Heads of Phospholipids but Does not Perturb Transversal Membrane Domains Located Deeper within the Bilayer

To further define azithromycin interactions with polar, interfacial and hydrophobic domains of phospholipids, ^{31}P NMR and fluorescence depolarization studies were performed. First, we measured by ^{31}P NMR spectroscopy the effective chemical shift anisotropy ($\Delta\sigma$) as a function of temperature and pH. Typical spectra obtained with multilamellar vesicles (MLV) for pH 7.0 at 33°C or 70°C are shown in Fig. 1 (upper panel). In control conditions, signal shapes are characteristic of a bilayer organization with a high-field maximum and a low field shoulder. Warming caused a decrease of the effective chemical shift anisotropy, $\Delta\sigma$. In the presence of azithromycin, the $\Delta\sigma$ value was higher than for control liposome spectra and similarly decreased upon warming. Again, comparable experiments performed with liposomes containing phosphatidylethanolamine showed similar results (*data not shown*). Glucosamine, a monocationic compound that does not interact with liposomes, used as negative control (Mingeot-Leclercq et al., 1989), had no effect as compared with control (*data not shown*). The lower panel of Fig. 1 shows the measured values of the effective chemical shift anisotropy ($\Delta\sigma$) as a function of temperature at pH 7.0. A sharp decrease was observed when control and azithromycin-treated liposomes were warmed from 33°C to 70°C. At the three temperatures investigated, $\Delta\sigma$ values were systematically higher for liposomes incubated with azithromycin as compared with control liposomes. There was no appreciable effect of pH between 5.4 and 7.0 (Table 1).

Second, to investigate the influence of azithromycin deeper within the bilayer, we examined its effect on the extent of fluorescence polarization of two probes: trimethylammoniumdiphenylhexatriene (TMA-DPH), and diphenylhexatriene (DPH). Both tracers become inserted within the bilayer, with a shallower depth for TMA-DPH, because of the cationic TMA group directly attached to one DPH phenyl ring (Kaiser & London, 1998). Figure 2 shows that the extent of polarization of the two probes in control LUV decreased linearly when temperature increased, albeit to a lesser extent for TMA-DPH than for DPH. This can be interpreted to indicate a lesser increase in mobility upon warming of the alkyl chains close to the interface in comparison to the deeper hydrophobic domain. Addition of azithromycin did not significantly alter the degree of polarization of DPH or TMA-DPH over the entire range of temperatures investigated. Similarly to pH 7.0, no effect of azithromycin was observed at pH 5.4 and 6.0

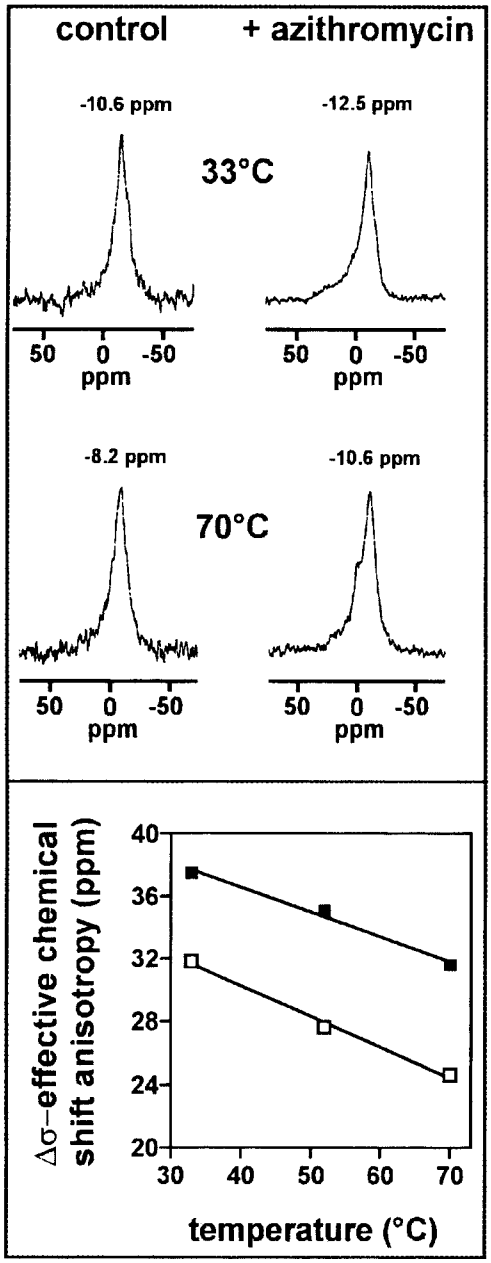

Fig. 1. *Upper panel:* Effect of azithromycin on typical ^{31}P nuclear magnetic resonance (NMR) spectra of large multilamellar vesicles (MLV). Liposomes (37.5 mM in lipids) were prepared at pH 7.0 and analyzed at 33°C and 70°C in the absence or presence of 5 mM azithromycin. The isotropic signal was set at 0 ppm as a reference. Figures show the chemical shift values of the peak at high-field. *Lower panel:* Effect of temperature on the effective chemical shift anisotropy ($\Delta\sigma$) of ^{31}P NMR signals of MLV. Liposomes were prepared at pH 7.0 and incubated in the presence of azithromycin (at drug:phospholipid molar ratio of 0.2; filled symbols) or without drug (empty symbols) at 37°C for 1 h. $\Delta\sigma$ values are means of three independent determinations with SD < 1.2 ppm. Undistinguishable values were observed at pH 6.0 and 5.4 (*see* Table 1).

(Table 1) and when phosphatidylethanolamine was added to liposomes (*data not shown*). In contrast, benzyl alcohol, used as positive control (Friedlander et al., 1987), induced a systematic decrease of polarization values of both TMA-DPH and DPH.

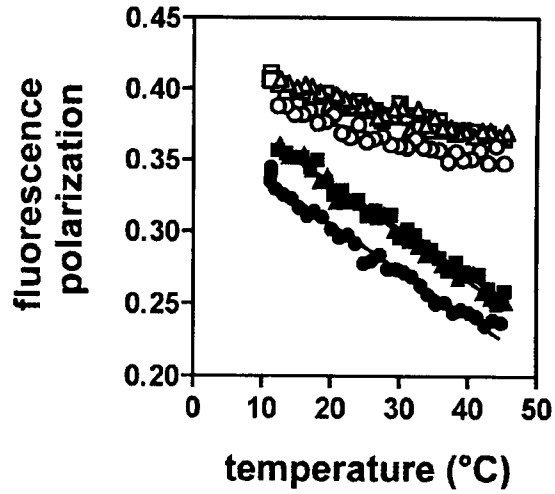

Fig. 2. Effect of azithromycin on DPH and TMA-DPH fluorescence polarization on large unilamellar vesicles (LUV). Liposomes (0.314 mM in lipids) were prepared at pH 7.0, and allowed to incorporate DPH (filled symbols) or TMA-DPH (empty symbols) at a molar ratio to the lipids of 1:250. Labeled LUV (*squares*) were then mixed with azithromycin (132 μM; *triangles*) or benzyl alcohol (30 mM; circles) and incubated at 37°C for 30 min. Liposomes were then brought to 45°C in 15 min, and stabilized at this temperature during 5 min before starting the measurements, during which the samples were cooled down to 10°C at a rate of 50°C/h. Data shown are representative of experiments that were reproduced four times for DPH and twice for TMA-DPH.

Azithromycin Does not Induce Membrane Fusion

Since azithromycin interacts with the polar head group of phospholipids, the effect of azithromycin/lipid interactions on lipidic membrane fusion was studied. As shown in Fig. 3, using fluorescence dequenching of R18, melittin caused an immediate (<15 s) and marked increase in the fluorescence signal that levelled off after ~5 min, as expected for a positive control (Van Bambeke et al., 1995). In complete opposition, addition of azithromycin (from 0.65 μM to 800 μM) caused no detectable increase in the fluorescent signal during the entire observation period (20 min). Similar results were obtained when phosphatidylethanolamine, a phospholipid known to promote membrane fusion (Düzgünes et al., 1987), was added to liposomes and when experiments were performed at pH 5.4 instead of pH 7.0 (*data not shown*).

Azithromycin Disrupts Lateral Membrane Domains in Langmuir-Blodgett Monolayers

To test whether the interaction between azithromycin and lipids could alter membrane organization in lateral domains, atomic force microscopy was used. This surface imaging technique, which operates by measuring forces between a probe and the sample, offers nanometer-scale lateral resolution and subna-

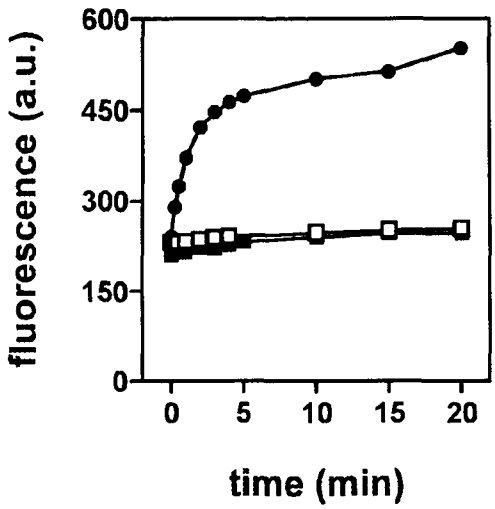

Fig. 3. Time course of octadecylrhodamine B (R18) fluorescence dequenching upon interaction between R18-labeled LUV and unlabeled LUV prepared as in Fig. 2. Labeled and unlabeled vesicles (5 μM in lipids) were mixed (ratio 1:4) and the fluorescence was followed during 20 min either without any addition (controls; *empty squares*), or after addition of melittin (0.635 μM; *filled circles*) or azithromycin (800 μM; *filled squares*). Experiments were reproduced twice.

nometer-scale normal resolution. Figure 4*A* shows a representative topographic image of mixed DPPC/cholesterol monolayers. Phase separation was observed with a step height between the lower and higher domains measured of 1.9 ± 0.1 nm. As shown in Figs. 4*B–D*, addition of azithromycin to the lipid mixture yielded very different morphologies. In most areas, monolayer domains were no longer seen and small aggregates of about 1 to 5 nm height were distributed across the mica surface (Fig. 4*B*). Furthermore, while domains observed in the absence of azithromycin were stable during iterative scanning, small aggregates formed in the presence of the drug were easily displaced by the scanning probe, even under minimal load (<1 nN; Fig. 4*C*). The low stability of the system is further illustrated in Fig. 4*D* in which a 2 μM × 2 μM image was first recorded at large force (several nN), followed by imaging a 4 μm × 4 μm image of the same area under normal load (<1 nN). This image shows that most of the aggregates had been pushed aside by the probe and had accumulated on the edges of the 4 μm^2 area. This result also confirms the absence of a continuous film on the mica substrate.

Azithromycin Differently Affects Plasma Membrane Insertion and Cellular Accumulation or Internalization of Three Membrane Tracers in J774 Macrophages and Perturbs Plasma Membrane Fluidity

Since azithromycin directly interacts with artificial membranes, we next evaluated its ability to perturb insertion into the pericellular membrane of three membrane tracers at a low temperature that prevents endocytosis, as well as their total cellular accumulation or internalization upon rewarming at the physiological temperature. We also checked the effect of azithromycin on plasma membrane fluidity. For these experiments, macrophages were preincubated with the drug for 3 h before addition of the various membrane tracers. This time of preincubation was selected because it corresponds to a plateau for maximal inhibition of horse radish peroxidase uptake (our unpublished results).

Plasma membrane insertion studies were performed using C_6-NBD-SM and TMA-DPH, which, respectively, label the outer interfacial zones (Koval & Pagano, 1989) and the deeper domain of the bilayer (Kaiser & London, 1998) as well as *N*-Rh-PE, a non-exchangeable tracer that spontaneously forms small lateral aggregates in the plane of the plasma membrane (Kok et al., 1990). Incorporation of these three membrane tracers into the plasma membrane increased linearly with the extracellular concentration in the range used for further studies (Fig. 5*A–C*). For TMA-DPH, the increment of fluorescence intensity at concentrations above 4 μM declined sharply (*data not shown*), which can be explained by self-quenching of the probe above a threshold value (Illinger et al., 1990; Illinger, Poindron & Kuhry, 1991). Concentrations of 4 μM C_6-NBD-SM, 2 μM TMA-DPH and 5 μM *N*-Rh-PE were selected for subsequent experiments. Azithromycin significantly decreased surface incorporation of C_6-NBD-SM, TMA-DPH, and *N*-Rh-PE by respectively ~20%, ~25%, and ~40%, over the entire range of tracer concentrations used (Fig. 5*A–C*). This effect seemed roughly proportional to extracellular azithromycin concentration for C_6-NBD-SM (10.0 ± 4.0% at 50 mg/L *vs.* 19.3 ± 2.1% at 100 mg/L) and for *N*-Rh-PE (21.3 ± 7.2% at 50 mg/L *vs.* 41.4 ± 3.6% at 100 mg/L), but not for TMA-DPH (23.2 ± 6.6% at 50 mg/L and 23.8 ± 5.0% at 100 mg/L).

The comparison of these three tracers could also be used to discriminate the fate of bulk *vs.* clustered membrane upon internalization. Indeed, the different supramolecular organization of these membrane tracers dictates their sorting in endosomes towards either the recycling route, in the absence of clustering, or their discharge as clusters to lysosomes along the degradation pathway (Kok et al., 1990; Mukherjee & Maxfield, 2000). Bulk-membrane internalization was measured using C_6-NBD-SM and TMA-DPH. Azithromycin had only a marginal effect on the kinetics of internalization for C_6-NBD-SM and no significant influence for TMA-DPH. Interestingly, C_6-NBD-SM was less efficiently internalized (by approx. 50%) than TMA-DPH in control cells (Fig. 5*D vs.* 5*E*). Membrane trafficking of a clustered tracer destined to lysosomes was evaluated using

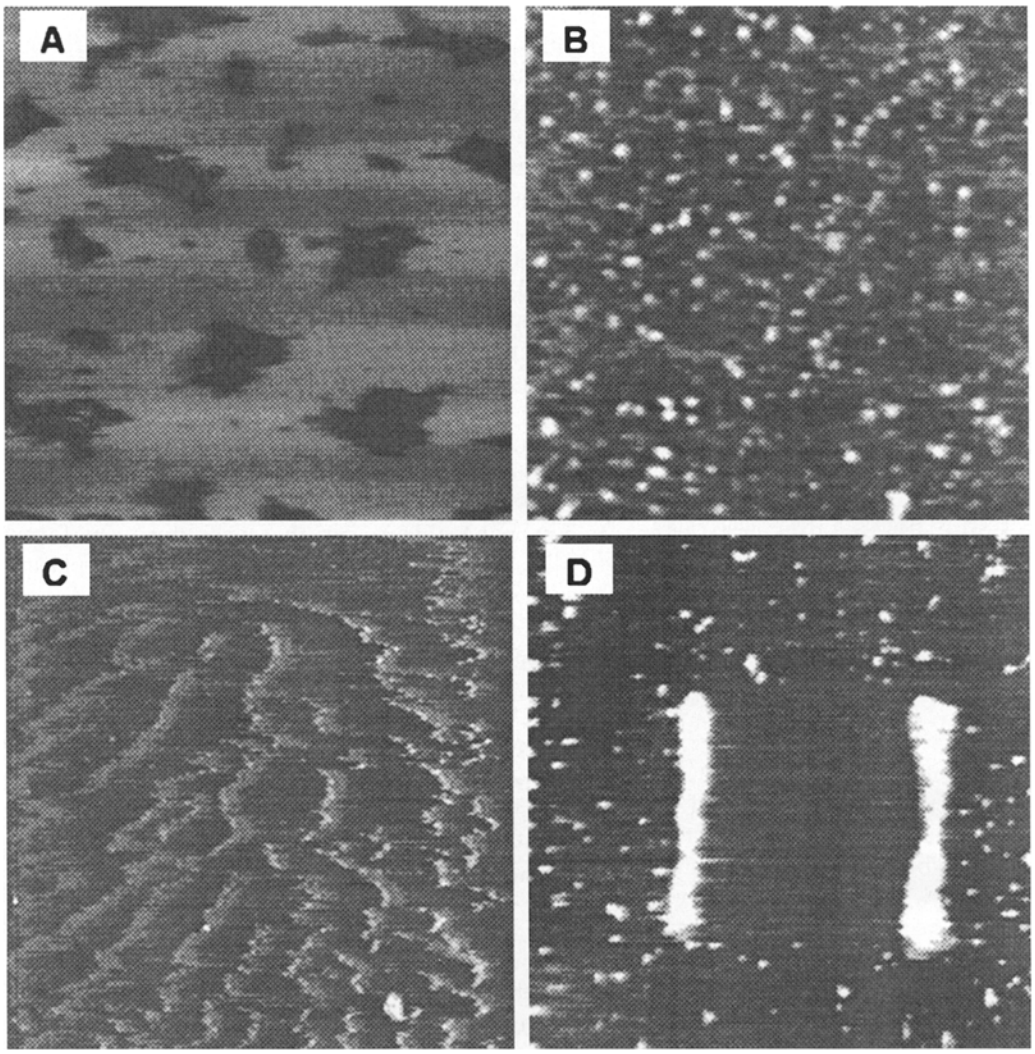

Fig. 4. Atomic force microscopy topographic images showing the influence of azithromycin on the nanoscale organization of mixed DPPC/cholesterol Langmuir-Blodgett monolayers supported on mica. (*A*) DPPC/cholesterol monolayer (2:1 molar ratio); (*B*, *C*, *D*) DPPC/cholesterol/azithromycin monolayers.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

N-Rh-PE. This probe 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 preexisting endosomes due to dilution with unlabeled membranes. The rate of self-quenching relief of *N*-Rh-PE was slowed down by ~40% upon azithromycin treatment (Fig. 5*F*).

A perturbation of membrane fluidity could explain the decrease of membrane tracer insertion and cellular accumulation induced by azithromycin. This hypothesis was directly investigated by measuring fluorescence polarization of TMA-DPH at the plasma membrane of J774 cells. Cells were either left untreated or were pretreated with azithromycin or benzyl alcohol, a local anesthetic known to alter membrane fluidity and used as a positive control (Friedlander et al., 1987), then incubated at 37°C for 30 s with TMA-DPH in the absence of drug. A polarization value (P) of 0.354 ± 0.003 was measured for the plasma membrane of control J774 cells. This value is similar to that reported by Coupin and Kuhry (1999) in mouse L929 fibroblasts and is thought to reflect a well-organized plasma membrane. Preincubation with 30 mM benzyl alcohol for 30 min significantly increased the polarization value (P = 0.381 ± 0.005; $P < 0.001$), confirming the marked effect of this drug on membrane fluidity. Similarly, increasing concentrations of azithromycin also increased TMA-DPH fluorescence polarization, to reach a plateau of 0.371 ± 0.003 at 50 mg/L azithromycin.

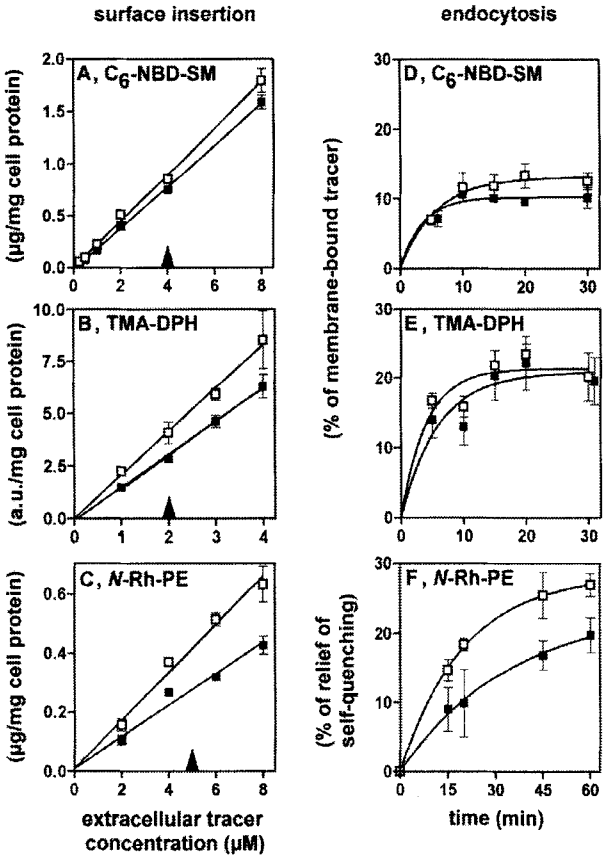

Fig. 5. Effect of azithromycin on the plasma membrane insertion (*A–C*), internalization (*D–E*) and cellular accumulation (*F*) of three membrane tracers in J774 mouse macrophages. (*A*, *D*) C_6-NBD-SM; (*B*, *E*) TMA-DPH; (*C*, *F*) *N*-Rh-PE. Cells were either left untreated (*empty squares*) or pretreated with 132 μM azithromycin at 37°C for 3 h (*filled squares*), prior to labeling. For surface labeling (*A–C*), cells were incubated at 4°C with the indicated concentrations of each lipid tracer. For internalization studies of C_6-NBD-SM and TMA-DPH (*D–E*), cells were exposed at 4°C to 4 μM probe for 30 min and 2 μM for 2 min, respectively, then incubated at 37°C in tracer-free medium for the indicated times, after which tracer remaining at the cell surface was removed by a back-exchange procedure at 4°C. Internalization was defined as the fraction of cell-associated tracer resisting surface removal. Statistical analysis showed no significant difference between untreated and azithromycin-treated cells for all data with TMA-DPH. For C_6-NBD-SM, only the steady-state values are significantly different ($P < 0.01$). For accumulation of *N*-Rh-PE (*F*), after labeling with 5 μM for 30 min at 4°C, cells were further incubated 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). All data are means ± SD of 3 dishes. Where not visible, error bars are included in the symbols. Experiments were reproduced three times in *A* and *D*, twice in *B* and *E* and four times in *C* and *F*, with similar results.

An effect of azithromycin on membrane fluidity was further confirmed by confocal microscopy at 4°C, by following induction of the clustering of membrane glycoproteins at the cell surface by fluorescent concanavalin A (Roberson, Neri & Oppenheimer, 1975). Lectin-induced clusters became clearly patched on the cell surface in control cells (Fig. 6*A*), but not, or to a much lesser extent, in azithromycin pretreated cells (Fig. 6*B*). This suggests that decreased membrane fluidity impaired the capacity of glycoproteins to move along the lateral plane of the plasma membrane to become aggregated by the lectin.

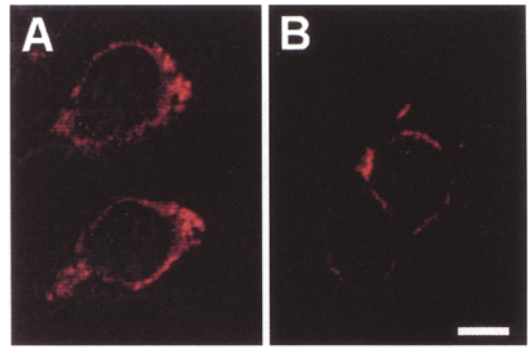

Fig. 6. Confocal microscopy of J774 cell surface-labeling by fluorescent concanavalin A. Cells were either left untreated (*A*) or treated with 132 μM azithromycin (*B*) at 37°C for 3 h, after which they were cooled down to 4°C and labeled with concanavalin A-TRITC for 1 h, fixed and examined by confocal microscopy. Panels *A* and *B* reflect the observations performed on more than 15 images of both control and azithromycin-treated cells. Bar is 10 μm.

Discussion

We have recently observed that, in J774 macrophages, azithromycin inhibited fluid-phase endocytosis and induced a delay between ligand binding to their receptors and subsequent internalization into clathrin-coated pits for receptor-mediated endocytosis, but did not affect phagocytosis (Tyteca et al., 2002). The strong effect of azithromycin on fluid-phase endocytosis of horseradish peroxidase was also observed in rat fetal fibroblasts, by biochemical and morphological studies. However, in this cell type, no effect was detected on receptor-mediated endocytosis of transferrin, neither on cell-surface binding nor on ligand internalization (Tyteca et al., 2001). This difference could be easily explained by the rate of internalization of transferrin, that is approximately four times faster in J774 macrophages than in rat fetal fibroblasts. It is indeed reasonable to assume that a faster process is more sensitive to a drug affecting a rate-limiting component of its machinery. Moreover, there are multiple examples where biological properties differ considerately between primary cultures (such as rat fetal fibroblasts) and immortalized cell lines (such as J774 macrophages).

These cell biological observations, which could result from decreased lateral diffusion (Draye et al., 1988) or impaired membrane partitioning (Mukherjee & Maxfield, 2000), suggested that azithromycin perturbs lateral mobility of receptors and/or budding

of endocytic pits into endocytic vesicles. The present biophysical study was performed to analyze interactions of azithromycin with artificial (liposomes and Langmuir-Blodgett monolayers) and cellular membranes (J774 mouse macrophages), in order to evaluate the consequences of these interactions on biomembrane properties and endocytic membrane trafficking. Azithromycin was found to: (i) directly interact with membrane phospholipids; (ii) perturb the organization of model membranes both in transversal and lateral planes without causing fusion of lipidic phase; (iii) decrease the fluidity of plasma membrane; and (iv) reduce the cellular accumulation of a lipid analog destined to lysosomes, but not the internalization of bulk-membrane lipid tracers.

The dicationic weak base, azithromycin, largely codistributes with lysosomal hydrolases where it accumulates by a diffusion-acidotropic sequestration process. About one-third of the cell-associated drug, however, remains not sedimentable (Carlier et al., 1994) and is presumably present in the cytosol. This suggested to us that azithromycin could also interact with the cytosolic leaflet of the plasma membrane and the limiting membrane of endocytic vesicles. Equilibrium dialysis and ^{31}P-NMR spectroscopy reported in this paper establish that azithromycin is indeed able to bind to negatively charged phospholipid bilayers, not only at pH 5.4 as shown earlier (Van Bambeke et al., 1996), but also at pH 6.0 and 7.0, i.e., at values that correspond to those of the pH of the endosomal lumen and cytosol, respectively. At all three pHs, the chemical shift anisotropy is increased in the presence of azithromycin in the whole range of measured temperatures, indicating that the antibiotic interacts at the level of the phosphate groups and reduces their motional freedom. Furthermore, the comparable binding of azithromycin to lipids between pH 5.4 and 7.0 strongly supports the conclusion that azithromycin interacts not only with phosphate groups of negatively-charged phospholipids but also with zwitterionic phospholipids, as already suggested by conformational analysis (Montenez et al., 1999).

This conclusion is in agreement with results obtained by atomic force microscopy, a high-resolution imaging technique recently established in biophysics to characterize lipid films at nanometer (z axis) and subnanometer (xy axis) scales (for a review, *see* Dufrêne & Lee, 2000) and to bring new light into drug/membrane interactions (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 measured between the two phases (1.9 ± 0.1 nm) is in good agreement with the film thickness, expected at ~3 nm for DPPC and 1.5 nm for cholesterol, respectively, but 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 not only aggregates, rather than a continuous film, were observed at the mica surface but the membrane was even destabilized when the material was reorganized perpendicularly to the scanning direction. These results thus suggest 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. Since the position of azithromycin is probably near the polar head and the interface, this superficial insertion in the hydrophobic domain would not affect the fluidity of the hydrophobic domain of phospholipids and not cause a profound lipidic reorganization to the extent that is required for membrane fusion.

Since azithromycin was found to perturb the mobility of the polar head groups of phospholipids in model membranes, the effect of the drug on biological membranes was further examined using whole cells. We showed that azithromycin decreased the plasma membrane insertion of three membrane tracers in J774 macrophages, and this, to a different extent for each tracer (C_6-NBD-SM [~20%] < TMA-DPH [~25%] < *N*-Rh-PE [~40%]). Several explanations can be offered to account for this difference. First, part of azithromycin could be intracellular, close to the lipid/water interface and interacting with acidic phospholipids, which are mostly located in the inner leaflet of the plasma membrane. This could explain why azithromycin affects more severely the insertion of TMA-DPH, a synthetic compound labeling the deep domain of the bilayer (Kaiser & London, 1998), than it affects that of C_6-NBD-SM, which labels the outer interfacial zone of the bilayer and does not translocate to the cytoplasmic surface during the endocytic process (Koval & Pagano, 1989). Second, azithromycin could preferably perturb the organization of the bilayer in specific lateral domains, namely those to which *N*-Rh-PE partitions. This could be of great significance and would explain why azithromycin induced only a small decrease in *bulk* fluidity of J774 plasma membrane, as measured by fluorescence polarization of TMA-DPH, a probe which evaluates the bulk membrane fluidity (Illinger et al., 1990; Bastiaanse et al., 1993). Intriguingly enough, the effect of azithromycin on the polarization of TMA-DPH inserted in a cellular model is different from that observed on liposomes, possibly due to a different location of the probe and/or differences in the experimental protocols used. More interestingly,

the decreased membrane fluidity in azithromycin-treated J774 cells is further supported by the apparent reduction of concanavalin A patching at the cell surface. Indeed, membrane fluidity is a result of both lipid microviscosity and mobility of proteins. In addition, protein patches are created by barriers to lateral diffusion and disappear during vesicular traffic to and from the plasma membrane (Gheber & Edidin, 1999). Our conclusion also corroborates the observations of Tang and Edidin (2001), who showed that conditions inhibiting vesicular trafficking (hypertonic treatment, GTPase-defective dynamin mutants) also increased the apparent size of patches whereas their intensities decreased.

Although our observations provide some interesting clues, they do not yet fully explain how azithromycin/membrane interactions inhibit fluid-phase endocytosis but not phagocytosis (Tyteca et al., 2001, 2002). Quite different from liposomes or Langmuir-Blodgett monolayers, the plasma membrane of a living cell undergoes continuous vesicular exchange with a dynamic reservoir of membrane constituents, due to constitutive endocytosis into, and recycling from, internal compartments. C_6-NBD-SM and TMA-DPH are adequate tracers to follow this bulk membrane flow (Koval & Pagano, 1989; Illinger et al., 1990). By contrast, *N*-Rh-PE, which forms small clusters, is preferentially delivered to the late endosomal pathway (Mukherjee & Maxfield, 2000). We observed that azithromycin does not affect internalization of C_6-NBD-SM and TMA-DPH but decreased *N*-Rh-PE endocytic accumulation. Two major forces driving local membrane curvature—and thereby endocytosis—are now recognized candidates for vesicle formation in living cells. One such force is the polymerization onto the cytosolic phospholipid leaflet of coat proteins such as clathrin, aided by an array of accessory proteins (Takei & Haucke, 2001). In contrast to the data reported for C_6-NBD-SM or TMA-DPH, which are both taken up to a comparable extent by clathrin-dependent and clathrin-independent pathways (Puri et al., 2001; Coupin & Kuhry, 1999), no data are published, to our knowledge, on the *N*-Rh-PE endocytosis pathway. Because azithromycin decreases more the insertion and endocytosis of *N*-Rh-PE than of C_6-NBD-SM and TMA-DPH, we suggest that azithromycin directly interacts with membranes, perturbs their organization and probably the fluidity of specific domains of the plasma membrane, namely those containing *N*-Rh-PE. The other driving force, observed in absence of clathrin, is the active generation of membrane asymmetry between the two leaflets of the plasma membrane (Farge, 1995; Farge et al., 1999; Huijbregts et al., 2000; Rauch & Farge, 2000; Heerklotz, 2001). Preferential addition of azithromycin to one leaflet might generate an asymmetry of bilayer area and thereby increase its curvature, causing membrane curling away from the side to which the drug molecule is added, thus expanding this leaflet with additional molecules while compressing the other. Our study suggests that membrane fluidity (Dai & Sheetz, 1995), a property that is strongly related to membrane asymmetry, is an additional important parameter. This hypothesis is in agreement with the fact that lipid ordering is related to membrane curvature and that clathrin-coated pits, which are surrounded by a cytoplasmic coat imposing a local constraint on membrane fluidity, are less perturbed by a change in membrane fluidity than other regions of the membrane (Coupin & Kuhry, 1999). If one leaflet of the plasma membrane could be preferentially ordered by azithromycin binding to lipids, the difference of fluidity should inhibit membrane curvature. In other words, preferential ordering of one leaflet would decrease vesicle budding (Huttner & Zimmerberg, 2001).

In conclusion, we have shown that azithromycin is able to interact with lipids and to modify some membrane biophysical properties at specific domains of the plasma membrane. It is likely that these effects are in relation with perturbation of endocytosis. Accordingly, we propose that further studies exploiting perturbation of the plasma membrane by azithromycin may contribute to elucidate the physicochemical properties underlying membrane budding and endocytosis.

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