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# Role of Extracellular Calcium in In Vitro Uptake and Intraphagocytic Location of Macrolides

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**We compared the uptakes and intracellular locations of four 14-membered-ring macrolides (roxithromycin, dirithromycin, erythromycin, and erythromycylamine) in human polymorphonuclear neutrophils (PMNs) in vitro. Intracellular location was assessed by cell fractionation and uptake kinetics in cytoplasts (granule-poor PMNs). Trapping of dirithromycin within PMN granules (up to 80% at 30 min) was significantly more marked than the intracellular trapping of the other drugs (erythromycylamine, 45% ± 5.1%; erythromycin, 42% ± 3.7%; roxithromycin, 35% ± 3.0%). A new finding was that, in the absence of extracellular calcium, the uptakes of all of the macrolides by PMNs and cytoplasts were significantly impaired, by about 50% (PMN) and 90% (cytoplasts). Furthermore, inorganic $Ca^{2+}$ channel blockers inhibited macrolide uptake in a concentration-dependent manner, with 50% inhibitory concentrations of 1.6 to 2.0 mM and 29 to 35 μM, respectively, for $Ni^{2+}$ and $La^{3+}$. The intracellular distributions of the drugs were unchanged in the presence of $Ni^{2+}$ and $La^{3+}$ and in $Ca^{2+}$-free medium supplemented with ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. The organic $Ca^{2+}$ channel blocker nifedipine had no effect on macrolide uptake, whereas verapamil inhibited it in a time- and concentration-dependent manner. These data show the importance of extracellular $Ca^{2+}$ in macrolide uptake by phagocytes and suggest a link with $Ca^{2+}$ channels or a $Ca^{2+}$ channel-operated mechanism.**

Infections caused by organisms which survive and multiply within host cells, such as *Legionella* spp., *Chlamydia* spp., and *Mycobacteria* spp., are difficult to cure, partly because of the intracellular locations of these pathogens, which are protected from the antimicrobial activities of non-cell-penetrating agents (20). However, the abilities of antibiotics to enter and concentrate within host cells, generally estimated from the intracellular concentration-to-extracellular concentration ratio, may not actually reflect their overall intracellular bactericidal activities (17). Another factor of importance is the respective intracellular locations of the drug and the pathogen. Possible interactions between the drug and host cell factors (inactivation, binding, synergy) may also result from the particular location of the drug. In addition, intracellular accumulation of antibacterial agents or the mechanism used for this cellular transport may in turn affect host cell metabolism and functions, ultimately modifying the overall course of infectious diseases. Among those cell-penetrating agents, macrolide antibiotics have been much studied (14, 27). Many publications examine their intracellular (particularly intraphagocytic) accumulation (for a review, see reference 29) and their possible interference with phagocyte activities (for a review, see reference 28). Despite abundant data, the mechanism underlying macrolide uptake by host cells is poorly understood. Various hypotheses have been put forward. These hypotheses involve liposolubility and active transport systems, particularly those used by nucleosides (18, 21). A likely mechanism for their intracellular accumulation involves the weakly basic nature of macrolides and the possibility of trapping by protonation within acidic cellular compartments, lysosomes, and polymorphonuclear neutrophil (PMN) granules (9, 36). This hypothesis has been verified in phagocytic and nonphagocytic human and animal cells (6, 9, 45, 50, 51). The possibility that the intragranular location alters the correct functioning of this cellular compartment (exocytosis, phagolysosomal fusion) has been little explored (1, 8, 30). Recently, we have demonstrated that various 14-membered-ring macrolides induce PMN degranulation (1, 30) and that extracellular calcium is required for this phenomenon (31). The intracellular locations of roxithromycin and erythromycin have been characterized (9) in human PMNs; to our knowledge that of dirithromycin has been analyzed in mouse macrophages only (6), and no data are available concerning its metabolite, erythromycylamine. This prompted us to determine the intracellular locations of dirithromycin and erythromycylamine (for which no data are available in human PMNs) in comparison with those of erythromycin and roxithromycin. In addition, we analyzed the influence of extracellular calcium on macrolide uptake and location in PMN.

(Part of this work was presented at the 94th General Meeting of the American Society for Microbiology [31].)

## MATERIALS AND METHODS

**Antibiotics and chemicals.** Erythromycin and roxithromycin (Roussel Uclaf, Paris, France), dirithromycin and erythromycylamine (Eli Lilly, Indianapolis, Ind.), and the radiolabelled drugs were provided by the respective manufacturers. [$^3$H]dirithromycin (20 mCi/mg), [$^3$H]roxithromycin (26.1 mCi/mg), and [$^3$H]erythromycin (27 mCi/mg) were solutions in ethanol-water (7/3; vol/vol), and [$^{14}$C]erythromycylamine (4.8 μCi/mg) was the base powder. Tritiated drugs (2.5 μl) were mixed with 25 μl of unlabelled drugs (0.001 mg/ml of ethanol-Hanks buffered salt solution [HBSS]; 1/9; Pasteur, Paris, France) and 222.5 μl of HBSS. [$^{14}$C]erythromycylamine was made up to a solution of 0.001 mg/ml of ethanol-HBSS (1/9). Stock solutions were further diluted in HBSS to the desired concentrations.

Nickel chloride ($Ni^{2+}$), lanthanum chloride ($La^{3+}$), verapamil hydrochloride, nifedipine, BAPTA-AM [1,2-bis-(*O*-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid tetra-(acetoxymethyl) ester], and cytochalasin B were from Sigma. Magnesium chloride ($Mg^{2+}$), nifedipine, and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] were from Merck. All reagents except verapamil and nifedipine were dissolved in HBSS; verapamil and nifedipine were dissolved in chloroform and were further diluted in HBSS to final concentrations

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of 100 to 500 μM in 0.1% chloroform. Because of the photosensitivity of nifedipine, experiments with this agent were performed in the dark. The solutions of all reagents were buffered at pH 7.4 to avoid any influence of pH on macrolide uptake. A solvent control (0.1% chloroform) was tested in parallel with nifedipine and verapamil.

**Human PMNs.** Human PMNs were obtained from the venous blood of healthy volunteers by Ficoll-Paque centrifugation and then 2% dextran sedimentation and osmotic lysis of residual erythrocytes.

**Macrolide uptake.** A radiometric assay was used to measure macrolide uptake (39). Briefly, $2.5 \times 10^6$ PMNs were incubated at 37°C with the radiolabelled drugs and were then centrifuged at $12{,}000 \times g$ for 3 min at 22°C through a water-impermeable silicone-paraffin (86 and 14%, respectively; vol/vol) oil barrier. The pellet was solubilized in Hionic fluor (Packard), and cell-associated radioactivity was quantified by liquid scintillation counting (Beckman LS-6000-S). Standard dilution curves were used to determine the amounts of cell-associated drugs.

The concentrations of macrolides used in the assays were 2.5 mg/liter (approximately 3 μM) for roxithromycin, dirithromycin, and erythromycin and 50 mg/liter for erythromycylamine (because of the poor labelling of this drug). For comparative uptake kinetic studies, an extracellular concentration of 10 mg/liter was used for all macrolides.

In agreement with the literature (18), roxithromycin uptake was rapid and plateaued after 60 min; erythromycin displayed a similar profile, although cell-associated drug amounts were far less than those with roxithromycin. By contrast, as we and others have already observed (16, 39), dirithromycin uptake increased as a function of time; a similar but less striking phenomenon was observed with erythromycylamine.

**Cellular location.** Drug-loaded PMNs (30 min at 37°C) were centrifuged through the silicone-paraffin oil barrier, and the cell pellet was sonicated in the presence of 0.5% Triton (three times for 15 s each time) or 0.73 M sucrose to protect granules (three times for 5 s each time) (1). After centrifugation ($100{,}000 \times g$, 30 min), the amounts of marker enzymes lactate dehydrogenase (LDH) (5), β-glucuronidase (47), and lysozyme (35) in the pellet and the supernatant, together with the amounts of radiolabelled drugs, were determined.

**Uptake kinetics in cytoplasts.** Enucleated and granule-poor PMNs (cytoplasts) were prepared as described by Roos et al. (43). PMNs ($3 \times 10^7$ cells) were suspended in 12.5% (wt/vol) Ficoll with 20 μM cytochalasin B. The cell suspension was preincubated at 37°C for 5 min and was then layered onto a prewarmed discontinuous density gradient (16% Ficoll on 25% Ficoll) containing 20 μM cytochalasin B. After centrifugation ($81{,}000 \times g$ at 33°C for 30 min), a band of cytoplasts was formed at the interface of the 12.5 and 16% Ficoll solutions. PMNs and cell debris were recovered at the interface of the 16 and 25% Ficoll solutions, and granules were obtained in the pellet. All three fractions were washed five times to remove cytochalasin B. The amounts of marker enzymes (lysozyme and β-glucuronidase) were measured in these fractions; cytoplasts contained less than 2% lysozyme and β-glucuronidase, PMNs and cell debris contained approximately 4%, and the granular pellet contained both markers at 95 to 96%. The cytoplasts were counted with a hematocytometer and were diluted ($2.5 \times 10^6$) before use as intact cells to measure drug uptake.

**Activation energy.** Activation energy was calculated by measuring the kinetics of macrolide uptake by PMNs incubated for 5 min at 4, 25, 30, 37, and 40°C (34). The temperature dependence of the antibiotic uptake rates was quantified by calculating Arrhenius activation energies given in equation 1: $\Delta G = -RT \ln K_{eq}$, where $\Delta G$ is the activation energy (in calories per mole), $T$ is the temperature (in degrees Kelvin), $R$ is a constant (equal to 1.98), and $\ln K_{eq}$ is the Napierian logarithm of the intracellular concentration-to-extracellular concentration ratio at 5 min (a time when uptake rates are maximal). By transforming calories per mole to joules per mole and Ln to log, equation 1 becomes equation 2: $\Delta G = -1.98 \times 4.18 \times 2.3\ T \log K_{eq}$. $\Delta G$ can be obtained from the slope of the curve by using the Van't Hoff plot representation of data: $\log K_{eq} = -[1/(4.18 \times 2.3 \times 1.98)]\ \Delta G\ (1/T)$ (equation 3).

**Effect of $Ca^{2+}$ chelators on macrolide uptake.** Intracellular $Ca^{2+}$ was chelated by loading PMNs with 30 μM BAPTA-AM for 30 min at 37°C (40) before their use in drug uptake experiments. Extracellular $Ca^{2+}$ was depleted by using nominally $Ca^{2+}$-free HBSS supplemented with 1 mM EGTA. Total depletion of $Ca^{2+}$ was obtained by combining the two treatments.

**Effect of $Ca^{2+}$ channel blockers.** PMNs in HBSS containing 1 mM $CaCl_2$ were pretreated for 10 min at 37°C with the inorganic $Ca^{2+}$ channel blockers (44) $Ni^{2+}$ (0.125 to 10 mM), $La^{3+}$ (20 to 100 μM), and $Mg^{2+}$ (1.25 to 5 mM) before measuring macrolide uptake. PMNs were also pretreated for 10 min in $Ca^{2+}$ containing HBSS with verapamil (250 to 500 μM) or nifedipine (100 to 500 μM), two organic antagonists of L-type voltage-dependent $Ca^{2+}$ channels (19).

**PMN viability.** PMN viability was assessed by measuring the amount of LDH released. None of the experimental conditions (EGTA at 1 mM in $Ca^{2+}$-free HBSS, BAPTA-AM at 30 μM, the combined treatment of BAPTA-AM plus EGTA, $Ca^{2+}$ channel blockers, and $Ca^{2+}$ antagonists, all with or without macrolides) altered PMN viability after incubation for 5, 30, or 60 min (LDH release, <10%). We also verified that none of the conditions altering extracellular and intracellular $Ca^{2+}$ concentrations or $Ca^{2+}$ influx into PMNs induced PMN degranulation. The percentage of lysozyme and β-glucuronidase release by PMNs incubated under the various experimental conditions for 30 min was always comparable to the percentage of spontaneous enzyme release in $Ca^{2+}$-containing HBSS (for lysozyme, 13 to 14% versus 14% for $Ca^{2+}$ depletion or $Ca^{2+}$ influx inhibition versus that for controls, respectively; the respective values for β-glucuronidase were 6 to 9% versus 8%.

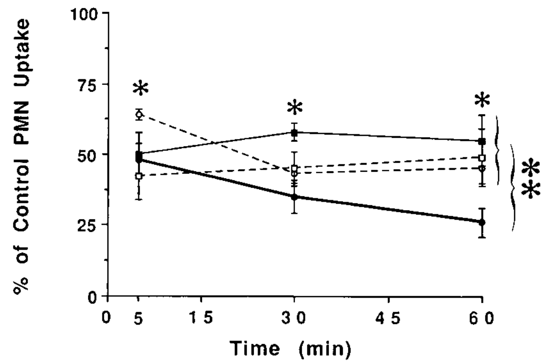


FIG. 1. Uptake kinetics of dirithromycin (●), roxithromycin (■), erythromycin (□) and erythromycylamine (○) by cytoplasts. Results are expressed as percentage of control uptake by PMNs from the same donors; values are the means ± standard errors of the means from three to six experiments. *, $P < 0.05$ for macrolide uptake by cytoplasts compared with control uptake by human PMNs; **, $P < 0.05$ for dirithromycin versus each other macrolide.

**Statistical analysis.** Results are expressed as means ± standard errors of the means of $n$ experiments conducted with PMNs from different volunteers. Analysis of variance, regression analysis, and Student's $t$ test for paired data were used to determine statistical significance. All tests were performed on the Statworks program, version 1.2, Cricket software, 1985).

## RESULTS

**Cellular location of macrolides.** The cellular locations of macrolides were assessed first by cell fractionation studies. Under conditions of maximal cell disruption (sonication with three 15-s bursts in the presence of 0.5% Triton), the cytoplasmic and granular membrane pellets contained less than 10% the cytosolic enzyme marker LDH and the granule enzyme markers lysozyme and β-glucuronidase. None of the macrolides was significantly associated with the membrane structure (dirithromycin, 5% ± 1.0%; roxithromycin, 2% ± 0.4%; erythromycylamine, 13% ± 6.1%; erythromycin, 4% ± 0.7%). When the granules were protected from disruption by the presence of 0.73 M sucrose, as indicated by the recovery of 89% ± 0.5% and 90% ± 0.8% β-glucuronidase and lysozyme, respectively, and less than 10% LDH, they contained most of the cellular dirithromycin. Also, more dirithromycin than the other drugs was found in the granular pellet (73% ± 3.9% versus 45% ± 5.1%, 35% ± 3.0%, and 42 ± 3.7%, respectively, for erythromycylamine [$P = 0.023$], roxithromycin [$P < 0.001$], and erythromycin [$P = 0.002$]).

In keeping with these data, macrolide uptake by cytoplasts (i.e., neutrophils devoid of granules) was significantly less marked than that by intact cells (Fig. 1). This was particularly so with dirithromycin, whose accumulation, unlike that of the other drugs, decreased significantly with time (regression analysis, $P = 0.026$ and $r = 0.538$). At 60 min, the percentage of dirithromycin uptake by cytoplasts differed significantly from those of the other macrolides ($P < 0.05$). These data strongly support the hypothesis that trapping of macrolides within intracellular granules drives their uptake in phagocytes and that this cellular compartment is the preferential location of dirithromycin.

Laufen and colleagues (33, 34) have suggested that for macrolides with similar cross-sectional areas and similar numbers of sites for the formation of hydrogen bonds, differences in activation energy could be linked either to the use of an active

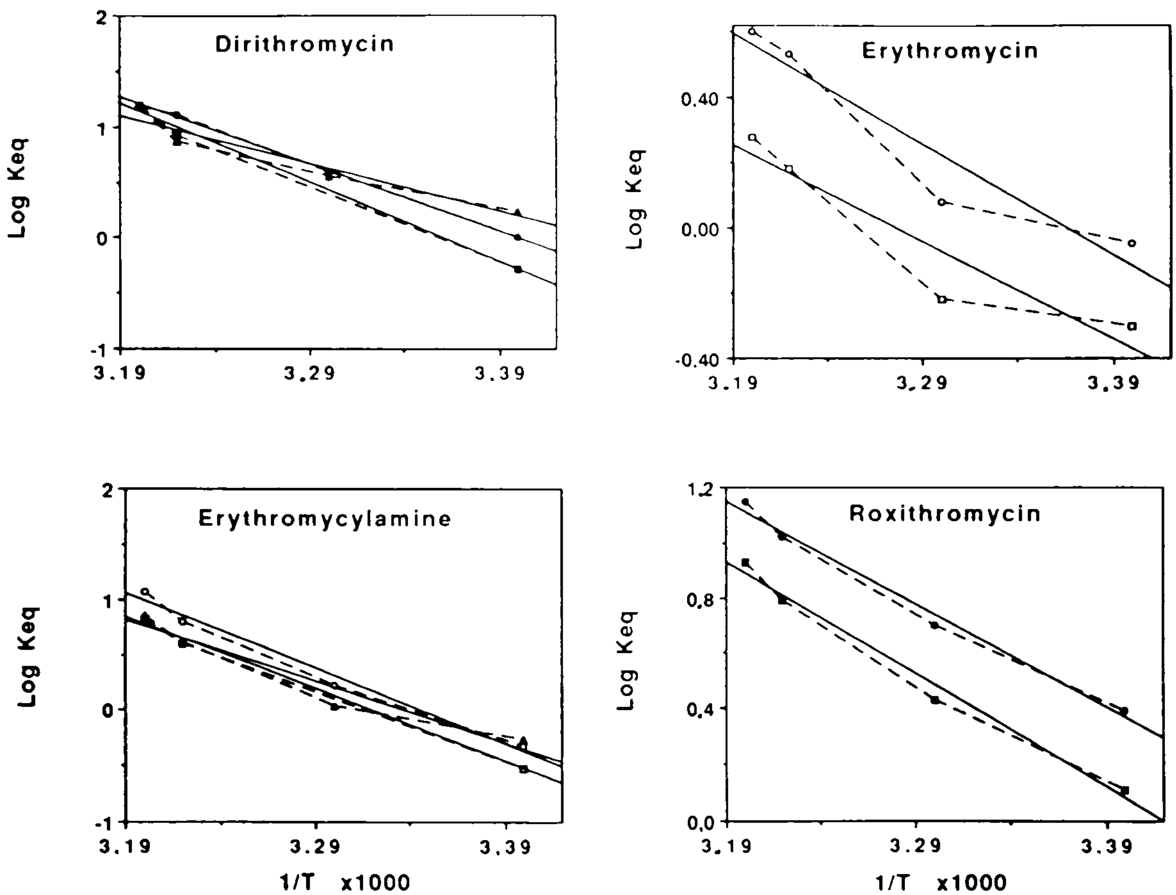


FIG. 2. Van't Hoff plots of temperature dependence of macrolide uptake kinetics by intact PMNs (dirithromycin and erythromycylamine were tested in three experiments each and roxithromycin and erythromycin were tested in two experiments each). For details of calculation of activation energy, see Materials and Methods.

transport system such as that of nucleosides or to the need to cross the lysosomal membrane in addition to the cell membrane. The activation energies of the four macrolides tested here (Fig. 2) were as follows: dirithromycin, 109 ± 15.6 kJ/mol; erythromycylamine, 119 ± 7.3 kJ/mol; roxithromycin, 75 ± 2.2 kJ/mol; and erythromycin 61 ± 4.1 kJ/mol. The high activation energy of dirithromycin is in keeping with that reported by Laufen et al. (34) for azithromycin and probably reflects its trapping within granules. However, erythromycylamine, which does not concentrate within granules substantially better than erythromycin and roxithromycin and which does not use a known active transport system (39), also had a high activation energy. It is noteworthy that compared with the drugs assessed here, erythromycylamine displays poor liposolubility (Kow's index, 0.1011 at pH 7 [34a]). The frictional resistance of this hydrophilic drug through the lipidic cell membrane could explain its requirement for a high activation energy to enter the cells.

**Effect of $Ca^{2+}$ on drug uptake.** The effect of $Ca^{2+}$ on macrolide uptake kinetics by PMNs is shown in Fig. 3. Dirithromycin uptake (Fig. 3A) was significantly impaired in $Ca^{2+}$-free medium. Inhibition was significant within the first 5 min ($P$ = 0.001) and was maximal at 30 min ($P$ = 0.02 versus that at 5 min), with no further modification up to 120 min. Intracellular chelation of $Ca^{2+}$ did not modify drug uptake except for slight inhibition at 120 min (76% ± 5.6% that of the control; $P$ = 0.003). In $Ca^{2+}$-free medium and in the presence of intracellular chelators of $Ca^{2+}$, there was no significant difference in macrolide uptake compared with the removal of extracellular $Ca^{2+}$ alone. Erythromycin (Fig. 3B) and roxithromycin (Fig. 3C) uptake showed sensitivities to $Ca^{2+}$ chelation similar to that of dirithromycin except for their sensitivities to intracellular $Ca^{2+}$ chelation at 120 min. Furthermore, the inhibition of roxithromycin uptake in $Ca^{2+}$-free medium was maximal within the first 5 min and then decreased, with no significant effect at 120 min.

Since EGTA and BAPTA-AM did not induce PMN degranulation (see Materials and Methods), it was unlikely that the effects of these agents on macrolide uptake were due to a decrease in the intracellular organelles where the macrolides accumulated.

**Effect of extracellular calcium on macrolide uptake by cytoplasts.** To differentiate between the effects of extracellular $Ca^{2+}$ on the entry of macrolides into cells and intragranular accumulation, we analyzed the kinetics of macrolide uptake by cytoplasts in HBSS or $Ca^{2+}$-free HBSS plus EGTA (Fig. 4). Only the results obtained with dirithromycin (Fig. 4A; location mainly intragranular) and roxithromycin (Fig. 4B; dual cytoplasmic and granular locations) are shown. Experiments performed with the other two macrolides gave similar results (data not shown). The amounts of cytoplast-associated macrolides were considerably lower in $Ca^{2+}$-free medium than in control buffer and were also lower than PMN-associated amounts of macrolides in $Ca^{2+}$-free buffer, suggesting an additive effect of granule and $Ca^{2+}$ deprivation on macrolide uptake and that extracellular calcium is important for drug entry into the cells.

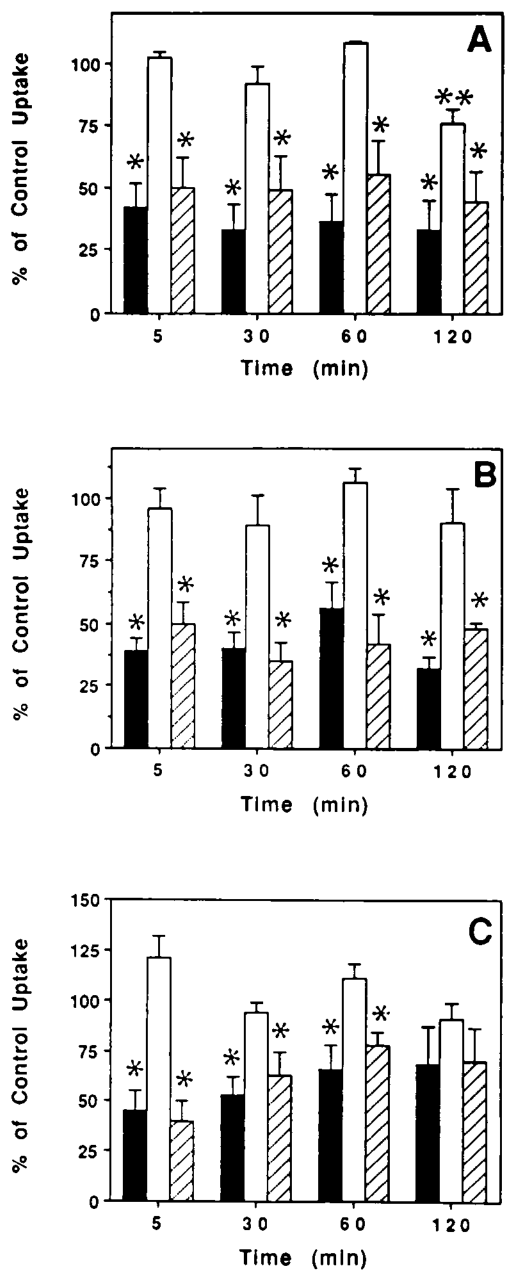


FIG. 3. Effects of $Ca^{2+}$ chelators on macrolide uptake. (A) Dirithromycin, five experiments; (B) erythromycin, five experiments; (C) roxithromycin, six experiments. Results are expressed as the percentage of control uptake by untreated PMNs (means ± standard errors of the means). Black bars, PMNs in $Ca^{2+}$-free medium plus 1 mM EGTA; open bars, PMNs treated with BAPTA-AM; hatched bars, combination of the two treatments. *, $P < 0.001$; **, $P < 0.01$.

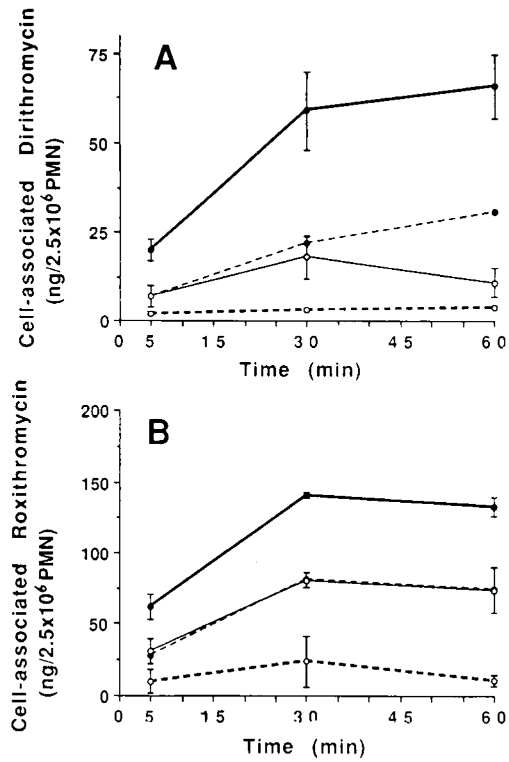


FIG. 4. Effects of extracellular calcium on macrolide uptake by PMNs and cytoplasts. (A) Dirithromycin; (B) roxithromycin. The extracellular concentrations of macrolides were 2.5 mg/liter. Results are expressed as the amount of cell-associated macrolides (ng/2.5 × $10^6$ cells) and are means ± standard errors of the means. Control PMNs (——●——), control cytoplasts (——○——) ($P < 0.01$ versus control PMN uptake), and cytoplasts in $Ca^{2+}$-free medium (– – –○– – –) ($P < 0.01$ versus control PMN uptake) were tested in three experiments, and PMNs in $Ca^{2+}$-free medium (– – –●– – –) were tested in two experiments.

**Effects of $Ca^{2+}$ channel blockers on macrolide uptake.** We first analyzed the effect of $Ni^{2+}$, an inorganic $Ca^{2+}$ channel blocker acting at the level of $Na^+$-$Ca^{2+}$ exchanges in resting neutrophils (46), on macrolide uptake by PMNs. $Ni^{2+}$ at 1.25 mM inhibited the entry of macrolides by about 40 to 50% (5 min of incubation; $P < 0.001$); the inhibition of accumulation did not vary during the 60-min incubation period (analysis of variance, $P = 0.73$, 0.290, and 0.288 for dirithromycin, erythromycin, and erythromycylamine, respectively), but it did vary for roxithromycin, whose accumulation was inhibited significantly more strongly by $Ni^{2+}$ at 60 min (analysis of variance and then Student's $t$ test, $P = 0.011$). The effect of $Ni^{2+}$ was concentration dependent. We used regression analysis to determine the concentrations inhibiting 50% of macrolide uptake at 5 min ($IC_{50}$) of $Ni^{2+}$, which were 2 mM dirithromycin (three to seven experiments; $r = 0.964$; $P < 0.001$) and 1.6 mM roxithromycin (two to five experiments; $r = 0.917$; $P < 0.001$). Approximation of the $IC_{50}$s of erythromycin and erythromycylamine from a single experiment gave values of 1.44 and 1.5 mM, respectively. Inhibition of accumulation by $La^{3+}$, which in general appears to be the most potent inhibitor of $Na^+$-$Ca^{2+}$ exchanges in human PMNs ($K_i$, 10 μM), and $Mg^{2+}$, which is poorly active in this system ($K_i$, 1.25 mM), was also assessed. $Mg^{2+}$ (up to 5 mM) did not inhibit macrolide uptake over a 60-min incubation period. The mean percent uptakes compared with that by the control (two experiments) were 98 and 105% for dirithromycin, 107 and 100% for erythromycin, 83 and 100% for roxithromycin, and 99 and 100% for erythromycylamine after 5- and 60-min incubations, respectively. $La^{3+}$ strongly inhibited macrolide uptake, with $IC_{50}$s of about 29 μM (roxithromycin, $r = 0.999$ and $P = 0.027$), 32 μM (erythromycin, $r = 0.956$ and $p = 0.025$) and 35 μM (dirithromycin, $r = 0.999$ and $P < 0.01$; erythromycylamine, $r = 0.999$; $P = 0.05$) (two experiments).

**Effect of $Ca^{2+}$ channel blockers and EGTA on intracellular locations of macrolides.** PMNs pretreated for 10 min with $Ni^{2+}$ (5 mM), $La^{3+}$ (50 μM), $Mg^{2+}$ (5 mM), or HBSS were further incubated with macrolides in control HBSS; in addition, $Ca^{2+}$-

TABLE 1. Effect of extracellular $Ca^{2+}$ deprivation and $Ca^{2+}$ channel blockers on intracellular location of macrolides

| Compound | % Granular pellet-associated macrolides and enzymes under the following experimental conditions[a]: | | | | |
|---|---|---|---|---|---|
| | HBSS | $Ca^{2+}$-free HBSS + EGTA (1 mM) | $Ni^{2+}$ (5 mM) | $La^{3+}$ (50 μM) | $Mg^{2+}$ (5 mM) |
| Macrolides | | | | | |
| Dirithromycin | 84 ± 3.1 (3) | 85 | 87 ± 5.7 (3) | 90 | 80 |
| Roxithromycin | 39 ± 1.0 (2) | 43 ± 2.9 (3) | 32 | 38 | 39 |
| Erythromycylamine | 58 | 57 | 56 | 61 | 57 |
| Enzymes | | | | | |
| LDH | 9 ± 0.6 (3) | 7 ± 0.9 (3) | 6 ± 0.9 (3) | 9 ± 1.8 (3) | 8 ± 0.6 (3) |
| Lysozyme | 87 ± 1.8 (3) | 87 ± 0.6 (3) | 86 ± 0.7 (3) | 85 ± 0.6 (3) | 83 ± 0.6 (3) |

[a] Results are expressed as means ± standard errors of the means and are the percentage of macrolides (enzymes) associated with the pellet obtained from drug-loaded neutrophils for 30 min sonicated (three times for 5 s each time) in the presence of 0.73 M sucrose (see Materials and Methods for details). Values in parentheses are numbers of experiments.

free HBSS-treated PMNs were incubated with macrolides in $Ca^{2+}$-free HBSS plus EGTA. After 30 min, the cells were centrifuged over the oil cushion and the pellet was sonicated in the presence of 0.5% Triton or 0.73 M sucrose. The amounts of marker enzymes and macrolides in the membrane or granular pellet were then determined. In the case of protection of granules by sucrose, although $Ca^{2+}$ deprivation, as well as the presence of $Ni^{2+}$ and $La^{3+}$, but not that of $Mg^{2+}$, drastically reduced the amounts of cell-associated macrolides, the overall distribution (cytoplasmic and granular) was unchanged compared with that for control cells (Table 1). We also checked that modification of the incubation medium (EGTA, $Ni^{2+}$, $La^{2+}$, $Mg^{2+}$) did not alter the amount of membrane-associated macrolides: the proportion of pellet-associated macrolides after neutrophil disruption (three times for 15 s each time) in the presence of 0.5% Triton was less than 5%, whatever the experimental conditions. Marker enzyme activity (LDH and lysozyme) was always less than 10%.

**Effects of organic $Ca^{2+}$ channel blockers.** Various investigators have observed that only relatively high concentrations of verapamil or nifedipine, two $Ca^{2+}$ antagonists, alter neutrophil function, whereas these drugs are inhibitory at $10^5$-fold lower concentrations in electroporated neutrophils (13), suggesting that $Ca^{2+}$ channels in the PMN cytoplasmic membrane are not sensitive to these inhibitors. In keeping with these data, we observed that nifedipine (100 to 500 μM) did not inhibit macrolide uptake over a 60-min incubation period (data not shown). However, verapamil inhibited macrolide uptake in a concentration- and time-dependent manner (Fig. 5). The maximal inhibitory effect was obtained with roxithromycin; this was followed by erythromycin; dirithromycin uptake was altered only by a high concentration (500 μM) of verapamil.

## DISCUSSION

Phagocytic uptake of macrolides is an area of active investigation. Most studies have defined the intracellular concentrations/extracellular concentration ratios and the cellular uptakes kinetics of older and newer macrolides, including those of the azalide azithromycin. Although the marked accumulation of these drugs is widely acknowledged, no clear interpretation of the entry mechanism has been given. There is a virtual scientific consensus that macrolide accumulation reflects lysosomal trapping by protonation of these weak bases. Although this hypothesis has been verified in various phagocytic and nonphagocytic cells of human and animal origin, there are few comparative data concerning human PMNs (9, 38, 51, 52). Understanding of the entry mechanisms of macrolides and their cellular locations is important for two reasons: first, a definition of a comprehensive approach to the study of their intracellular bioactivities and, second, for the analysis of the consequences for cellular function. It is widely acknowledged that macrolides modulate host cell, particularly phagocyte, functions (28). Most 14-membered-ring macrolides are able to decrease oxidant production by phagocytes in vitro at therapeutically achievable concentrations (23, 32), and this could be one of the mechanisms supporting their anti-inflammatory activities observed in vivo and in some animal models (2, 10, 37). In parallel, these molecules possess a prodegranulating activity (1, 30). This phenomenon has also been demonstrated with various drugs which display in vitro antioxidant properties (such as staurosporine and mefloquine) (4, 12) and, in some cases, anti-inflammatory activity (chloroquine) (15). A common mechanism for both antioxidant and prodegranulating activity has not yet been proven, but it could reflect a particular cellular location and/or entry mechanism. To check this hypothesis, we first investigated the comparative intracellular locations of four macrolides which display similar prodegranulating (30) and antioxidant properties (32) in the cellular target, the human PMN. Dirithromycin was the only drug to display a strong intragranular location, which may be linked to its dibasic nature. Interestingly, its metabolite erythromycylamine, which is also dibasic in nature, was significantly less strongly accumulated within PMN granules. The results of cell

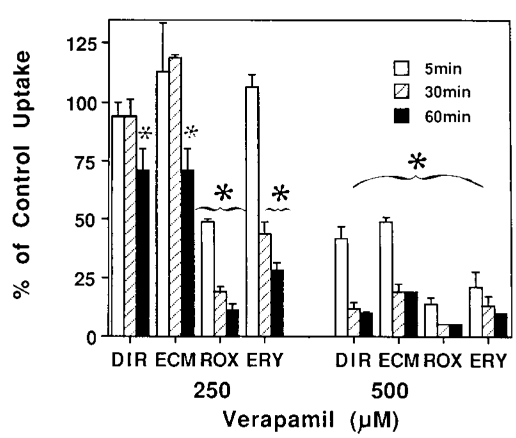


FIG. 5. Effect of verapamil on macrolide uptake at 5, 30, and 60 min. Results are expressed as the percentage of control uptake by untreated PMNs and are from three to five experiments. *, $P < 0.001$; DIR, dirithromycin; ECM, erythromycylamine; ROX, roxithromycin; ERY, erythromycin.

fractionation procedures were confirmed by uptake kinetics with cytoplasts (Fig. 1) and were also correlated with the calculated activation energy, except in the case of erythromycylamine. It is widely admitted that dirithromycin quickly hydrolyzes in solution to generate erythromycylamine. However, when erythromycylamine was compared with its parent drug, it displayed significantly poorer cellular pharmacokinetics (39). There is no clear explanation for this observation. The poor liposolubility of erythromycylamine could account for its difficulty in crossing the cytoplasmic membrane, as reflected by its high activation energy, whereas dirithromycin, which is more hydrophobic by nature, should be rapidly trapped by cytoplasmic membranes and driven into organelles.

Since intragranular location by itself is not sufficient to explain the similar prodegranulating effects of the macrolides assessed here (30), we further investigated the role of extracellular calcium in the entry processes of these drugs. The present investigation was based on the observation that degranulation kinetics triggered by macrolides are similar to those induced by the calcium ionophore A23187 but not formylmethionylleucylphenylalanine (FMLP) (1). Furthermore, FMLP- but not A23187-stimulated PMN degranulation was additive with that stimulated by macrolides (30), and lastly, extracellular $Ca^{2+}$ is required for macrolide-induced degranulation (31). Indeed, a major finding in the present study was that extracellular (not intracellular) calcium plays a determining role in macrolide uptake. $Ca^{2+}$-free HBSS supplemented with EGTA significantly impaired macrolide uptake by PMNs and cytoplasts (Fig. 3 and 4), suggesting a role for this cation in macrolide entry into cells. In keeping with this hypothesis, we observed no effect of $Ca^{2+}$ chelators or $Ca^{2+}$ channel blockers on the intracellular distribution of the drug (Table 1). The mechanism by which extracellular $Ca^{2+}$ favors macrolide uptake was not elucidated. In resting PMNs, $Ca^{2+}$ entry is mediated by $Na^+$-$Ca^{2+}$ exchanges (46) which are inhibited by inorganic cations in the order $La^{3+} > Cd^{2+} >> Mn^{2+} > Ni^{2+} >> Mg^{2+}$. The data reported here support a role for this exchanger in macrolide uptake since various cations ($Ni^{2+}$, $La^{3+}$) but not $Mg^{2+}$ inhibited this uptake in a concentration-dependent manner, with $IC_{50}$s slightly greater than the $K_i$ of the cation for this exchanger (29 to 35 versus 10 μM, respectively, for $La^{3+}$ and 1.6 to 2 mM versus 0.67 mM, respectively, for $Ni^{2+}$). Verapamil and nifedipine, two $Ca^{2+}$ antagonists which block voltage-operated $Ca^{2+}$ channels of the L type (19, 44), do not act on the entry of $Ca^{2+}$ into resting neutrophils mediated by the $Na^+$-$Ca^{2+}$ exchanger (46). Accordingly, nifedipine did not inhibit macrolide uptake by PMNs, but surprisingly verapamil did so strongly in a time- and concentration-dependent manner (Fig. 5). It must be pointed out that organic $Ca^{2+}$ channel blockers have been reported to inhibit various PMN functions elicited by FMLP, bacteria, or latex in vitro and ex vivo (3, 13, 25, 26, 53). According to Jaconi et al. (22), the mechanism of calcium influx in stimulated PMNs does not appear to involve voltage-operated, receptor-operated, or second messenger-operated $Ca^{2+}$ channels but correlates with the filling state of intracellular $Ca^{2+}$ stores and is referred to as capacitative $Ca^{2+}$ influx (42). This store-operated channel is stimulated by depletion of intracellular $Ca^{2+}$ pools by inositol 1,4,5-triphosphate (IP3) or the $Ca^{2+}$-ATPase inhibitor thapsigargin (48), but the precise nature of this pathway is unknown. However, Simchowitz and Cragoe (46) suggest that, directly or indirectly, receptor agonists activate the $Na^+$-$Ca^{2+}$ exchanger.

Although Azuma et al. (3) and Wright et al. (53) have observed an inhibition of $Ca^{2+}$ influx in stimulated neutrophils and macrophages by verapamil and nifedipine, respectively, $Ca^{2+}$ intake by the receptor-operated $Ca^{2+}$ channel is generally insensitive to classical $Ca^{2+}$ antagonists (49). Furthermore, functional inhibition by nifedipine and verapamil was obtained at significantly higher concentrations than those required to inhibit $Ca^{2+}$ uptake (3). In addition, Elferink et al. (13) observed that in electroporated neutrophils verapamil and nifedipine were inhibitory in the nanomolar range, whereas $10^5$-fold higher concentrations were required in intact cells. Della Bianca et al. (11) have shown that besides the blocking of $Ca^{2+}$ channels, many mechanisms (including protein kinase C inhibition and an increase in cyclic AMP levels) are responsible for inhibition of the PMN response by verapamil. All of these results suggest that the inhibitory effect of organic $Ca^{2+}$ antagonists on cell function is not mediated by an impairment of $Ca^{2+}$ influx but by an inhibition of a $Ca^{2+}$-dependent intracellular target. It is likely that verapamil-mediated inhibition of macrolide uptake is not directly linked to the inhibition of cytoplasmic membrane $Ca^{2+}$ channels but reflects an interaction with another cellular target responsible for the activation of the putative macrolide transporter. Whatever the nature of the active transporter of macrolides, our data strongly suggest that its activity is linked to the correct functioning of the $Na^+$-$Ca^{2+}$ exchanger in PMNs. Calcium deprivation or blockade of the $Na^+$-$Ca^{2+}$ exchanger could also modify cell functions related to macrolide uptake. However, PMN viability was not impaired by these conditions, and there was no triggering of exocytosis. Whereas $Ca^{2+}$ transients appear to be essential for neutrophil responses to receptor agonists (leukotriene B4, FMLP, platelet-activating factor), intracellular sources of $Ca^{2+}$ generally appear to be sufficient (41). Furthermore, some agents activate PMNs without altering the intracellular $Ca^{2+}$ concentration. Only $Ca^{2+}$ ionophore-mediated PMN activation is highly linked to the presence of extracellular calcium. That macrolides act as $Ca^{2+}$ ionophores or $Ca^{2+}$ antagonists was not investigated in the present study, but this possibility is under study in our laboratory. Since $Ca^{2+}$, whether from intracellular or extracellular sources, appears to be of major importance in mediating various PMN functions, our data also provide some basis for exploration of the interference of macrolides with exocytosis and oxidant production by PMNs.

**Conclusion.** In conclusion, our data shed light on the mechanism of macrolide uptake by PMNs. From the results reported here it is clear that this uptake is linked to the correct functioning of the $Na^+$-$Ca^{2+}$ exchanger. We are studying the effects of macrolides on this plasmalemmal transporter and the direct modulation of $Na^+$-$Ca^{2+}$ homeostasis by this class of antibiotics. Since the $Na^+$-$Ca^{2+}$ exchanger is clearly operational in many cells (24), and particularly in cardiac myocytes, the relevance of our in vitro findings to various clinical observations of macrolide-induced cardiotoxic effects (7) should be investigated. It remains to be determined if the effects observed here with four 14-membered-ring macrolides can be extended to other members of this antibiotic family, particularly 16-membered-ring macrolides and azalides. Further work is required to determine the mechanism by which the $Na^+$-$Ca^{2+}$ exchanger permits macrolide entry. The characterization of an intracellular target responsible for macrolide uptake is under investigation.

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## REFERENCES

1. **Abdelghaffar, H., E. M. Mtairag, and M. T. Labro.** 1994. Effects of dirithromycin and erythromycylamine on human neutrophil degranulation. Antimicrob. Agents Chemother. **38:**1548–1554.

2. **Agen, C., R. Danesi, C. Blandizzi, M. Costa, B. Stacchini, P. Favini, and M. Del Tacca.** 1993. Macrolide antibiotics as anti inflammatory agents: roxithromycin in an unexpected role. Agents Action **38:**85–90.
3. **Azuma, Y., T. Tokunaga, Y. Takeda, T. Ogawa, and N. Takagi.** 1986. The effect of calcium antagonists on the activation of guinea pig neutrophils. Jpn. J. Pharmacol. **42:**243–251.
4. **Bates, E. J., and A. Ferrante.** 1988. Stimulation of human neutrophil degranulation by mefloquine. Int. Arch. Allergy Appl. Immunol. **86:**446–452.
5. **Bergmeyer, H. U.** 1963. Methods in enzymatic analysis, p. 737–739. Academic Press, Inc., New York.
6. **Blais, J., D. Croteau, and S. Chamberland.** 1992. Uptake and subcellular distribution of dirithromycin (LY 237216) in cultured mouse macrophages, abstr. 1704, p. 395. *In* Program and abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
7. **Bryskier, A., and C. Agouridas.** 1994. Macrolide antibiotics and the heart. Exp. Opin. Invest. Drugs **3:**1213–1219.
8. **Carevic, O., and S. Djokic.** 1988. Comparative studies on the effects of erythromycin A and azithromycin upon extracellular release of lysosomal enzymes in inflammatory processes. Agents Action **25:**124–131.
9. **Carlier, M. B., A. Zenebergh, and P. M. Tulkens.** 1987. Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. J. Antimicrob. Chemother. **20**(Suppl. B)**:**47–56.
10. **Dalziel, K., P. S. Dykes, and R. Marks.** 1987. The effect of tetracycline and erythromycin in a model of acne-type inflammation. Br. J. Exp. Pathol. **68:**67–70.
11. **Della Bianca, V., M. Greskowiak, P. De Togni, M. Cassatella, and F. Rossi.** 1985 Inhibition by verapamil of neutrophil responses to formylmethionylleucylphenylalanine and phorbol myristate acetate. Mechanisms involving $Ca^{2+}$ changes, cyclic AMP and protein kinase C. Biochim. Biophys. Acta **845:**223–236.
12. **Dewald, B., M. Thelen, M. P. Wymann, and M. Baggiolini.** 1989. Staurosporine inhibits the respiratory burst and induces exocytosis in human neutrophils. Biochem. J. **264:**879–884.
13. **Elferink, J. G. R., G. J. J. C. Boonen, and B. M. de Koster.** 1992. The role of calcium in neutrophil migration; the effect of calcium and calcium antagonists in electroporated neutrophils. Biochem. Biophys. Res. Commun. **182:** 864–869.
14. **Fernandes, R. B.** 1987. The macrolide revival: thirty five years after erythromycin. Antimicrob. Newsl. **4:**25–34.
15. **Fontagne, J., M. Roch-Arveiller, J. P. Giroud, and P. Lechat.** 1989. Effects of some antimalarial drugs on rat inflammatory polymorphonuclear leukocyte function. Biomed. Pharmacother. **43:**43–51.
16. **Hand, W. L., and D. L. Hand.** 1993. Interactions of dirithromycin with human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **37:** 2557–2562.
17. **Hand, W. L., and N. L. King-Thompson.** 1986. Contrast between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity. Antimicrob. Agents Chemother. **29:**135–140.
18. **Hand, W. L., N. King-Thompson, and J. W. Holman.** 1987. Entry of roxithromycin (RU 965), imipenem, cefotaxime, trimethoprim, and metronidazole into human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **31:**1553–1557.
19. **Henry, P. D.** 1980. Comparative pharmacology of calcium antagonists: nifedipine, verapamil, and diltiazem. Am. J. Cardiol. **46:**1047–1058.
20. **Holmes, B., P. G. Quie, D. B. Windhorst, B. Pollara, and R. A. Good.** 1966. Protection of phagocytized bacteria from the killing action of antibiotics. Nature (London) **210:**1131–1132.
21. **Ishiguro, M., H. Koga, S. Kohno, T. Hayashi, K. Yamaguchi, and M. Hirota.** 1989. Penetration of macrolides into human polymorphonuclear leucocytes. J. Antimicrob. Chemother. **24:**719–725.
22. **Jaconi, M. E. E., D. P. Lew, A. Monod, and K.-H. Krause.** 1993. The regulation of store-dependent $Ca^{2+}$ influx in HL-60 granulocytes involves GTP-sensitive elements. J. Biol. Chem. **268:**26075–26078.
23. **Jooné G., K., C. E. J. Van Rensburg, and R. Anderson.** 1992. Investigation of the in-vitro uptake, intraphagocytic biological activity and effects on neutrophil superoxide generation of dirithromycin compared with erythromycin. J. Antimicrob. Chemother. **30:**509–523.
24. **Kaczorowski, G. J., R. S. Slaughter, V. F. King, and M. L. Garcia.** 1989. Inhibitors of sodium-calcium exchange: identification and development of probes of transport activity. Biochim. Biophys. Acta **988:**287–302.
25. **Kalra, A., M. L. Dubey, N. K. Ganguly, and R. C. Mahajan.** 1993. Effect of nifedipine on calcium status and chemiluminescence response of phagocytes during *Plasmodium berghei* infection in mice. J. Pharm. Pharmacol. **45:**540–544.
26. **Kazanjian, P. H., and J. E. Pennington.** 1985. Influence of drugs that block calcium channels on the microbicidal function of human neutrophils. J. Infect. Dis. **151:**15–22.
27. **Kirst, H. A., and G. D. Sides.** 1989. New directions for macrolide antibiotics: pharmacokinetics and clinical efficacy. Antimicrob. Agents Chemother. **33:** 1419–1422.
28. **Labro, M. T.** 1993. Effect of macrolides on host natural defences, p. 389–408. *In* A. Bryskier, J. P. Butzler, H. C. Neu, and P. M. Tulkens (ed.), Macrolides: chemistry, pharmacology and clinical use. Arnette-Blackwell, Paris.
29. **Labro, M. T.** 1993. Intraphagocytic penetration of macrolide antibiotics, p. 379–388. *In* A. Bryskier, J. P. Butzler, H. C. Neu, and P. M. Tulkens (ed.), Macrolides: chemistry, pharmacology and clinical use. Arnette-Blackwell, Paris.
30. **Labro, M. T., H. Abdelghaffar, and A. Bryskier.** 1993. Effect of macrolides on human neutrophil degranulation, abstr. 309, p. 176. *In* Program and abstracts of the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
31. **Labro, M. T., H. Abdelghaffar, and E. M. Mtairag.** 1994. Extracellular Ca++ is required for macrolide uptake by human neutrophils (PMN) and subsequent PMN exocytosis, abstr. E110, p. 162. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
32. **Labro, M. T., J. El Benna, and H. Abdelghaffar.** 1993. Modulation of human polymorphonuclear neutrophil function by macrolides: preliminary data concerning dirithromycin. J. Antimicrob. Chemother. **31**(Suppl. C)**:**51–64.
33. **Laufen, H., and A. Wildfeuer.** 1989. Kinetics of the uptake of antimicrobial agents by human polymorphonuclear leucocytes. Arzneim. Forsch. **39**(I)**:** 233–235.
34. **Laufen, H., A. Wildfeuer, and P. Lach.** 1990. Mechanism of azithromycin uptake in human polymorphonuclear leucocytes. Arzneim. Forsch. **40**(I)**:** 686–689.
34a. **Eli Lilly & Co.** Data on file. Eli Lilly & Co., Indianapolis, Ind.
35. **Litwack, G.** 1955. Photometric determination of lysozyme activity. Proc. Soc. Exp. Biol. Med. **89:**401–403.
36. **MacIntyre, A., and D. J. Cutler.** 1988. The potential role of lysosomes in tissue distribution of weak bases. Biopharm. Drug Dispos. **9:**513–526.
37. **Mikasa, K., E. Kita, M. Sawaki, K. Kunimatsu, K. Hamada, M. Konishi, S. Kashiba, and N. Narita.** 1992. The anti-inflammatory effect of erythromycin in zymosan induced peritonitis of mice. J. Antimicrob. Chemother. **30:**339–348.
38. **Miller, M. F., J. R. Martin, P. Johnson, J. T. Ulrich, E. J. Rdzok, and P. Billing.** 1984. Erythromycin uptake and accumulation by human polymorphonuclear leukocytes and efficacy in killing ingested *Legionella pneumophila*. J. Infect. Dis. **149:**714–718.
39. **Mtairag, E. M., H. Abdelghaffar, and M. T. Labro.** 1994. Investigation of dirithromycin and erythromycylamine uptake by human neutrophils *in vitro*. J. Antimicrob. Chemother. **33:**523–536.
40. **Niessen, H. W. M., T. W. Kuijpers, D. Roos, and A. J. Verhoeven.** 1991. Release of azurophilic granule contents in FMLP-stimulated neutrophils requires two activation signals, one of which is a rise in cytosolic free $Ca^{2+}$. Cell. Signalling **3:**625–633.
41. **O'Flaherty, J. T., A. G. Rossi, D. P. Jacobson, and J. F. Redman.** 1991. Roles of $Ca^{++}$ in human neutrophil responses to receptor agonists. Biochem. J. **278:**705–711.
42. **Putney, J. W., Jr. and G. St. Bird.** 1993. The signal for capacitative calcium entry. Cell **75:**199–201.
43. **Roos, D., A. A. Voetman, and L. J. Meerhof.** 1983. Functional activity of enucleated human polymorphonuclear leukocytes. J. Cell Biol. **97:**368–377.
44. **Schramn, M., and R. Towart.** 1985. Modulation of calcium channel function by drugs. Life Sci. **37:**1843–1860.
45. **Schwab, J. C., Y. Cao, M. R. Slowik, and K. A. Joiner.** 1994. Localization of azithromycin in *Toxoplasma gondii*-infected cells. Antimicrob. Agents Chemother. **38:**1620–1627.
46. **Simchowitz, L., and E. J. Cragoe, Jr.** 1988. $Na^{+}$-$Ca^{2+}$ exchange in human neutrophils. Am. J. Physiol. **254** (Cell Physiol. **23**)**:**C150–C164.
47. **Talalay, P., W. H. Fishman, and C. Huggins.** 1946. Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. Biol. Chem. **166:**756–772.
48. **Thastrup, O., A. P. Dawson, O. Scharff, B. Foder, P. J. Cullen, B. K. Drobak, P. J. Bjerrum, S. B. Christensen, and M. R. Hanley.** 1989. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents Action **27:**17–23.
49. **Tsunoda, Y.** 1993. Receptor-operated $Ca^{2+}$ signaling and crosstalk in stimulus secretion coupling. Biochim. Biophys. Acta **1154:**105–156.
50. **Villa, P., D. Sassela, M. Corada, and I. Bartosek.** 1988. Toxicity, uptake and subcellular distribution in rat hepatocytes of roxithromycin, a new semisynthetic macrolides and erythromycin base. Antimicrob. Agents Chemother. **32:**1541–1546.
51. **Wildfeuer, A., H. Laufen, and K. Räder.** 1986. Activity of josamycin in human tissue and neutrophils, p. 7–9 *In* Yamanouchi pharmaceutical (ed.). Proceedings of the 14th International Congress of Chemotherapy. Josamycin book II. Yamanouchi Pharmaceutical Co. Ltd., London.
52. **Wildfeuer, A., I. Reisert, and H. Laufen.** 1993. Uptake and subcellular distribution of azithromycin in human phagocytic cells. Arzneim. Forsch **43**(I)**:**484–486.
53. **Wright, B., I. Zeidman, R. Greig, and G. Poste.** 1985. Inhibition of macrophage activation by calcium channel blocker and calmodulin antagonists. Cell. Immunol. **95:**46–53.