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Cellular Accumulation of the New Ketolide RU 64004 by Human Neutrophils: Comparison with That of Azithromycin and Roxithromycin

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We analyzed the uptake of RU 64004 by human neutrophils (polymorphonuclear leukocytes [PMNs]) relative to those of azithromycin and roxithromycin. RU 64004 was strongly and rapidly accumulated by PMNs, with a cellular concentration/extracellular concentration ratio (C/E) of greater than 200 in the first 5 min, and this was followed by a plateau at 120 to 180 min, with a C/E of 461 ± 14.8 (10 experiments) at 180 min. RU 64004 uptake was moderately sensitive to external pH, and activation energy was also moderate (63 ± 3.8 kJ/mol). RU 64004 was mainly located in PMN granules (about 70%) and egressed slowly from loaded cells, owing to avid reuptake. The possibility that PMN uptake of RU 64004 and other macrolides occurs through a carrier-mediated system was suggested by three key results. First, there existed a strong interindividual variability in uptake kinetics, suggesting variability in the numbers or activity of a transport protein. Second, macrolide uptake displayed saturation kinetics characteristic of that of a carrier-mediated transport system: RU 64004 had the highest V_{max} value (3,846 ng/2.5 × 10^6 PMNs/5 min) and the lowest K_m value (about 28 μM), indicating a high affinity for the transporter. Third, as observed previously with other erythromycin A derivatives, Ni^{2+} (a blocker of the Na^+/Ca^{2+} exchanger which mediates Ca^{2+} influx in resting neutrophils) impaired RU 64004 uptake by PMNs, with a 50% inhibitory concentration of about 3.5 mM. In addition, we found that an active process is also involved in macrolide efflux, because verapamil significantly potentiated the release of all three macrolides tested. This effect of verapamil does not seem to be related to an inhibition of Ca^{2+} influx, because neither EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] nor Ni^{2+} modified macrolide efflux. The nature and characteristics of the entry- and efflux-mediating carrier systems are under investigation.

RU 64004 is a new member of the ketolide family (2), a subgroup of erythromycin A derivatives characterized by a 3-keto function in place of the cladinose moiety. RU 64004 was synthesized from the 3-keto-6-methoxy erythromycin skeleton and has a quinoline side chain linked to the 11-12 position by a cyclic hydrazono-carbamate function. RU 64004 possesses a broad spectrum of activity against many clinically important gram-positive, gram-negative, and atypical respiratory pathogens and is active against intracellular pathogens (e.g., *Chlamydia* spp., *Legionella*, and atypical mycobacteria) (7, 15, 18, 35). In addition, RU 64004 has been reported to accumulate strongly in the monocytic cell line THP-1 (1) and to display intracellular bioactivity. We have previously proposed a preliminary classification of erythromycin A derivatives according to the characteristics of their cellular pharmacokinetics (kinetics of accumulation and release, activation energy, susceptibility to extracellular pH variation, and intracellular location) in human neutrophils (21); group I includes all dibasic compounds (azithromycin, dirithromycin, and erythromycylamine) and is characterized by gradual accumulation without saturation for up to 3 h, slow release from loaded cells, high activation energy, and preferential intragranular location; group II includes all other erythromycin A derivatives (monobasic compounds) and is characterized by saturable accumulation kinetics, rapid efflux, and dual (granular and cytoplasmic) location. Whatever the groups, competitive and metabolic inhibitors had little effect on drug uptake. By contrast, a common characteristic is the influence of extracellular Ca^{2+}, which is necessary for the intracellular accumulation (but not the granular location) of erythromycin A derivatives (28). RU 64004 is more lipophilic than erythromycin A and possesses two substituents with pK_as above and below the physiological pH (pK_1 for the quinoline ring, 5.3; pK_2 for D-desosamine, 8.6). It was therefore of interest to compare in our model this new drug to two macrolides representative of group I (azithromycin) and group II (roxithromycin).

(These results have been presented in part at the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy New Orleans, La., 15 to 18 September 1996 [22].)

MATERIALS AND METHODS

Macrolides. RU 64004 and roxithromycin (Hoechst-Marion-Roussel, Romainville, France) and azithromycin (Laboratoire Pfizer, Paris, France) were provided by the respective manufacturers. The radiolabelled drugs [^{3}H]RU 64004 (25.2 Ci/mmol), [^{3}H]roxithromycin (21.9 Ci/mmol), and [^{3}H]azithromycin (23.48 Ci/mmol), in ethanol-water (7/3; vol/vol), were provided by Hoechst-Marion-Roussel. Tritiated drugs (2.5 μl; about 30 μg/ml) were mixed with 25 μl of unlabelled drugs (1 mg/ml of Hanks buffered salt solution [HBSS]; Diagnostic Pasteur, Paris, France) and 222.5 μl of HBSS. Stock solutions were further diluted in HBSS to the desired concentrations.

Human neutrophils (PMNs). Polymorphonuclear leukocytes (PMNs) were obtained from the venous blood of healthy volunteers by Ficoll-Paque centrifugation followed by 2% dextran sedimentation and osmotic lysis of residual erythrocytes. The viability and purity of the PMN preparation, as assessed by Trypan blue exclusion, were greater than 96%.

Macrolide uptake. A radiometric assay was used to measure macrolide uptake (27, 28). Briefly, 2.5 × 10^6 PMNs were incubated at 37°C with the radiolabelled drugs and were then centrifuged at 12,000 × *g* for 3 min at 22°C through a water-impermeable silicone-paraffin (86 and 14% [vol/vol], respectively) oil barrier. The pellet was solubilized in Hionic fluor (Packard), and the cell-associated radioactivity was quantified by liquid scintillation counting (LS-6000-S; Beck-

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man). Standard dilution curves were used to determine the amounts of cell-associated drug.

The results were expressed as nanograms per 2.5×10^6 PMNs. The concentration of macrolides used in the assays was 2.5 mg/liter except where indicated otherwise. A previously determined intracellular volume of 0.6 μl/2.5×10^6 PMNs (27) was used to determine the cellular concentration/extracellular concentration ratio (C/E). We verified that the various experimental conditions used here (temperature, pH, inhibitors) did not significantly modify this value.

Characteristics of macrolide uptake. We first analyzed the kinetics of uptake over a 3-h incubation period. The influences of extracellular pH and temperature were assessed after incubation for 5 and 30 min. The effects of metabolic inhibitors (10 min of pretreatment with sodium cyanide [NaCN; 1 mM], potassium fluoride [KF; 1 mM]), sodium azide [NaN_3; 1 mM], and 2,4-dinitrophenol [2,4-DNP; 1 mM] were assessed over a 60-min incubation period. All chemical solutions were buffered to pH 7.4 to avoid any influence of pH on macrolide uptake (27). The influences of extracellular concentrations (1 to 100 mg/liter) were assessed in the first 5 min of incubation, a time when the rate of uptake is optimal.

Cellular location. Macrolide-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 X-100 (three 15-s bursts) or 0.73 M sucrose (three 5-s bursts) to protect the granules (28). After centrifugation (100,000 × *g* for 30 min), the amounts of marker enzymes, lactate dehydrogenase (LDH) (5), β-glucuronidase (34), and lysozyme (26), in the pellet and the supernatant together with the amounts of radiolabelled drugs were determined.

Macrolide efflux. Aliquots of macrolide-loaded PMNs were centrifuged through the silicone-paraffin oil barrier; one aliquot was used to quantify the amount of cell-associated macrolides (total associated drug). The other cell pellets were placed in drug-free HBSS, and at various time intervals, they were again centrifuged through the oil barrier; the radioactivity in the cell pellet and the supernatant was then measured. We verified that the sum of the radioactivity (that in the pellet plus that in the supernatant) did not significantly differ from the total load. Efflux of macrolides was expressed as the percentage of drug remaining associated with the cell pellet compared to the sum of the radioactivity (radioactivity in the pellet plus radioactivity in the supernatant).

Influence of Ca^{2+} on macrolide uptake, efflux, and intracellular location. The uptake kinetics of the macrolides were assessed first in Ca^{2+}-depleted HBSS (Gibco) supplemented with 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (Merck), 1 mM magnesium chloride (Merck), and 1.2 mM sodium bicarbonate (Diagnostic Pasteur). Uptake kinetics were also assessed in the presence of nickel chloride (Ni^{2+}; 5 to 0.5 mM; Sigma) or verapamil hydrochloride (250 to 5 μM; Sigma). All reagents were buffered at pH 7.4 before use. Efflux of macrolides was measured in Ca^{2+}-free HBSS or in the presence of verapamil (250 to 125 μM) or in the presence of Ni^{2+} (5 mM).

PMN viability. PMN viability was assessed by measuring the amount of LDH released. None of the experimental conditions altered PMN viability (LDH release, <10%).

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

RESULTS

Comparative accumulation of RU 64004, azithromycin, and roxithromycin. RU 64004 was rapidly and strongly accumulated by PMNs, with C/Es as high as 253 ± 15.0 (mean of 27 experiments) in the first 5 min (Fig. 1). This rapid uptake was followed by a slower accumulation, with C/Es of about 461 ± 14.8 at 180 min (mean of 10 experiments). In agreement with published data (6, 16), roxithromycin uptake was rapid (C/E, 29 ± 3.5 at 5 min; nine experiments) and plateaued after 60 min (C/E, 136 ± 8.8 at 60 min and 138 ± 21.7 at 120 min; four experiments), whereas azithromycin accumulation increased without saturation for up to 3 h (C/E, 393 ± 37.9; four experiments). The uptake kinetics of all macrolides differed among PMN samples from different individuals (Fig. 2), a phenomenon already observed with dirithromycin and erythromycylamine (27) and, to a lesser extent, with azithromycin (25). The individual variance in the speed of macrolide uptake was controlled at least two to three times after intervals of 1 to 6 months without significant variations. This phenomenon was not specific for any macrolide; i.e., PMN samples displaying slow uptake kinetics for RU 64004 also accumulated azithromycin and roxithromycin slowly. This suggests that a carrier protein whose numbers or activity differs according to PMN samples is involved in the uptake of these drugs.

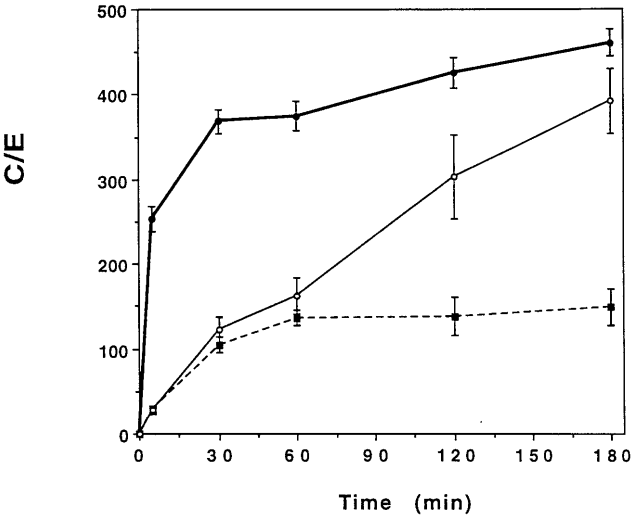

FIG. 1. Uptake kinetics of macrolides. Results are expressed as mean ± SEM C/EO ●, RU 64004 (10 to 27 experiments); ■, roxithromycin (4 to 9 experiments); ○, azithromycin (5 to 14 experiments).

Factors influencing macrolide uptake. (i) Extracellular pH. The uptake of RU 64004 was little influenced by modification of the extracellular pH (Fig. 3). At 5 min, accumulation of this drug was increased only at pH 9 compared to the level of accumulation at pH 7.4 (C/Es, 409 ± 39.3 versus 260 ± 40.5; $P = 0.028$; five experiments), whereas after 30 min of incubation there was no difference in the level of accumulation over the entire pH range (pH 7 to 9; ANOVA, 0.92; four experiments). A similar profile was observed with roxithromycin (Fig. 3). By contrast, azithromycin uptake was more dependent on the extracellular pH at 5 and 30 min of incubation (Fig. 3).

(ii) Metabolic inhibitors. None of the metabolic inhibitors (KF, an inhibitor of anaerobic glycolysis; NaCN, an inhibitor of mitochondrial oxidative respiration; 2,4-DNP, a phosphorylative oxidation uncoupler; NaN_3, an inhibitor of cytochrome electron transfer) inhibited macrolide uptake (data not shown).

(iii) Effects of temperature. Macrolides were incubated at temperatures ranging from 4 to 40°C for 5 to 180 min (Fig. 4). At 4°C, RU 64004 uptake was low, with a maximal C/E of 21 ± 4.6 at 30 min without a further increase ($P < 0.001$ versus that at 37°C). At 22°C, uptake was moderate and gradual, with no saturation up to 180 min. At 40°C, RU 64004 uptake was not different from that observed at 37°C. By contrast, azithromycin uptake was zero at 4°C (C/E, about 0.5 throughout the incubation period), was moderate at 22°C (C/Es, from 2.4 ± 0.9 to 6.1 ± 0.5 at 180 min), and was increased at 40°C compared to that at 37°C (C/Es, 428 ± 18.2 versus 338 ± 9.6; $P < 0.02$; three experiments at 180 min). Roxithromycin displayed a profile similar to that of azithromycin, with C/Es of about 1 at 4°C, 3 to 10 at 22°C, 25 to 95 at 37°C, and 38 to 195 at 40°C (data not shown).

Activation energy was calculated by measuring the uptake of the macrolides by PMNs incubated for 5 min at 4, 20, 22, 30, 33, 37, and 40°C as described previously (25, 28) by using the Arrhenius equation: $\Delta G = -RT \ln K_{eq}$, where ΔG 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 C/E at 5 min (a time when uptake rates are maximal). ΔG can be obtained from the slope of the

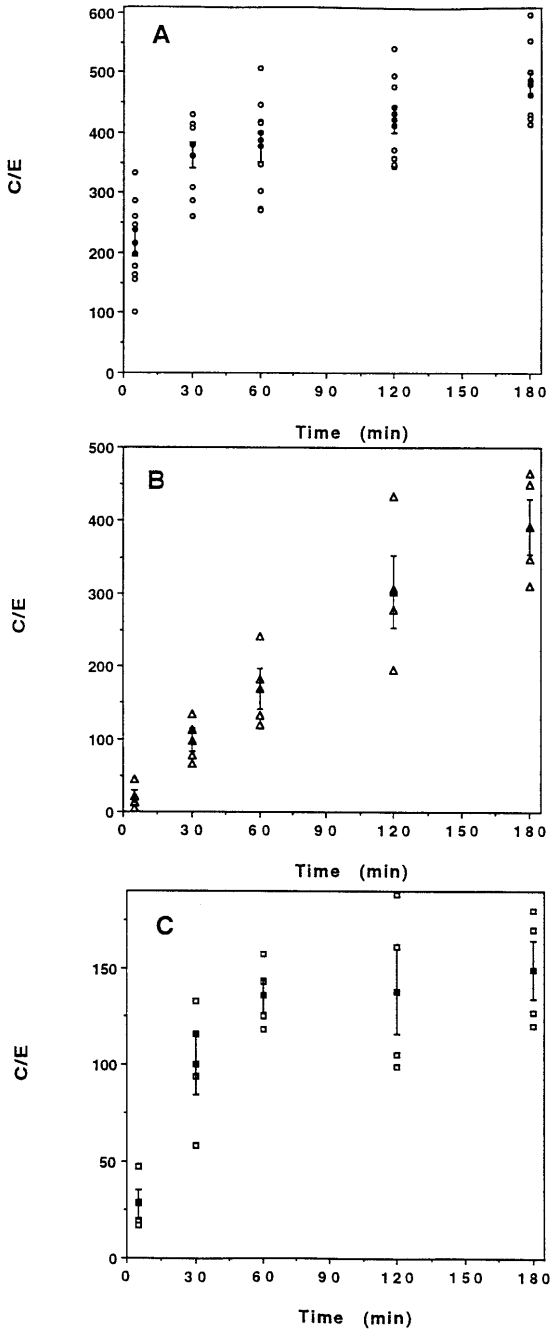

FIG. 2. Interindividual variability in uptake kinetics. (A) RU 64004 and PMN samples from 10 different individuals; (B) azithromycin and PMN samples from 4 different individuals; (C) roxithromycin and PMN samples from 4 different individuals. Open symbols indicate individual values, closed symbols indicate the mean ± SEM.

curve by using the Van't Hoff plot representation of data: $\ln K_{eq} = -\Delta G/RT$.

The activation energy of RU 64004 was moderate (68 ± 3.8 kJ/mol; mean of six experiments), as was that of roxithromycin (75 ± 3.1 kJ/mol; mean of two experiments). By contrast, and in agreement with published data (25), that of azithromycin was higher (122 ± 17.2 kJ/mol; mean of four experiments).

(iv) Effect of extracellular concentrations on RU 64004 uptake. The influence of extracellular concentrations in the range of 1 to 100 mg/liter was studied in the first 5 min of incubation (Fig. 5A). RU 64004 uptake displayed saturation kinetics characteristic of those of a carrier-mediated transport system. Azithromycin and roxithromycin displayed similar profiles. The results of kinetic analyses of macrolide uptake (Lineweaver-Burk reciprocal plots) are presented in Fig. 5C. Calculation of constants from the curve gave a mean V_{max} of 3,846 ng/2.5 × 10^6 PMNs/5 min and a mean K_m of 22 mg/liter (28 μM) for RU 64004. For azithromycin and roxithromycin, the mean values of the constants were 926 and 1,539 ng/2.5 × 10^6 PMNs/5 min, respectively, for V_{max} and 51 mg/liter (62 μM) and 91 mg/liter (108 μM), respectively, for K_m. It was interesting that, as with uptake kinetics (Fig. 2), there was a strong interindividual variability for the saturation kinetics of RU 64004 (Fig. 5B). Individual values of kinetic constants that were calculated resulted in V_{max} values ranging from 2,632 to 7,143 ng/2.5 × 10^6 PMNs/5 min and K_m values ranging from 14 to 66 mg/liter. A similar aspect was observed with roxithromycin (V_{max}, 740 to 5,000 ng/2.5 × 10^6 PMNs/5 min; K_m, 34 to 375 mg/liter) and azithromycin (V_{max}, 407 to 3,624 ng/2.5 × 10^6 PMNs/5 min; K_m, 24 to 263 mg/liter).

We further studied whether macrolide transport into PMNs was mediated by a common carrier system. The uptake of RU

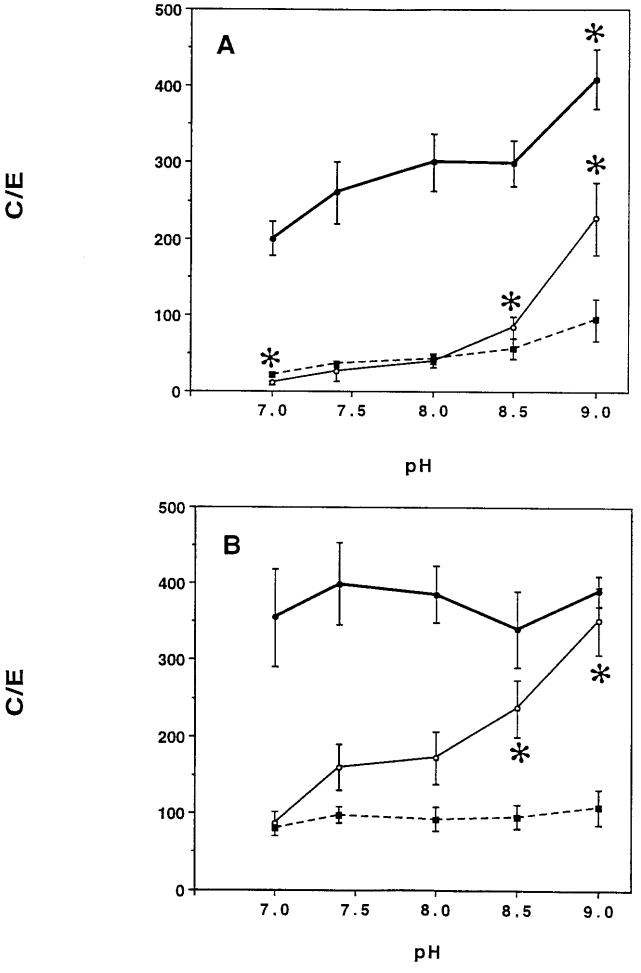

FIG. 3. Effect of extracellular pH on macrolide uptake at 5 min of incubation (A) and 30 min of incubation (B). ●, RU 64004 (four to six experiments); ○, azithromycin (four experiments); ■, roxithromycin (four experiments). *, $P < 0.05$ (by ANOVA followed by Student's t test for paired data) versus uptake at pH 7.4.

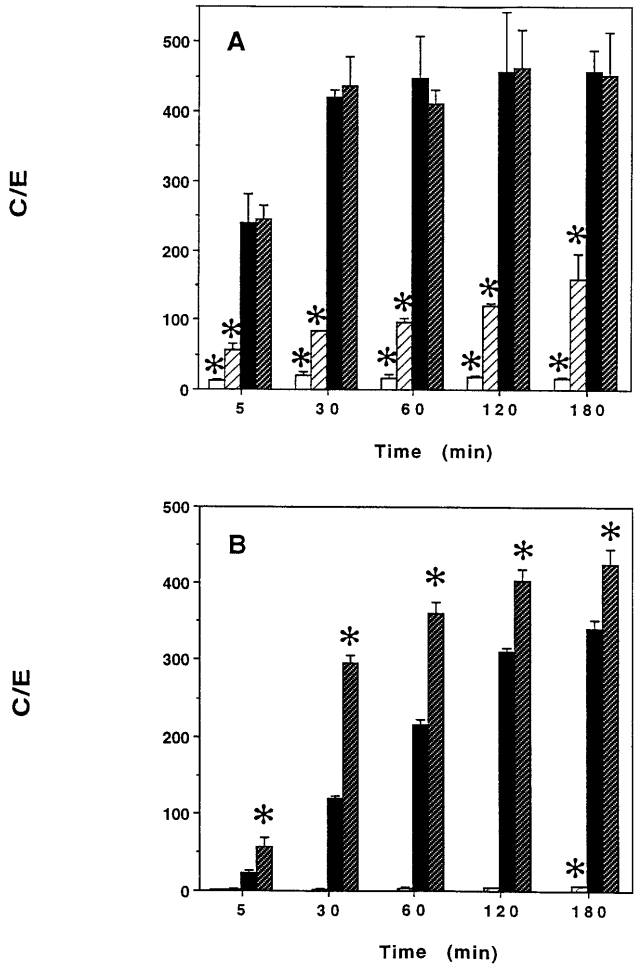

FIG. 4. Effect of temperature on uptake kinetics. (A) RU 64004 (four to six experiments); (B) azithromycin (two to four experiments). White bars, 4°C; lightly hatched bars, 22°C; black bars, 37°C; heavily hatched bars, 40°C. *, $P < 0.05$ (by ANOVA followed by Student's t test for paired data) versus uptake at 37°C.

64004 (28 μM, i.e., the mean K_m found in our experiments) was measured in the presence of either azithromycin (62 μM, which is equal to the K_m) or roxithromycin (108 μM, which is equal to K_m) at 5 and 15 min. Compared to the control uptakes (1,802 ± 129.4 ng/2.5 × 10^6 PMNs/5 min and 2,100 ± 41.4 ng/2.5 × 10^6 PMNs/15 min), azithromycin and roxithromycin significantly impaired RU 64004 uptake by about 50%: for azithromycin (62 μM), 53% ± 18.4% (5 min) and 45% ± 3.0% (15 min) of control uptake; for roxithromycin (108 μM), 60% ± 13.6% (5 min) and 71% ± 15.2% (15 min) of control uptake ($P < 0.05$; three experiments). Competitive inhibitors of known transport systems on the PMN membrane (1 mM glucose, 1 mM the amino acids L-phenylalanine and L-cysteine, or 1 mM puromycin) did not inhibit RU 64004 uptake; 91% ± 1.4 to 106% ± 13.6% of the control uptake at 5 and 15 min (glucose) and 94% (L-phenylalanine), 81% (L-cysteine), and 93% (puromycin) of the control uptake at 5 min.

Intracellular location of RU 64004. The intracellular locations of the macrolides were studied after 5 and 30 min of incubation (Fig. 6). In the presence of 0.5% Triton X-100, sonication resulted in the breakage of all cytoplasmic and granular membranes, resulting in the release of about 97% of the lysozyme, β-glucuronidase, and LDH in the supernatant. None of the macrolides was strongly associated with the membrane pellet after 5 and 30 min (about 4 to 6% of the total amount of cell-associated macrolides). By contrast, in the granular pellet obtained in the presence of 0.73 M sucrose, there was a strong accumulation of the macrolides, as indicated by the release of less than 10% granule enzymes and more than 95% of LDH in the supernatant. The intragranular accumulation of these compounds was maximal as early as the first 5 min and was about 70% of the total intracellular RU 64004, a value not significantly different from that for azithromycin (about 60%) but significantly higher than that for roxithromycin (49%; $P = 0.032$).

Efflux of macrolides. RU 64004 slowly egressed from the loaded cells, as did azithromycin, with 27% of total macrolide released from the cells at 5 min without further changes (Fig. 7). In agreement with published data (6, 8, 9), the level of efflux of azithromycin was low, with about 28% released in the first 5 min, with no further changes, whereas that of roxithromycin was gradual, with less than 30% of the initial load being associated with the cell pellet after 1 h of incubation (Fig. 7). RU 64004 and azithromycin were not firmly bound to the cells. When PMNs incubated in drug-free medium for 60 min were centrifuged and again placed in fresh drug-free medium, RU 64004 and azithromycin egressed from the cells at a rate (about 5%/min) comparable to that after the first wash. This suggests that the equilibrium reached in the first drug-free medium was due to avid reuptake of the drugs by neutrophils.

Effects of Ca^{2+} chelators and Ca^{2+} channel blockers on macrolide accumulation. We have previously reported that the intracellular accumulation of various erythromycin A derivatives is highly dependent on the presence of extracellular Ca^{2+}, and particularly on the correct functioning of the Na^+ and Ca^{2+} exchanger (28). In addition, verapamil (but not other organic Ca^{2+} channel blockers) impaired roxithromycin and erythromycin uptake. We therefore investigated whether RU 64004 accumulation also depended on Ca^{2+} entry into PMNs relative to the levels of accumulation of azithromycin (which has not previously been analyzed in this context) and roxithromycin as control (Table 1). External Ca^{2+} chelation by EGTA did not significantly alter RU 64004 accumulation, but Ni^{2+} (a blocker of the Na^+ and Ca^{2+} exchanger) impaired it by about 70% in the first 5 min without further modification; in addition, 250 μM verapamil strongly inhibited RU 64004 uptake, an effect that increased with the incubation time. Azithromycin uptake was susceptible to Ca^{2+} chelation and the presence of Ni^{2+}. Interestingly, 250 μM verapamil significantly increased azithromycin uptake at 5 min, whereas it significantly impaired it at 30 and 60 min in a concentration-dependent manner. We further determined the concentration which inhibits 50% of macrolide uptake (IC_{50}) for Ni^{2+}. By regression analysis the IC_{50} of Ni^{2+} for RU 64004 uptake was 3.5 mM, whatever the incubation time ($P < 0.001$; $r = 0.823$, 0.863, and 0.919, at 5, 30, and 60 min, respectively). This value was slightly higher than those obtained with roxithromycin (1.6 mM [28]) and azithromycin (2 mM). The effect of verapamil was also analyzed over a wide concentration range (Fig. 8). Verapamil inhibited RU 64004 accumulation in a concentration-dependent manner (Fig. 8A). At the high concentration of 250 μM, verapamil-induced inhibition increased with time, whereas at concentrations of <125 μM, the effect of verapamil was optimal at 5 min, without further changes. IC_{50}s of 73 μM ($P < 0.01$; $r = 0.860$), 60 μM ($P < 0.001$; $r = 0.876$), and 60 μM ($P < 0.001$; $r = 0.867$) were obtained at 5, 30, and 60 min, respectively. Regression analysis of verapamil-induced inhibition of roxithromycin uptake gave IC_{50}s of 225 μM (5 min; $P < 0.001$; $r = 0.796$), 27 μM (30 min; $P < 0.001$; $r = 0.955$), and 21 μM (60 min; $P < 0.001$; $r = 0.985$). By contrast, azithromycin

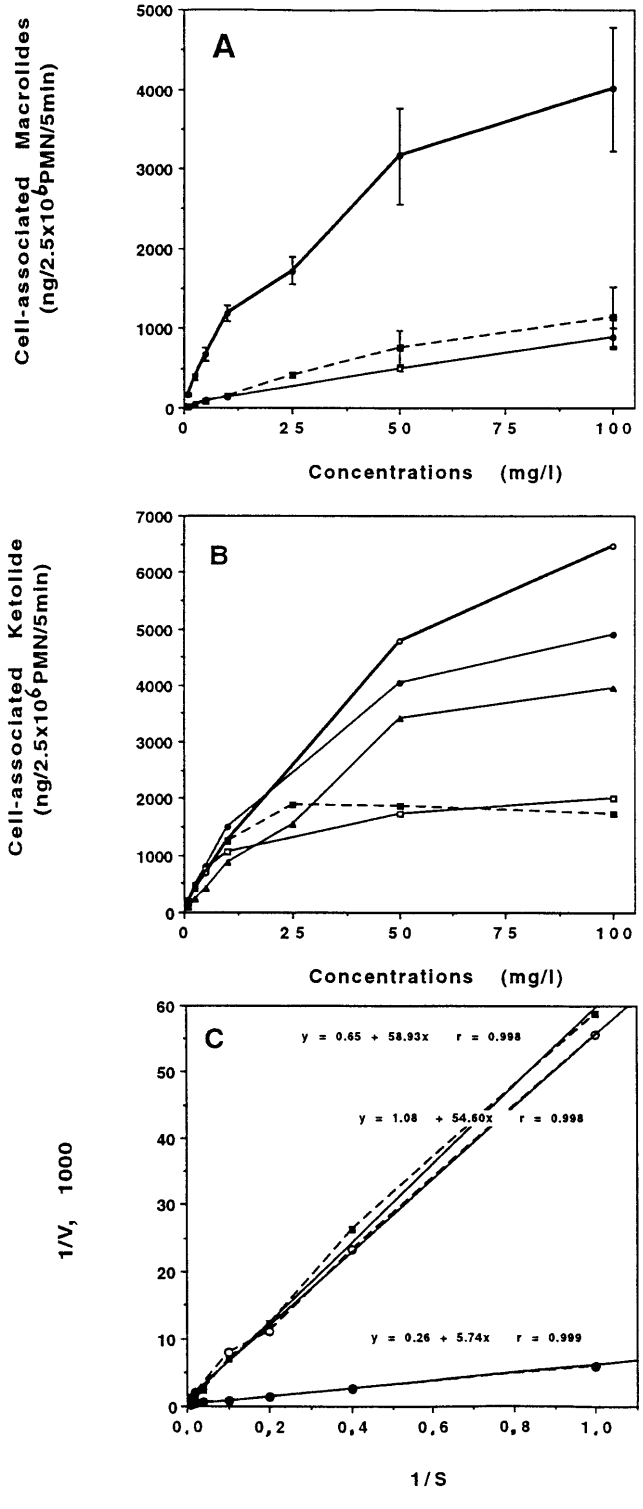

FIG. 5. Effect of extracellular concentration on macrolide uptake at 5 min. (A) Comparative accumulation of RU 64004 (●; five experiments), azithromycin (○; four experiments), and roxithromycin (■; four experiments). (B) Interindividual variability in RU 64004 accumulation by PMN samples from five different individuals. (C) Lineweaver-Burk plots of Fig. 5A.

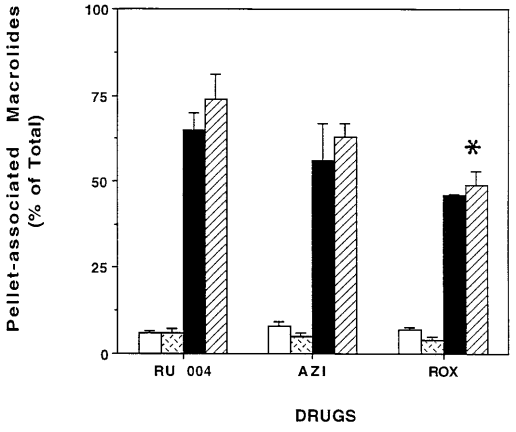

FIG. 6. Intracellular location of macrolides (RU 004, RU 64004 [six experiments]; AZI, azithromycin [three experiments]; ROX, roxithromycin [four experiments]). White bars, membrane pellet, 5 min; cross-hatched bars, membrane pellet, 30 min; black bars, granular pellet, 5 min; hatched bars, granular pellet, 30 min; *, $P = 0.032$ versus RU 64004. For technical details, see Materials and Methods.

uptake increased in the presence of 250 μM verapamil at 5 min, but lower concentrations did not significantly alter it (regression analysis; $P = 0.063$) (Fig. 8B). At 30 and 60 min, verapamil decreased azithromycin uptake with IC_{50}s of 241 μM (30 min; $P = 0.004$; $r = 0.643$) and 155 μM (60 min; $P < 0.001$; $r = 0.921$), respectively. Because the overall cellular accumulation of drugs reflects the balance between entry and efflux, we investigated the effects of Ni^{2+}, EGTA, and verapamil on macrolide efflux. Cells loaded for 30 min in control HBSS medium were recovered after velocity gradient centrifugation and were placed in drug-free medium: Ca^{2+}-deprived and 1 mM EGTA-supplemented HBSS, 5 mM Ni^{2+}-containing HBSS, or verapamil (250 or 125 μM)-containing HBSS.

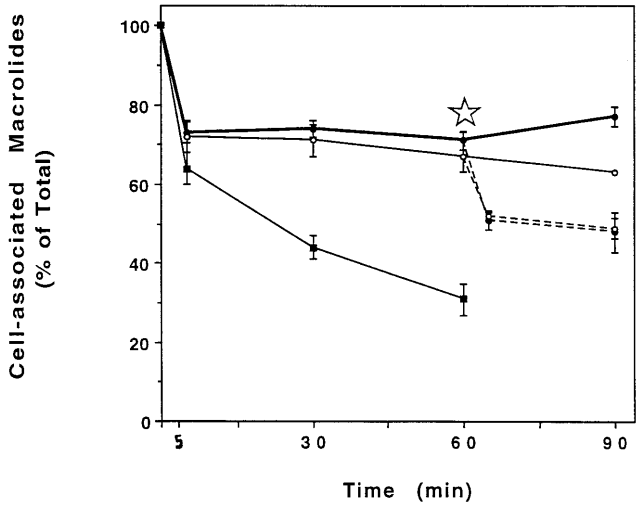

FIG. 7. Efflux of macrolides. PMNs were loaded for 30 min in the presence of macrolides (2.5 mg/liter) before centrifugation and incubation in drug-free medium (see Materials and Methods). Results are expressed as percent cell-associated macrolides at various time intervals in drug-free medium. The star indicates when PMNs were again centrifuged and incubated in fresh drug-free medium. —●—, RU 64004, first wash, 10 experiments; —●—, RU 64004, second wash, 2 experiments; —○—, azithromycin, first wash, 8 experiments; —○—, azithromycin second wash, 2 experiments; ■, roxithromycin, one wash, 4 experiments.

TABLE 1. Effects of extracellular Ca^{2+} deprivation and Ca^{2+} channel blockers on macrolide uptake

Macrolides	Experimental conditions	% (mean ± SEM) of control uptake at the following incubation times (min):		
		5	30	60
RU 64004	EGTA (1 mM)	103 ± 12.5 (8)[a]	112 ± 7.5 (8)	108 ± 7.1 (4)
	Ni^{2+} (5 mM)	36 ± 5.3 (10)[b]	34 ± 3.2 (10)[b]	30 ± 5.2 (6)[b]
	Verapamil	28 ± 4.7 (9)[b]	10 ± 2.4 (8)[b,d]	0.8 ± 0.2 (2)
Azithromycin	EGTA	32 ± 4.6 (7)[c]	38 ± 8.1 (7)[c]	32 ± 8.1[d] (6)
	Ni^{2+}	27 ± 11.7 (6)[c]	10 ± 1.6 (6)[c]	9 ± 1.2 (5)[c]
	Verapamil	196 ± 40.0 (8)[c]	46 ± 14.5 (7)[c,d]	9 ± 1.5 (3)[b,e]
Roxithromycin	EGTA	56 ± 17.4 (2)	69 ± 6.4 (2)	50 (1)
	Ni^{2+}	18 ± 1.7 (2)	16 ± 4.4 (2)	22 (1)
	Verapamil	45 ± 7.7 (6)[b]	13 ± 3.3 (6)[b,d]	11 ± 2.5 (3)[b]

[a] Number of experiments are given in parentheses.
[b] $P < 0.001$ versus control (by Student's t test for paired data).
[c] $P < 0.01$ versus control.
[d] $P < 0.01$ versus 5 min of incubation.
[e] $P < 0.01$ versus 30 min of incubation.

The percentage of drug remaining associated with the cell pellet was measured as described in Materials and Methods. Neither Ca^{2+} deprivation nor the presence of Ni^{2+} modified the efflux of any of the three macrolides in the first 5 min of incubation (Fig. 9); however, with longer incubation times, the percentage of total associated drug was reduced (about 50% of that of the control at 60 min). These data suggest, first, that extracellular Ca^{2+} and blockade of the Na^+ and Ca^{2+} exchanger are not required for efflux and, second, that the decrease in the level of cell-associated drug observed later is due to the impairment of reuptake of the drugs by neutrophils. By contrast, verapamil rapidly (5 min) and significantly, increased drug efflux in a concentration-dependent manner (Fig. 10). The effect was particularly marked with roxithromycin: IC_{50}s (the concentrations which reduced the amount of cell-associated drug by 50%) were 19 μM at 5 min ($P < 0.001$; $r = 0.923$) and 24 μM at 30 min ($P = 0.007$; $r = 0.648$). For azithromycin, IC_{50}s were 425 μM at 5 min ($P = 0.025$; $r = 0.542$) and 174 μM at 30 min ($P < 0.001$; $r = 0.847$). For RU 64004, IC_{50}s were 155 at 5 min ($P = 0.001$; $r = 0.860$) and 177 μM at 30 min ($P = 0.005$; $r = 0.805$).

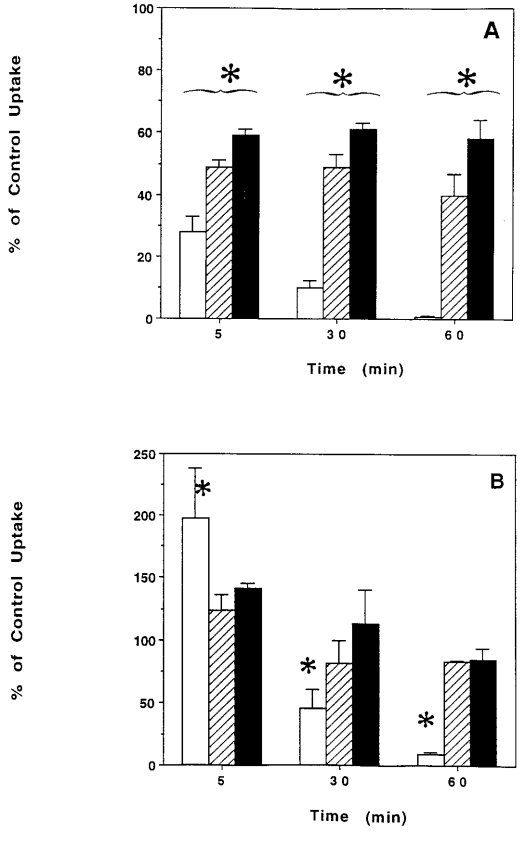

FIG. 8. Effect of verapamil on macrolide accumulation in PMNs. (A) RU 64004 (three to nine experiments); (B) azithromycin (three to eight experiments). White bars, verapamil at 250 μM; hatched bars, verapamil at 125 μM; black bars, verapamil at 50 μM. *, $P < 0.05$ versus control.

DISCUSSION

The study of the intracellular accumulation and release of macrolide antibiotics is an area of active research aimed at better determining their intracellular bioactivity and their peculiar pharmacokinetic profile (so-called tissue-directed pharmacokinetics) (31). The revival of macrolides in the last 15 years has mainly benefited from the development of hemisynthetic erythromycin A derivatives from which almost all modern therapeutic macrolides, including the azalide azithromycin, are derived (14, 19). A chemical innovation in the field of macrolides has recently been proposed i.e., the substitution of L-cladinose by a 3-keto group, the substituent that defines the ketolides (2). RU 64004, a new ketolide compound, displays intracellular bioactivity (7, 15, 35), suggesting that this drug does enter eukaryotic cells. Whether or not the absence of L-cladinose modifies the characteristics of the intracellular accumulation of erythromycin A derivatives has never been investigated. It was thus interesting to analyze the cellular pharmacokinetics of RU 64004 in a model, the neutrophil, which has been widely studied by our group and other groups of investigators. The uptake of RU 64004 was compared to those of classical erythromycin A derivatives, whose cellular pharmacokinetic parameters have been determined previously (21).

The main feature of the cellular kinetics of RU 64004 was the very strong and rapid accumulation, with C/Es largely exceeding those obtained with azithromycin, the drug most active in this context. As observed with roxithromycin (a group II erythromycin A derivative), accumulation kinetics were rapid

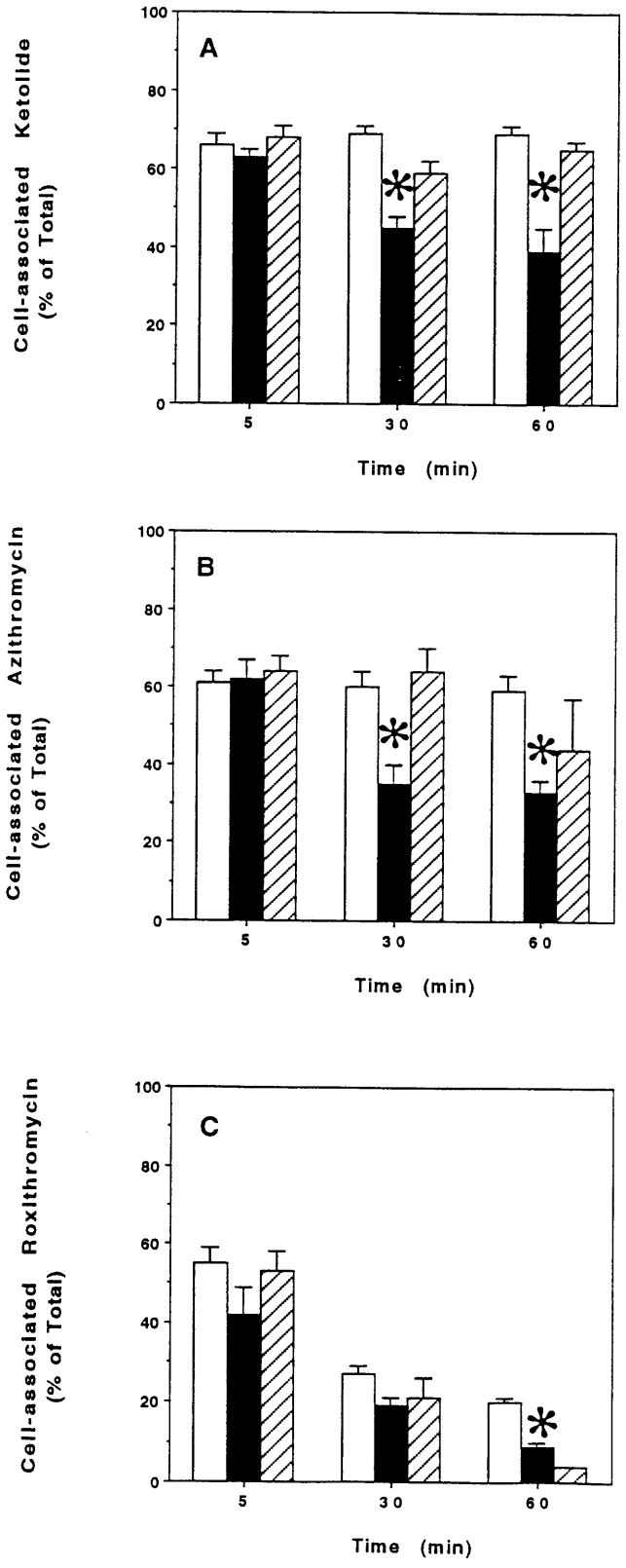

FIG. 9. Effect of nickel (5 mM) and EGTA (1 mM in Ca^{2+}-free medium) on macrolide efflux. (A) RU 64004 (3 to 8 experiments); (B) azithromycin (3 to 7 experiments); (C) roxithromycin (3 to 10 experiments). White bars, control (efflux in HBSS); black bars, efflux in the presence of Ni^{2+}; hatched bars, efflux in Ca^{2+}-free medium. *, $P < 0.05$ versus control efflux in HBSS. ANOVA followed by Student's t test.

but saturable over a 3-h incubation period, pH susceptibility was low, and activation energy was moderate. However, slow efflux from loaded cells and a preferential intragranular location confer on RU 64004 two main features of group I erythromycin A derivatives (represented by azithromycin). These two characteristics are likely related to the dibasic nature of the compounds and should reflect intragranular trapping by protonation, a phenomenon often forwarded to explain the intracellular accumulation of macrolides (8, 9). Laufen and colleagues (24, 25) have suggested that for macrolides with similar chemical structures, a high activation energy could reflect either the use of an active transport system or the crossing of two membranes (cytoplasmic and granular). The low activation energy of RU 64004, despite granular trapping to the same extent as azithromycin, may be explained by its high lipophilicity, which favors the crossing of membranes. The lipophilicity of RU 64004 may also explain its greater uptake at 4 and 22°C compared to the uptake of other macrolides (Fig. 4).

Despite abundant, mainly descriptive, information in the literature on the cellular accumulation of macrolides, the mechanisms underlying this phenomenon are poorly understood. Lipophilicity or intragranular trapping by protonation has often been forwarded as the essential passive mechanism(s) responsible for drug uptake. Also, the absence of strong inhibitory effects of various metabolic inhibitors on macrolide uptake has been used to support the strictly passive uptake of these drugs. Consequently, the existence of a macrolide carrier system has rarely been explored. The nucleoside transport system has been proposed to play a role in josamycin uptake (24), and Hand and colleagues (16) also suggested that an unidentified transport system could be involved in roxithromycin uptake by neutrophils, as shown by the saturation kinetics of this drug. The data presented here support the existence of an active transport system, for some macrolides at least. First, passive, lipophilicity-mediated accumulation is unlikely to explain the rapid and excessive accumulation of RU 64004 (≥260-fold the extracellular concentration within 5 min). Second, the interindividual variability in drug accumulation observed here (Fig. 2) and in previous studies (25, 27) is more likely to reflect interindividual variations in carrier proteins (numbers and activities) than in membrane lipid composition. Third, concentration dependence is a factor that favors the existence of a carrier-mediated transport system. The K_m of RU 64004 was low (about 30 μM), indicating a high affinity for the transporter, whereas the K_m values of azithromycin and roxithromycin (62 to 108 μM) indicated a lower affinity for the carrier. In addition, the fact that roxithromycin and azithromycin at concentrations of the mean K_m calculated in our system impaired by about 50% the uptake of RU 64004, whereas other competitive inhibitors of carrier systems existing on the PMN membrane were ineffective, strongly supports the existence of a macrolide carrier in PMNs.

The reason why metabolic poisons do not modify macrolide uptake remains unexplained. ATP production in neutrophils is mediated mainly by anaerobic glycolysis and should be decreased by fluoride. The concentration of NaF (1 mM) and the preincubation time (10 min) were chosen so that they did not interfere with cell viability, but they could have been insufficient to substantially decrease ATP production. This needs further investigation.

A major argument for the existence of an active process in the uptake of erythromycin A derivatives by neutrophils comes from a previous report that extracellular Ca^{2+} and, particularly, its entry by the Na^+ and Ca^{2+} exchanger were required for maximal uptake of erythromycin A derivatives by neutrophils (28). The data reported here confirm that work in the

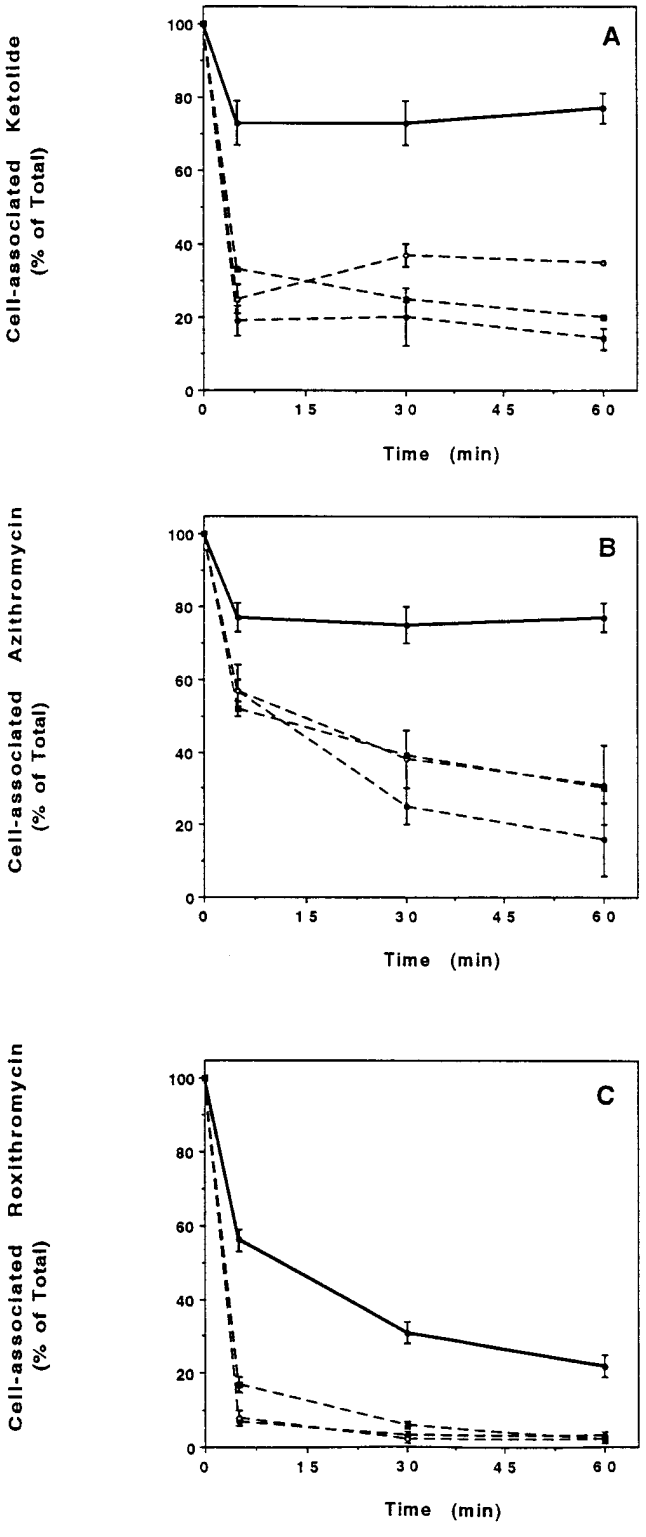

FIG. 10. Effect of verapamil on macrolide efflux. (A) RU 64004 (3 to 4 experiments); (B) azithromycin (3 to 7 experiments); (C) roxithromycin (3 to 11 experiments). Solid lines, control efflux; broken lines, efflux in the presence of verapamil; ●, 250 μM verapamil; ○, 125 μM verapamil; ■, 50 μM verapamil.

case of azithromycin and RU 64004 and extend it by demonstrating that macrolide efflux does not require extracellular Ca^{2+} or a functional Na^+ and Ca^{2+} exchanger. These results strongly suggest that Na^+ and Ca^{2+} exchanges are required for the entry process for macrolides derived from erythromycin A. The observation that RU 64004 uptake was not modified in the presence of 1 mM EGTA (Table 1) is intriguing. It is possible that some traces of Ca^{2+} not chelated by EGTA would be sufficient for the binding of RU 64004 to the macrolide transporter, owing to the high affinity of this drug for this carrier, but blockage of Ca^{2+} entry by Ni^{2+} impaired RU 64004 uptake by about 70%. The intracellular accumulation of macrolides reflects the balance between entry and efflux. Despite reports of the possibility of an active entry process for these drugs (see above), the mechanism by which they are released from host cells has received little attention. However, this process is crucial in explaining both intracellular bioactivity (maintenance of high intracellular concentrations) and tissue-targetted kinetics. The fact that azithromycin and other dibasic macrolides, including RU 64004, are slowly released from cells is compatible with the fact that they are trapped inside acidic compartments by a protonation mechanism, as first demonstrated by De Duve et al. (11). This has also been advocated as a mechanism for the concentration of some quinolone-containing antimalarial drugs inside the digestive vacuole of *Plasmodium* spp. (3) and in the azurophilic granules of neutrophils (33). A passive and slow egress of such protonated drugs from loaded cells was suggested to be the main (or exclusive) mechanism of efflux. The data reported here suggest for the first time that macrolide efflux from loaded cells may also occur via a carrier-mediated transport system. Here and elsewhere (25) we observed that the overall accumulation of various erythromycin A derivatives, particularly roxithromycin and RU 64004, is strongly impaired by verapamil in time- and concentration-dependent manners. The IC_{50}s of verapamil for drug uptake at 30 min were in the following order: roxithromycin (27 μM) < RU 64004 (60 μM) < azithromycin (241 μM). At 5 min, the effect of verapamil was greater on RU 64004 uptake (IC_{50}, 73 μM) than on roxithromycin uptake (IC_{50}, 226 μM), whereas it did not inhibit azithromycin uptake. However, the inhibitory effect of verapamil on drug uptake could be explained by a stimulatory action on macrolide efflux (Fig. 10). These data argue against a strictly passive efflux of these drugs and suggest the existence of a carrier system activated by verapamil. Besides its effect on L-type Ca^{2+} channels (17, 32), whose existence has not been demonstrated in the cytoplasmic membrane of the neutrophils, verapamil has many other effects, including inhibition of various protein kinases and elevation of cyclic AMP levels (12). Also, verapamil has been shown to reverse the multiple drug resistance (MDR) phenotype of various tumor cells by acting on the membrane pump, a protein belonging to the P-glycoprotein (PgP) family involved in the active efflux of various hydrophobic compounds (13, 30). Some macrolides, as well as the macrolidic immunosuppressants FK 506 and rapamycin, have been shown to reverse the MDR phenotype of tumor cell lines (4, 10), while erythromycin A does not kill *Listeria monocytogenes* growing in cells with the MDR phenotype (29). Both results suggest that macrolides might also use this PgP system to egress from cells. Our results do not fully agree with this hypothesis, however, because the PgP inhibitor verapamil did not inhibit macrolide efflux but, rather, increased macrolide efflux. It is thus impossible to link macrolide efflux to a PgP, whose existence on the PMN cytoplasmic membrane is still to be demonstrated (20). It is noteworthy that P glycoprotein (and the relevant *MDR1* mRNA) is expressed in granulocytes but does not show any measurable transport func-

tion (20). That verapamil mediates its stimulating effect by inhibiting kinase activity (and thus the phosphorylation of an egress transport protein) or by increasing cyclic AMP levels remains to be determined. However, preliminary data from our laboratory (23) indicate that roxithromycin efflux from neutrophils is greatly potentiated by H7 (a nonspecific kinase inhibitor) and H89 (a quasispecific protein kinase A inhibitor), suggesting that inhibition of protein phosphorylation is involved in roxithromycin efflux. Analysis of other macrolides is under way.

Lastly, the effect of verapamil may in fact be more complex than a single action on the efflux carrier system of macrolides, given its comparative effect on overall accumulation (Table 1) and macrolide release (Fig. 10). In particular, the verapamil-mediated increase in macrolide efflux is always stronger than the decrease in drug accumulation, suggesting that verapamil could have a stimulating effect on the entry mechanism of these drugs. This was particularly clear in the case of azithromycin, whose accumulation was almost doubled at 5 min by 250 μM verapamil, whereas efflux was significantly stimulated over the same incubation period.

In summary, RU 64004, a new macrolide, appears to have an exceptional ability to rapidly enter PMNs; this may explain its apparent slow egress from the cells due to the near equilibrium between efflux and reuptake. Although no carrier systems mediating entry and efflux of macrolides inside PMNs were identified here, we have obtained new evidence that such an active process exists. Further work is required to identify these carriers and their normal functions in cells as well as the macrolide chemical structures involved in binding to these carriers.

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