Transport, Uptake, and Metabolism of the Bis(pivaloyloxymethyl)-Ester Prodrug of 9-(2-Phosphonylmethoxyethyl)Adenine in an *In Vitro* Cell Culture System of the Intestinal Mucosa (Caco-2)

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Purpose. To evaluate intestinal transport, uptake and metabolism characteristics of the bis(pivaloyloxymethyl)-ester [bis(POM)-ester] of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine [PMEA].

Methods. Intestinal transport, uptake and metabolism of bis(POM)-PMEA were studied using an *in vitro* cell culture system of the intestinal mucosa (Caco-2 monolayers). Concentrations of bis(POM)-PMEA and its metabolites mono(POM)-PMEA and PMEA were determined using a reversed-phase HPLC method. Enzymatic stability of bis(POM)-PMEA was evaluated by incubation with purified liver carboxylesterase, homogenates of Caco-2 cells and scraped pig small intestinal mucosa.

Results. The use of bis(POM)-PMEA as a prodrug of PMEA resulted in a significant increase in transport of total PMEA [bis(POM)-PMEA, mono(POM)-PMEA and PMEA] across Caco-2 monolayers. While transepithelial transport of PMEA (500 μM) was lower than 0.1% during a 3 hr incubation period, transport of total PMEA after addition of bis(POM)-PMEA (100 μM) amounted to 8.8% over the same incubation period. Only 23% of the amount transported appeared as intact bis-ester at the basolateral side, while 33% of this amount was free PMEA and 44% was mono(POM)-PMEA, suggesting susceptibility of the prodrug to chemical and enzymatic degradation. Uptake studies revealed that only negligible amounts of bis(POM)-PMEA (< 0.2%) were present inside the cells. Very high intracellular concentrations of PMEA were found (≈ 1.2 mM, after a 3 hr incubation with 50 μM bis(POM)-PMEA), which suggests that PMEA was trapped inside the cells probably due to its negative charge. This explains that efflux of PMEA was relatively slow (25% of the intracellular amount in 3 hr). Enzymatic degradation of the prodrug by carboxylesterase was confirmed by incubation of bis(POM)-PMEA with purified enzyme (K_m = 87 μM and V_{max} = 9.5 μM/min). Incubation of bis(POM)-PMEA (10 μM) with cell homogenate of Caco-2 monolayers and pig small intestinal mucosa produced similar degradation profiles.

Conclusions. The use of the bis(POM)-prodrug significantly enhances the intestinal permeability of PMEA. Intracellular trapping of PMEA in the intestinal mucosa may result in slow release of PMEA to the circulation after oral administration of bis(POM)-PMEA.

KEY WORDS: Caco-2; prodrug; intestinal permeability; intestinal metabolism; bis(POM)-PMEA; antiviral.

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INTRODUCTION

9-(2-phosphonylmethoxyethyl)adenine (PMEA) is an acyclic nucleoside phosphonate analog with antiviral activity against retro-viruses (e.g. human immunodeficiency virus types 1 and 2 [HIV-1 and -2]), as well as several DNA-viruses (e.g. herpes simplex virus-1, Epstein-Barr virus) (1). Because of the presence of a phosphonate group, this compound shows activity against *thymidine kinase* deficient viruses and in thymidine kinase deficient cell lines. This is an important advantage over other well-known antiviral agents, such as zidovudine and stavudine; these compounds are not active against *thymidine kinase* deficient viruses in *thymidine kinase* deficient cells due to a lack of the enzyme required for initial phosphorylation and, thus, activation of these nucleoside analogs (2). Unfortunately, the permeation of PMEA across biological membranes is very low due to the negative charge associated with this phosphonate group at physiological pH (pK_{a1} = 2.0 and pK_{a2} = 6.8; (3)). This results in limited oral bioavailability of PMEA, which was reported to be less than 1% in rhesus monkeys (4), only 4.0% in cynomolgus monkeys (5) and 7.8% in rats (6). Therefore, esters of PMEA were synthesized in order to mask the negative charge and to obtain membrane permeable prodrugs. The bis(pivaloyloxymethyl)-ester of PMEA [bis(POM)-PMEA] is one of those prodrugs, which is currently undergoing phase II/III clinical trials in HIV-1 infected patients. The bis(pivaloyloxymethyl)-ester of PMEA was initially selected as a potential membrane permeable prodrug of PMEA by Shaw and Cundy (7), because of its enhanced transport across Caco-2 monolayers. *In vitro* studies in MT-2 cells showed an increased anti-HIV-1 activity by using the bis(POM)-prodrug of PMEA, due to higher intracellular levels of PMEA after enhanced uptake of the prodrug (8). Bioavailability of PMEA after oral administration of a formulation of its bis(POM)-ester in cynomolgus monkeys varied between 22% and 27% (3). Studies with bis(POM)-PMEA administered in single doses of 125 to 500 mg in men revealed an oral bioavailability for PMEA of approx. 35% (9).

We now investigated the transport, uptake and metabolism of the bis(POM)-ester of PMEA in an *in vitro* cell culture system of the intestinal mucosa (Caco-2) in order to gain insight into the mechanisms involved in the intestinal absorption of the prodrug ester.

MATERIALS AND METHODS

Materials

PMEA was kindly provided by Dr.A Holý (Czech Academy of Sciences, Prague, Czech Republic); bis(POM)-PMEA and mono(POM)-PMEA were obtained from Gilead Sciences (Foster City, CA, USA). Tetrabutylammonium hydrogen sulfate (Fluka, Switzerland), potassium dihydrogenphosphate (Merck, Darmstadt, Germany), methanol and acetonitrile (BDH, Poole, UK) were HPLC grade. All chemicals used for culturing the cells were purchased from Gibco Inc. (Life Technologies, Belgium). D-(+)-glucose, carboxylesterase (19 U/mg, from porcine liver), sodium azide, 2-deoxy-glucose and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium fluorescein, sodium hydroxide and ammo-

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nia 25% solution were obtained from UCB (Leuven, Belgium), while DMSO was obtained from Janssen Chimica (Geel, Belgium). Transport medium (TM) consisted of 500 ml HBSS containing 25 mM glucose and 10 mM Hepes. Cell culture medium consisted of DMEM supplemented with 10% Fetal Bovine Serum, 1% MEM-non-essential amino acids solution and 1% penicillin–streptomycin solution.

Test Solutions

Solutions of bis(POM)-PMEA (10–200 μM) in TM were prepared by spiking TM with a concentrated stock solution made up in DMSO. Final concentrations of DMSO were always adjusted to 0.4% DMSO. Preliminar experiments had shown that concentrations up to 2% DMSO did not affect cell monolayer integrity.

Caco-2 Cell Culture

Caco-2 cells were kindly provided by Prof. Dr. Y. Schneider (UCL, Belgium). Cells were grown in 75 cm^2 culture flasks at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1 to 7. Cells were negative for Mycoplasma infection.

Growth of Cells on Membrane Inserts and in Wells

For transport experiments, Caco-2 cells were plated at a density of 40,000 cells/cm^2 on Anopore membrane inserts (0.2 μm pore diameter, 25 mm diameter; Nunc, Roskilde, Denmark). For uptake experiments, the cells were seeded directly in 6-well plates at the same density. Confluence was reached within 3–4 days after seeding and the monolayers were used between days 18–24 post-seeding. Cell passages between 114 and 127 were used in experiments.

HPLC-Analysis of bis(POM)-PMEA and Its Metabolites

Bis(POM)-PMEA and its metabolites mono(POM)-PMEA and PMEA were analyzed using a high-performance liquid chromatographic system equipped with a Model 600 Controller and Pump, a Model 717plus autosampler and a Model 480 Lambda-Max UV detector at 260 nm (Waters, Milford). UV-signals were monitored and the obtained peaks integrated using a personal computer running Waters Maxima 820 chromatography software. The column used was a Waters Symmetry C8 (4.6 × 150 mm). The flow rate was 1 ml/min and the volume injected amounted to 25 μl. Mobile phase A consisted of a mixture of 900 ml buffer [10 mM potassium dihydrogenphosphate and 2 mM tetrabutylammonium hydrogensulfate (as a counter-ion for PMEA) adjusted to pH 6.0 with sodium hydroxide] and 50 ml acetonitrile, while mobile phase B consisted of acetonitrile. Separation was carried out with a linear gradient from 0 to 35% mobile phase B over 10 min, followed by an isocratic stage of 7.5 min, and return to the initial conditions (100% mobile phase A) over 0.5 min. Column re-equilibration in mobile phase A took 17 min. The pH of the samples (containing solutions of TM at pH 7.4) was adjusted to 3.3 by adding HCl 0.05 N. Samples were stored at 4°C in order to minimize degradation of bis(POM)-PMEA during storage.

Concentrations of PMEA and bis(POM)-PMEA were determined using calibration graphs of each compound. Mono-(POM)-PMEA concentrations were determined using bis (POM)-PMEA calibration curves; this was allowed because both esters had the same UV response at 260 nm as observed by monitoring the UV response (260 nm) during chemical breakdown of the bis-ester. The intra-day as well as inter-day reproducibility of the method, expressed as the relative standard deviation, for measuring PMEA and bis(POM)-PMEA was lower than 5% for concentrations ranging from 0.25 to 10 μM.

Transport Experiments

Transport of PMEA, mono(POM)-PMEA or bis(POM)-PMEA across Caco-2 monolayers was studied using monolayers 18–24 days post-seeding. Before experiments, the monolayers were rinsed twice with TM and preincubated for 30 min. When the effect of metabolic inhibitors on transport was investigated, 1 mM sodium azide and 50 mM 2-deoxyglucose were included in both compartments during the preincubation period. After the preincubation, transepithelial electrical resistance (TEER) of the monolayers was measured at 37°C using a Millicel ERS apparatus (Millipore) to check cell monolayer integrity. Only monolayers displaying TEER values above 400 $\Omega \cdot cm^2$ were used in experiments. Transport was initiated by adding 2 ml of TM to the acceptor side and 2 ml of a solution of test compound to the donor side. The inserts were transferred in wells containing fresh TM at 1 and 2 hr after initiation of the transport. At the end of the experiment (3 hr), samples from both apical and basolateral side were taken. TEER values as well as sodium fluorescein flux (determined by measuring the absorption of the basolateral solution at 490 nm, 1 hr after adding a solution of 1 mg/ml to the apical side) of monolayers incubated with test compound were compared with those of blank monolayers for evaluation of the influence of the test compounds on cell monolayer integrity. Transport was expressed as a percentage of the initial amount added to the apical side.

Uptake Experiments

Uptake of PMEA, mono(POM)-PMEA and bis(POM)-PMEA was determined using Caco-2 monolayers grown in 6-well plates. Before the experiments, the monolayers were rinsed twice with TM (37°C), preincubated for 30 min, followed by incubation with test compound. At various time points, three monolayers were washed twice with ice-cold TM to immediately arrest uptake and/or efflux. Immediately afterwards, three milliliters of a mixture of 70% methanol in water (−20°C) were then added to the wells in order to denature proteins and to arrest enzymatic as well as chemical hydrolysis. Preliminary experiments had shown that no degradation of bis(POM)-PMEA occurred during processing. The suspension obtained was centrifuged for 5 min at 3,000 g and −20°C. The supernatant was analyzed according to the procedure described. Uptake was expressed either as a percentage of the initial amount of the parent compound added to the donor side or by calculating intracellular concentrations with an estimated cell height of 30 μm (10).

Efflux of PMEA and Mono(POM)-PMEA from Caco-2 Monolayer

Caco-2 monolayers (grown on inserts) were incubated with 100 μM bis(POM)-PMEA at the apical side for 1 hr ("loading"). The monolayers were washed twice with ice-cold TM and incubated with fresh TM (37°C). The last washing medium was analyzed for the absence of bis(POM)-PMEA. The efflux of PMEA and mono(POM)-PMEA by the cells was measured as a function of time by analyzing apical and basolateral incubation media; the intracellular amount after loading was determined according to the procedure described under uptake experiments. Efflux was expressed as percentage of the total intracellular amount immediately after loading.

Degradation of Bis(POM)-PMEA in the Presence of Purified Carboxylesterase

Solutions with substrate concentrations of 3, 5, 10, 30, 50, 100, 300 μM of bis(POM)-PMEA and a fixed concentration of carboxylesterase (from porcine liver) of 0.1 U/ml were prepared. The solutions were incubated at 37°C for three min; samples (0.5 ml) were collected in Eppendorf test tubes (Hamburg, Germany) containing 0.5 ml ice-cold methanol, vortexed, and centrifuged for 5 min at 3,000 g. The supernatant was injected into the HPLC for analysis of bis(POM)-PMEA and metabolites. The kinetic parameters K_m and V_{max} were determined according to the Michaelis-Menten equation after linear transformation (Lineweaver-Burk).

Degradation of Bis(POM)-PMEA by Cell Homogenates

Freshly scraped Caco-2 monolayers or pig small intestinal mucosa (excised within 30 min after sacrificing the animal) were homogenized in TM using a cell disruptor (Branson Sonifier B15) for 10 sec at 4°C. After centrifugation at 5,000 g for 5 min, the supernatants were harvested and the protein content determined according to the method of Lowry *et al.* (11) using BSA as a standard. The protein content of both homogenates was then adjusted to 0.39 mg/ml by dilution with fresh TM. The initial degradation rate (37°C, 3.5 min) of bis(POM)-PMEA (10 μM) was determined (n = 3) in each homogenate and expressed as nmol/min/mg protein. Preliminar experiments had shown a linear concentration-time profile for the formation of mono(POM)-PMEA in the 0–5 min time range.

RESULTS AND DISCUSSION

In this study, transport, uptake, efflux and metabolism of the bis(POM)-prodrug of PMEA in Caco-2 monolayers were examined. Results of transport experiments of bis(POM)-PMEA across Caco-2 monolayers showed a strong increase in the intestinal permeability of PMEA by using the bis(POM)-ester: 8.8% of the initial amount of bis(POM)-PMEA (100 μM) appeared at the basolateral side after 3 hr (Fig. 1) as compared to less than 0.1% (which corresponds to the detection limit) when a solution of 500 μM free PMEA was administered. A contribution of mono(POM)-PMEA transport to total transport was also negligible, since transport of the mono-ester (200 μM) amounted to less than 0.1% over 3 hr. These transport data are in accordance with the lipophilicity of the various compounds as estimated by the logarithm of their octanol-water partition coefficient (log P): log P = −4.11, −2.95 and 2.48 for PMEA, mono(POM)-PMEA and bis(POM)-PMEA, respectively (12). The results also illustrate that the ester is rapidly metabolized, with only 23% of the amount transported appearing as intact bis(POM)-PMEA (1.99%), while 33% was free PMEA (2.92%) and 44% was mono(POM)-PMEA (3.90%). This observation is consistent with the previously reported findings that no intact bis(POM)-PMEA is detected in plasma of mice (2) or monkeys (3) after oral administration of the prodrug. Since the formation of mono(POM)-PMEA was faster than expected based on chemical degradation data ($t_{1/2}$ = 9.8 hr in TM at 37°C and pH 7.4) (13), it was concluded that bis(POM)-PMEA was degraded by enzyme-catalyzed hydrolysis (probably esterase, EC 3.1.1.1.). As PMEA itself was not observed after chemical degradation, its appearance must also be enzyme-catalyzed (probably phosphodiesterase, EC 3.1.4.1.). The role of esterase and phosphodiesterase is suggested based on the reported enzyme-catalyzed degradation of the bis(POM)-ester of the nucleotide analogue 2′-deoxy-5-fluorouridine 5′- monophosphate [bis(POM)-FdUMP] to its mono(POM)-ester and further to free FdUMP (14).

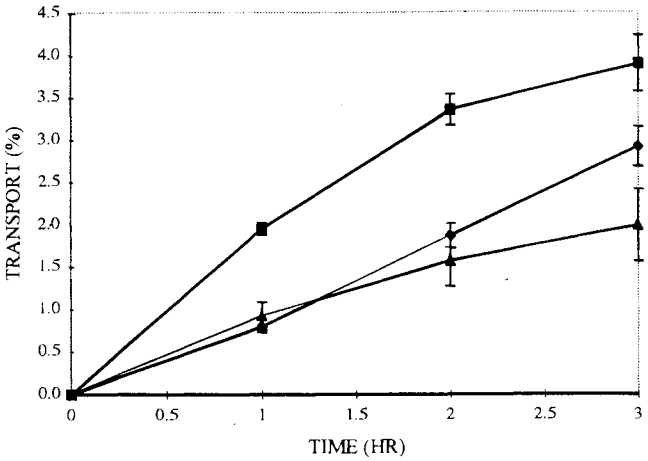

Fig. 1. Time course of transport of bis(POM)-PMEA across Caco-2 monolayers represented as cumulative basolateral percentages of PMEA (◆), mono(POM)-PMEA (■) and bis(POM)-PMEA (▲) after addition of 100 μM bis(POM)-PMEA to the apical side of the monolayers. Inserts were placed in wells containing fresh TM every hour. Values shown represent the average cumulative percentages ± SD of four independent determinations.

Uptake studies of bis(POM)-PMEA in Caco-2 cells (Fig. 2) further confirmed the rapid hydrolysis of the prodrug in the intestinal mucosal cells. No bis-ester was detected intracellularly after a 3 hr incubation period, irrespective of the initial concentration (25–200 μM) (data not shown).

The role of esterases in the generation of mono(POM)-PMEA from the bis-ester was confirmed by incubation of bis(POM)-PMEA with purified carboxylesterase (K_M = 87 μM and V_{max} = 9.5 μM/min). Incubation of bis(POM)-PMEA with Caco-2 homogenate or homogenized scraped pig small intestinal mucosa produced similar degradation profiles, i.e. rapid hydrolysis of bis(POM)-PMEA to its mono-ester and slow formation of PMEA (data not shown). Initial degradation rates of bis(POM)-PMEA (10 μM) in Caco-2 homogenate and scraped pig small intestinal homogenate were 1.10 ± 0.03 and 0.63 ±

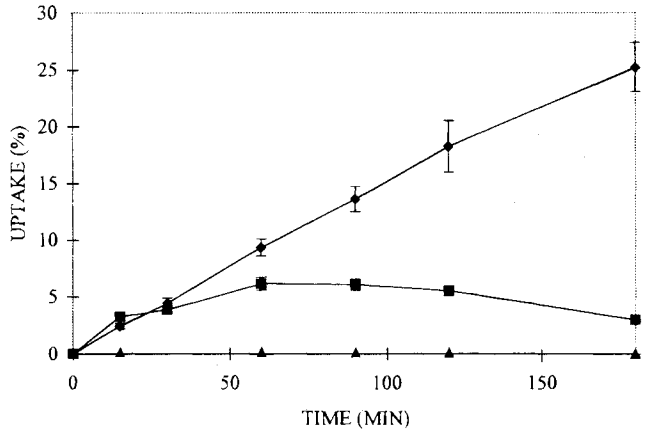

Fig. 2. Time course for the uptake of bis(POM)-PMEA (100 μM) into Caco-2 monolayers represented by the intracellular presence of bis(POM)-PMEA (▲) and of the metabolites PMEA (◆) and mono(POM)-PMEA (■). Uptake is expressed as percentage (± SD) of the initial amount of bis(POM)-PMEA added to the apical side (n = 3).

0.03 nmol/min/mg protein respectively. Mono(POM)-PMEA was found inside the Caco-2 cells after an incubation of 3 hr for initial concentrations of at least 100 μM bis(POM)-PMEA (Fig. 2) and even at lower concentrations (50 μM) at earlier time points (data not shown). This indicates that, at high initial concentration of bis(POM)-PMEA, there may have been saturation of the phosphodiesterases thought to be responsible for the conversion of the mono(POM)-ester into free PMEA (14). Similar conclusions could be drawn from the experiment where the concentration-dependent transport of the prodrug was evaluated (Fig. 3). The fact that bis(POM)-PMEA appeared at the basolateral side of the monolayers after 3 hr (albeit at a low concentration), and that no bis(POM)-PMEA was observed intracellularly after 3 hr, suggests that intact bis(POM)-PMEA that appeared at the basolateral side had crossed the monolayers by the paracellular pathway. This transport of intact bis(POM)-PMEA was slightly underestimated due to chemical degradation

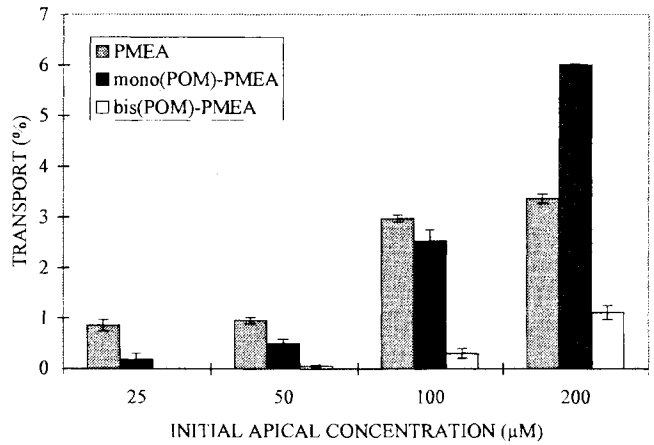

Fig. 3. Effect of the initial apical bis(POM)-PMEA concentration on transport across Caco-2 monolayers. Transport was measured for 3 hr and is expressed as percentage of the initial amount of bis(POM)-PMEA added to the apical side. Values shown represent the percentages of PMEA, mono(POM)-PMEA and bis(POM)-PMEA ± SD of three independent determinations.

during and after transport. Total paracellular transport may have been enhanced at high initial concentrations of bis(POM)-PMEA due to toxic effects of the ester on the monolayers at high concentrations. TEER values were indeed affected by the presence of bis(POM)-PMEA, i.e. TEER values decreased to 65 ± 7% of the initial value after a 3 hr incubation of the monolayers with 200 μM bis(POM)-PMEA as compared to a decrease to 80 ± 4 % of the initial value for control monolayers. A possible enhanced paracellular transport at high initial ester concentration as well as the saturation of enzymes involved in the hydrolysis of the prodrug are suggested for the observed concentration dependency of total transport. The amount of intact bis(POM)-PMEA transport was relatively low and of the same order of magnitude as transport of sodium fluorescein (paracellular leakage marker), determined afterwards (0.66 ± 0.17%/hr), which is consistent with the hypothesis that intact bis(POM)-PMEA appeared at the basolateral side by the paracellular pathway.

No effect of metabolic inhibitors [sodium azide (1 mM) and 2-deoxy-glucose (50 mM)] on transport of bis(POM)-PMEA was observed (data not shown), which suggests that no active transport mechanism is involved; surprisingly, transport could be completely inhibited by decreasing the temperature to 4°C. Influence of temperature on physicochemical properties of the ester and on membrane fluidity are possible explanations for these observations. An additional explanation for the temperature-induced reduction in transport may be the lower intracellular levels of total PMEA (PMEA, mono(POM)-PMEA and bis(POM)-PMEA) at 4°C due to a reduction of intracellular trapping of PMEA and mono(POM)-PMEA, which is caused by inhibition of enzyme-catalyzed degradation of bis(POM)-PMEA at lower temperature. A possible reduction of exocytosis may also contribute to a reduced efflux of PMEA and mono(POM)-PMEA to the basolateral compartment at 4°C over a 3 hr incubation period. Intracellular trapping of PMEA and mono(POM)-PMEA was confirmed by uptake experiments with bis(POM)-PMEA. Estimating a cell height of 30 μm (10), intracellular levels of PMEA amounted to approximately 1.2 mM for an initial concentration of 50 μM added to the apical side of the monolayers. The intracellular accumulation of PMEA has the advantage that the intestinal mucosa may serve as reservoir for PMEA, resulting in slow release of the drug to the systemic circulation, leading towards a prolonged effect. Fig. 4 shows that, after loading of the monolayers with 100 μM bis(POM)-PMEA for 1 hr, apical efflux of PMEA and mono(POM)-PMEA over a 3 hr incubation period amounted to 20.7 ± 1.6% and 1.8 ± 0.1% of the amount loaded, respectively. Fig. 4 also illustrates that the efflux of PMEA was 6-fold higher in the apical direction than in basolateral direction. Preliminary experiments with bis(POM)-PMEA (25 μM) had shown polarity in transport of total PMEA; the directional secretion of PMEA and the concomitant metabolism of bis(POM)-PMEA preclude however to draw any conclusions concerning the transport mechanism of bis(POM)-PMEA. The mechanisms involved in these observations are presently under investigation.

We conclude that the use of the bis(POM)-prodrug significantly enhances the intestinal permeability of PMEA, but that relatively low chemical and enzymatic stability are limiting its transport. High intracellular concentrations of free PMEA are observed in the intestinal cells due to trapping of the negatively

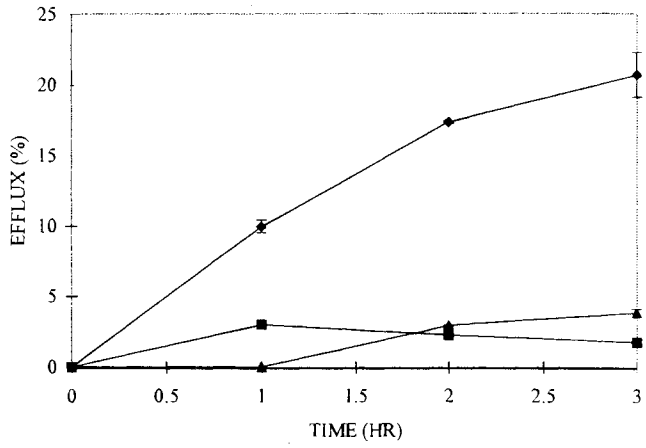

Fig. 4. Time course of the release of PMEA and mono(POM)-PMEA from Caco-2 monolayers grown on inserts as a function of time. Caco-2 mololayers were first "loaded" with a 100 μM bis(POM)-PMEA for 1 hr. Efflux towards the apical and basolateral compartment is expressed as the percentages PMEA and mono(POM)-PMEA (± SD; (n = 3)) relative to the total intracellular amount immediately after loading (t = 0 hr), [◆ = apical PMEA, ▲ = basolateral PMEA, and ■ = apical mono(POM)-PMEA].

charged PMEA, and may result in slow release of the drug into the systemic circulation.

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