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Modulation of Uptake of Organic Cationic Drugs in Cultured Human Colon Adenocarcinoma Caco-2 Cells by an Ecto-Alkaline Phosphatase Activity

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Abstract Alkaline phosphatase (ALP) refers to a group of nonspecific phosphomonoesterases located primarily in cell plasma membrane. It has been described in different cell lines that ecto-ALP is directly or indirectly involved in the modulation of organic cation transport. We aimed to investigate, in Caco-2 cells, a putative modulation of 1-methyl-4-phenylpyridinium (MPP^+) apical uptake by an ecto-ALP activity. Ecto-ALP activity and ^3H-MPP^+ uptake were evaluated in intact Caco-2 cells (human colon adenocarcinoma cell line), in the absence and presence of a series of drugs. The activity of membrane-bound ecto-ALP expressed on the apical surface of Caco-2 cells was studied at physiological pH using *p*-nitrophenylphosphate as substrate. The results showed that Caco-2 cells express ALP activity, characterized by an ecto-oriented active site functional at physiological pH. Genistein (250 μM), 3-isobutyl-1-methylxanthine (1 mM), verapamil (100 μM), and ascorbic acid (1 mM) significantly increased ecto-ALP activity and decreased ^3H-MPP^+ apical transport in this cell line. Orthovanadate (100 μM) showed no effect on ^3H-MPP^+ transport and on ecto-ALP activity. On the other hand, okadaic acid (310 nM) and all *trans*-retinoic acid (1 μM) significantly increased ^3H-MPP^+ uptake and inhibited ecto-ALP activity. There is a negative correlation between the effect of drugs upon ecto-ALP activity and ^3H-MPP^+ apical transport ($r = -0.9$; $P = 0.0014$). We suggest that apical uptake of organic cations in Caco-2 cells is affected by phosphorylation/dephosphorylation mechanisms, and that ecto-ALP activity may be involved in this process. J. Cell. Biochem. 87: 408–416, 2002.

Key words: Caco-2 cells; 1-methyl-4-phenylpyridinium (MPP^+); uptake; ecto-alkaline phosphatase

The primary function of the intestinal epithelium is to absorb molecules that are produced from digestion of food. In addition, this epithelium constitutes the major route for drug entry and is an important site of secretion of many substances.

Because biological membranes prevent transmembrane diffusion of the majority of organic molecules that bear net charges and many of the organic cations are polar and positively charged at physiological pH, membrane-bound transport systems are generally involved in the absorption, distribution, and elimination of these compounds. So, intestinal transporters may play a critical role in limiting and/or promoting the absorption or secretion of organic cations.

Various xeno- and endobiotics belong to the class of organic cations. Drugs from a wide array of clinical classes (representing the majority of drugs for therapeutic uses)—including antihistamines, skeletal muscle relaxants, antiarrhythmics, and β-adrenoceptor blocking agents—are organic cations. In addition, several endogenous bioactive amines—such as catecholamines, 5-hydroxytryptamine and histamine;

Abbreviations used: AA, ascorbic acid; ALP, alkaline phosphatase; ASF, amphiphilic solute facilitator; EMT, extraneuronal monoamine transporter; Hek293 cells, human embryonic kidney cells; IBMX, 3-isobutyl-1-methylxanthine; MPP^+, 1-methyl-4-phenylpyridinium; OA, okadaic acid; OCT1, organic cation transporter 1; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenylphosphate; RA, all *trans*-retinoic acid; RBE4 cells, rat brain endothelial cells.

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and some vitamins, such as thiamine and riboflavin—are also organic cations. Many organic cations are incompletely absorbed after oral administration and may also be actively secreted in the intestine [Hardman et al., 1996].

Current knowledge concerning intestinal transport of organic cations indicates that multiple mechanisms seem to be involved in the transport of these compounds at the brush-border membrane level. These transport systems include: (1) a sodium-independent thiamine transporter, involved in absorption of thiamine; (2) a sodium-independent, potential-dependent transporter that mediates absorption of tyramine, tryptamine, and disopyramide; (3) a sodium- and potential-independent transporter that mediates absorption of choline (and probably also organic cation secretion, as it may perform organic cation exchange); (4) a proton/cation antiporter, involved in guanidine secretion; (5) an ATP-dependent, polyspecific transporter (P-glycoprotein) that mediates secretion of hydrophobic organic cations; and (6) a polyamine transporter, involved in the absorption of spermine, spermidine, and putrescine [Koepsell, 1998; Zhang et al., 1998].

It was recently described by our group that the model organic cation MPP^+ is efficiently transported by Caco-2 cells in the apical-to-basolateral (absorptive) direction [Martel et al., 2000] and that absorption of MPP^+ seems to occur through two distinct Na^+-independent transporters belonging to the amphiphilic solute facilitator (ASF) family: extraneuronal monoamine transporter (hEMT; also known as OCT3 [Kekuda et al., 1998]) and organic cation transporter 1 (hOCT1) [Martel et al., 2001a]. These organic cation transporters have been shown to be regulated by phosphorylation/dephosphorylation mechanisms [Hohage et al., 1998; Mehrens et al., 2000, 2001b].

Alkaline phosphatase (ALP) refers to a group of nonspecific phosphomonoesterases located primarily in cell plasma membrane. ALP anchored to the plasma membrane may function as an ecto-phosphatase, dephosphorylating extracellular substrates [Anagnostou et al., 1996; Whyte et al., 1988; Ohkubo et al., 2000; Scheibe et al., 2000] or cell-surface proteins [Chan and Stinson, 1986]. Ecto-ALP was shown to be directly or indirectly involved in the modulation of organic cation transport in different cell types (rat hepatocytes, Hek293 and RBE4 cells) [Martel et al., 1996a, 1998a,b, 2001b; Calhau et al., 2000a, 2002b]. Thus, we considered the hypothesis of an involvement of ecto-ALP on MPP^+ intestinal uptake modulation. For this purpose, we used Caco-2 cells, an enterocyte-like cell line derived from a human colonic adenocarcinoma, as an intestinal model [Hidalgo et al., 1989; Artursson, 1991; Artursson and Karlsson, 1991; Lennernas, 1997; Yee, 1997]. Ecto-ALP activity and MPP^+ uptake were evaluated in intact Caco-2 cells.

MATERIALS AND METHODS

Materials

^3H-MPP^+ (*N*-[methyl-^3H]-4-phenylpyridinium acetate; specific activity 82 Ci/mmol) (New England Nuclear Chemicals, Dreieich, Germany); MPP^+ (1-methyl-4-phenylpyridinium iodide) (Research Biochemicals International, Natick, MA); Triton X-100 (Merck, Darmstadt, Germany); ascorbic acid (AA), genistein, 3-isobutyl-1-methylxanthine (IBMX), *p*-nitrophenol (pNP), *p*-nitrophenylphosphate (pNPP), okadaic acid (OA), all *trans*-retinoic acid (RA), sodium orthovanadate, Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), verapamil hydrochloride (all from Sigma, St. Louis, MO). IBMX, genistein, OA, and RA were dissolved in DMSO. The final concentration of these solvents in the buffer was 1%. Control for these drugs were run in the presence of the solvent.

Cell and Culture Conditions

The Caco-2 cells, a human colon adenocarcinoma cell line with colon epithelial origin was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and was used between passage number 27–43. Caco-2 cells (ATCC 37-HTB) were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO) supplemented with 20% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; ⌀ 60 mm; Corning Costar, Corning, NY). For the experiments, the Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ⌀ 16 mm;

Corning Costar). For 24 h before the experiment, the cell medium was free of fetal calf serum. Uptake and enzymatic studies were generally performed 9–11 days, after the cells formed a monolayer. Each square centimeter contained about 200–300 μg cell protein.

Transport Studies

The transport experiments were performed in Hanks' medium with the following composition (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 1.0 $MgCl_2$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 0.15 Tris-HCl, and 1.0 sodium butyrate, pH 7.4. Initially, the growth medium was aspirated and the cells were washed with Hanks' medium at 37°C; then the cell monolayers were preincubated for 20 or 60 min in Hanks' medium at 37°C.

Uptake studies were performed in cells cultured on plastic supports, ^{3}H-MPP$^+$ being applied from the apical cell membrane. Incubation was initiated by the addition of 0.3 ml medium at 37°C containing 200 nM ^{3}H-MPP$^+$, and stopped after 5 min by placing the cells on ice and rinsing them with 0.5 ml ice-cold Hanks' medium. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4), and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Assay of Phosphohydrolase Activity of ALP on Intact Caco-2 Cells

ALP phosphohydrolase activity was assessed by measuring *p*-nitrophenol (pNP) release from *p*-nitrophenylposphate (pNPP) at physiological pH by absorbance spectrophotometry at 410 nm.

The growth medium was removed, and cell monolayers were washed once with Hanks' medium at 37°C; thereafter, the cell monolayers were preincubated for 20 or 60 min in Hanks' medium at 37°C. The enzyme reaction was carried out in 0.3 ml of Hanks' medium containing 2.86 mM of pNPP. After incubation, the supernatant solution was transferred into hemolyse tubes containing 0.02 M NaOH at 4°C to stop the reaction, and released pNP was measured.

The results are expressed as milliunits (mU) of ecto-pNPPase activity per milligram of cell protein. One unit (U) is the enzyme activity which hydrolyses 1 μmol pNPP/min at pH 7.4 and at 37°C.

Effect of drugs. Drugs to be tested were present during both the preincubation and incubation periods.

Protein Determination

The protein content of cell monolayers was determined as described by Bradford [1976], with human serum albumin as standard.

Calculations and Statistics

Values are expressed as the arithmetic mean ± SEM and geometric means are given with 95% confidence limits. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA test) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

RESULTS

Ecto-Alkaline Phosphatase Activity in Caco-2 Cells

In preliminary experiments, the time course of pNP formation was determined, using a substrate (pNPP) concentration of 2.86 mM. Formation of pNP increased linearly with time for at least 80 min (results not shown). Thus, in all subsequent experiments in which ALP activity was determined, cells were incubated for 60 min with substrate.

In experiments aimed at determining the kinetic parameters of ALP activity, intact cells were incubated with increasing concentrations of substrate (10 μM–20 mM) (Fig. 1). ALP activity was found to follow nonlinear kinetics and to be nearly saturated at 2.86 mM. The kinetic parameters K_m and V_{max} values, obtained by nonlinear analysis of the saturation curves, were 762.5 ± 453 μM and 1.35 ± 0.1 nmol mg^{-1} protein min^{-1} ($n = 4$), respectively.

Thus, in subsequent experiments performed to evaluate the effect of drugs on ecto-ALP activity, intact cells were incubated with 2.86 mM pNPP.

Effect of Drugs on ^{3}H-MPP$^+$ Uptake and Ecto-ALP Activity in Caco-2 Cells

In previous experiments, it was demonstrated that uptake of ^{3}H-MPP$^+$ in Caco-2 cells was linear with time for up to 5 min of incubation [Martel et al., 2000, 2001a]. So, in the

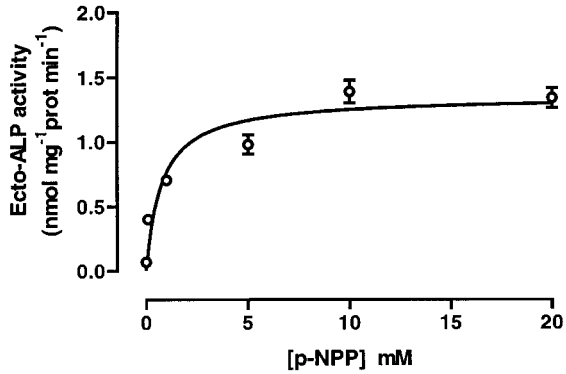

Fig. 1. Formation of pNP in intact Caco-2 cells. Cells were incubated for 60 min at 37°C with increasing (10 μM–20 mM) concentrations of the substrate (pNPP) at pH 7.4. Symbols are means of four experiments in triplicate and vertical lines show SEM.

present study, cells were incubated with ^{3}H-MPP^+ for 5 min, to determine initial rates of uptake.

As can be seen in Figure 2 ascorbic acid (100 μM and 1 mM), verapamil (100 μM), IBMX (1 mM), and genistein (250 μM) were found to significantly inhibit ^{3}H-MPP^+ uptake and to increase ecto-ALP activity in Caco-2 cells. On the other hand, orthovanadate (100 μM) showed no effect on transport of ^{3}H-MPP^+ or on ecto-ALP activity (Fig. 3), and all *trans*-retinoic acid (1 μM) and okadaic acid were able to significantly increase ^{3}H-MPP^+ uptake and to inhibit ecto-ALP activity in these cells (Fig. 4).

Finally, Figure 5 shows the negative correlation between the effect of these drugs on ecto-ALP activity and ^{3}H-MPP^+ uptake into Caco-2 cells ($P = 0.0014$; $r = -0.9$, $n = 8$).

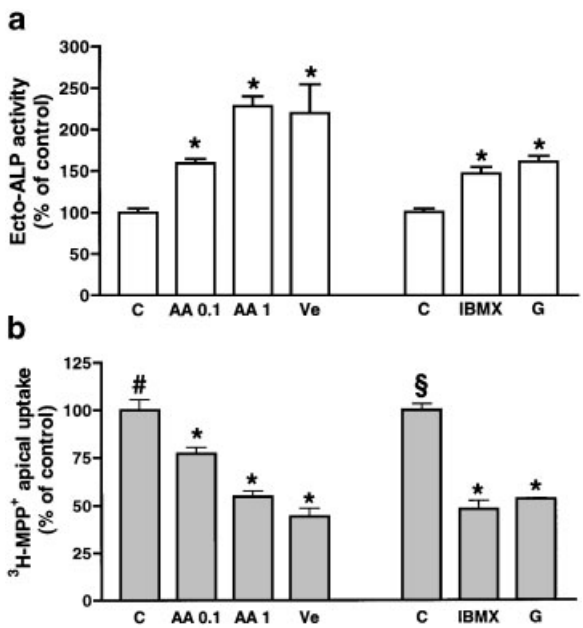

Fig. 2. Effect of ascorbic acid (AA 0.1; 100 μM or AA 1; 1 mM), verapamil (Ve; 100 μM), IBMX (1mM) and genistein (G; 250 μM) on **(a)** ecto-ALP activity and **(b)** ^{3}H-MPP^+ uptake into Caco-2 cells. Cells were preincubated for 20 min and incubated at 37°C with 2.86 mM pNPP for 60 min or with 200 nM ^{3}H-MPP^+ for 5 min, in the absence or presence of these drugs. Control values were 3.5 ± 0.2 (#, $n = 8$) and 2.6 ± 0.4 (§, $n = 7$) pmol mg protein^{-1}. Each value represents the mean ± SEM ($n = 3$–8). *significantly different from control ($P < 0.05$).

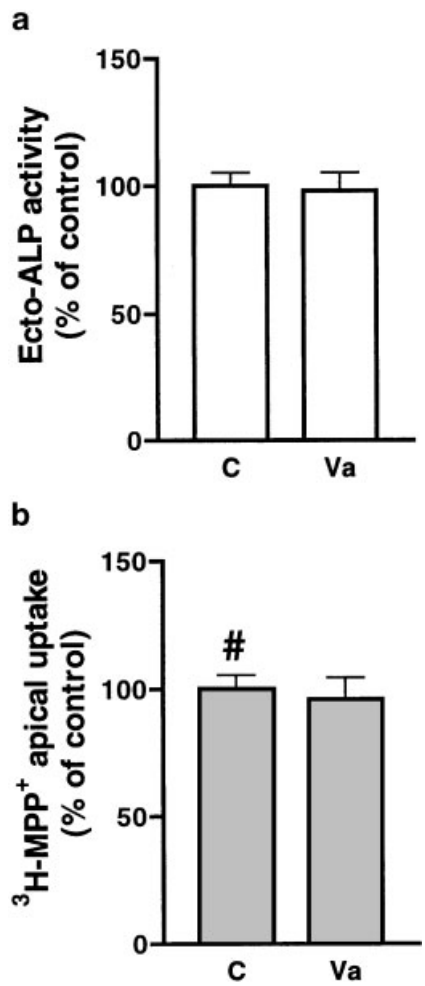

Fig. 3. Effect of orthovanadate (Va;100 μM) on **(a)** ecto-ALP activity and **(b)** ^{3}H-MPP^+ uptake into Caco-2 cells. First, cells were preincubated for 20 min in the absence or presence of this drug. Secondly, cells were incubated at 37°C with 2.86 mM pNPP for 60 min or with 200 nM ^{3}H-MPP^+ for 5 min, in the absence or presence of this drug. Control value was 3.5 ± 0.2 (#, $n = 8$) pmol mg protein^{-1}. Each value represents the mean ± SEM ($n = 4$–12). *Significantly different from control ($P < 0.05$).

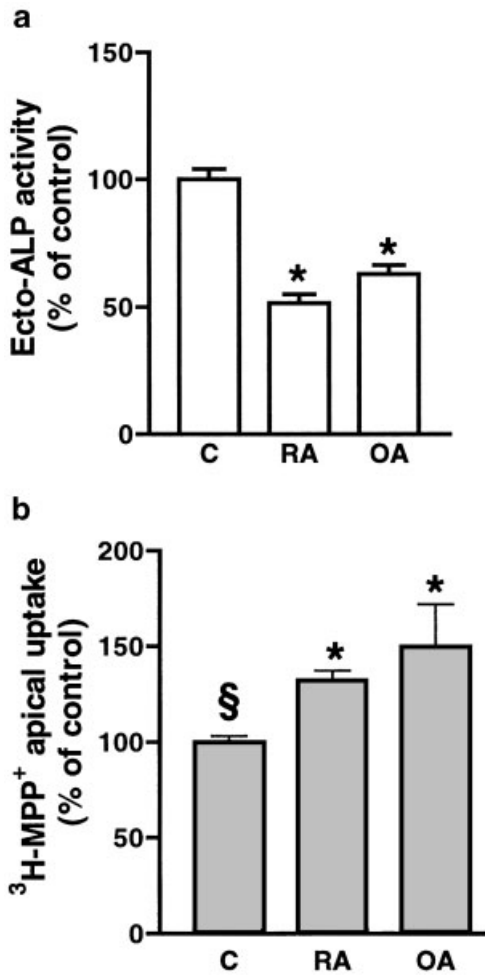

Fig. 4. Effect of all *trans*-retinoic acid (RA; 1 μM) and okadaic acid (OA; 310 nM) on **(a)** ecto-ALP activity and **(b)** ^{3}H-MPP$^+$ uptake into Caco-2 cells. First, cells were preincubated for 20 min (RA) or 60 min (OA) in the absence or presence of these drugs. Secondly, cells were incubated at 37°C with 2.86 mM pNPP for 60 min or with 200 nM ^{3}H-MPP$^+$ for 5 min, in the absence or presence of these drugs. Control value was 2.6 ± 0.4 (§, $n = 7$) pmol mg protein^{-1}. Each value represents the mean ± SEM ($n = 4–12$). *Significantly different from control ($P < 0.05$).

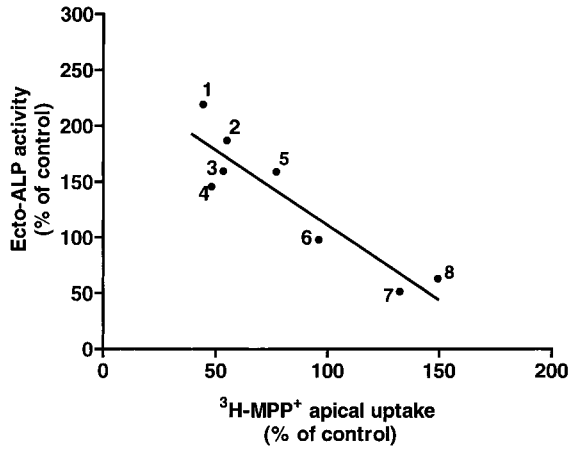

Fig. 5. Correlation between the effect of compounds in relation to ^{3}H-MPP$^+$ uptake and ecto-ALP activity in Caco-2 cells. Drugs used were: (1) verapamil 100 μM; (2) ascorbic acid 1 mM; (3) genistein 250 μM; (4) IBMX 1 mM; (5) ascorbic acid 100 μM; (6) vanadate 100 μM; (7) okadaic acid 310 nM; and (8) retinoic acid 1 μM. Each value represents the mean ± SEM. There is a significant negative correlation ($P = 0.0014$; $r = -0.9$, $n = 8$).

DISCUSSION

Most research effort concerning phosphorylation/dephosphorylation mechanisms has focused on understanding the biochemical regulation and physiological importance of intracellular phosphorylation pathways. However, during the past few years, numerous reports described the presence of ecto-kinases on the outer cell surface of a wide variety of cells. These ecto-enzymes were shown to phosphorylate both extracellular (soluble) substrates and cell-surface proteins [Redegeld et al., 1999]. Thus, they might play an important role in the regulation of ligand binding, signal transduction, and cell-to-cell interactions. It has been suggested that both protein kinases and phosphatases are required for reversible control of extracellular phosphorylation processes, acting in a manner similar to that in which they control cytoplasmatic phosphorylation/dephosphorylation events. However, although activation by protein kinases has been studied in some detail, the dephosphorylation step has received little attention.

ALP belongs to a class of proteins that are anchored to plasma membrane via covalent linkage to glycosylphosphatidylinositol. The physiological function of ALP as an ecto-phosphatase remains controversial, mainly because early studies with purified ALP describe this enzyme as exhibiting a non-physiological alkaline pH optimum [McComb et al., 1979]. However, a pH optimum of 7–8 was determined for purified and plasma membrane-bound human liver ALP [Chan and Stinson, 1986], as well as for purified bovine hepatic and calf intestinal ALP [Swarup et al., 1981].

It was recently demonstrated that ALP concentration in different tissues is positively correlated with the surface of apical membrane per unit volume of the tissue, pointing to a possible association between ALP and transport systems [Calhau et al., 1999, 2000b]. Moreover,

evidence has been obtained for an involvement of ALP in the modulation of organic cation transmembrane transporters. These include P-glycoprotein in liver [Martel et al., 1996a, 1998a,b; Calhau et al., 2000a] and organic cation transporters at the renal, hepatic and blood–brain barrier levels [Calhau et al., 2000a, 2002b; Martel et al., 2001b].

The present experiments were performed using an established epithelial cell line derived from a human colon adenocarcinoma (Caco-2 cells). Caco-2 cells have been proved as an appropriate model system for intestinal epithelial permeability studies [Delie and Rubas, 1997]. These advantages include a simple epithelial monolayer structure, free of mucus or underlying submucosal and muscle tissues, ease of maintenance of tissue viability as well as its human origin [Delie and Rubas, 1997]. The purpose of the present investigation was twofold: (1) to characterize the ecto-ALP activity in this intestinal cell line; (2) to determine whether organic cation transport in Caco-2 cells is modulated by ecto-ALP modulators.

This study demonstrates that cultured Caco-2 cells possess a phosphatase activity which is able to hydrolize pNPP in the external medium under physiological pH conditions. The experimental conditions used in this study allowed us to characterize this activity as an ecto-phosphatase activity, mainly because: (1) the substrate was present in the external medium; (2) the substrate used (pNPP) does not penetrate into plasma membranes [DePierre and Karnovsky, 1974]; (3) a pNPPase activity was not detected in the external medium (data not shown), ruling out the possible enzymatic action of soluble contaminants or the leakage of intracellular enzymes; (4) substrate hydrolysis was linear with time, demonstrating that cell breakage did not occur during the incubation. Our results are, thus, in agreement with the involvement of ecto-ALP as an ecto-protein phosphatase [Chan and Stinson, 1986; Anagnostou et al., 1996; Scheibe et al., 2000] and/or as an ecto-phosphohydrolase that hydrolyzes a variety of organic phosphates (e.g., ATP, 3′-AMP, ribose-5-phosphate) [Lemmens et al., 1996; Ohkubo et al., 2000; Vollmayer et al., 2001].

In the transport experiments, we used radiolabeled MPP^+ as the organic cation probe, in part because this compound has been extensively employed to study the handling of organic cations by other organs or cells such as the kidney [Sokol et al., 1987; Lazaruk and Wright, 1990], liver [Martel et al., 1996a,b,c, 1998a,b, 1998b, 1999], catecholaminergic neurons [Javitch et al., 1985; Melamed et al., 1985], glial cells [Streich et al., 1996; Russ et al., 1996] and Caki-1 cells [Russ et al., 1992], and because MPP^+ is not subjected to metabolism in vivo [Sayre, 1989].

We have previously demonstrated that MPP^+ is efficiently transported by Caco-2 cells in the apical-to-basolateral direction (absorption). These functional studies indicated that absorption of MPP^+ occurs through two distinct Na^+-independent transporters belonging to the ASF family: hEMT and hOCT1 [Martel et al., 2000, 2001a]. More recently, we verified that Caco-2 cells express mRNAs for hEMT and hOCT1 [Martel et al., 2001a]. OCT1 is most likely active in the phosphorylated state [Hohage et al., 1998; Mehrens et al., 2000] and EMT in the dephosphorylated state [Martel et al., 2001b].

In this study, we used several drugs known to be ALP modulators, namely verapamil [Sjoden et al., 1990; Calhau et al., 2000a], genistein [Basson et al., 1998; Calhau et al., 2000a], ascorbic acid [Morton et al., 2001; Yamagiwa et al., 2001], and RA [El Hafny et al., 1996; Calhau et al., 2002a]. On the other hand, we also used IBMX, known to be an organic cation transporter modulator [Said et al., 1999; Martel et al., 2001b]. Compounds such as verapamil and genistein have been also described as organic cation transport modulators [Martel et al., 1996a, 2000, 2001b; Hooijberg et al., 1997]. Orthovanadate [Seargeant and Stinson, 1979; Charbonneau and Tonks, 1992; Calhau et al., 2000a; Calhau et al., 2002a] and okadaic acid [Charbonneau and Tonks, 1992] were used as protein phosphatase inhibitors at tyrosine and serine/threonine residues, respectively.

Acute treatment of Caco-2 cells with RA, one of the biologically active forms of vitamin A, increased the amount of ^3H-MPP^+ transported, and decreased ecto-ALP activity. The same results were observed with okadaic acid, the main toxin produced by dinoflagellates, which cause diarrheic shellfish poisoning. On the other hand, drugs such as verapamil and IBMX inhibited ^3H-MPP^+ uptake. The water-soluble vitamin, ascorbic acid, and the bioflavonoid genistein markedly inhibited this transport. Thus, all these drugs and nutrients may interfere with the transport of organic cationic drugs, at the intestinal mucosa. Additionally, all these

drugs were able to modulate ecto-ALP: drugs that inhibited MPP^+ uptake were ecto-ALP activators (verapamil, IBMX, ascorbic acid, and genistein); okadaic acid and RA had a stimulatory effect on MPP^+ uptake and an inhibitory effect on ecto-ALP activity.

Bioflavonoids have multiple effects, including effects on phosphorylation/dephosphorylation mechanisms. The results presented here show that genistein produced an increase in ecto-ALP activity and a decrease in ^{3}H-MPP^+ transport. These results on ALP activity agree with those of Basson et al. [1998], Calhau et al. [2000a], and Sugimoto and Yamaguchi [2000]. The inhibitory effect of genistein and IBMX on ^{3}H-MPP^+ transport is in agreement with our results obtained with 293_{hEMT} cells [Martel et al., 2001b].

It is possible that the reported effects of drugs on ^{3}H-MPP^+ transport may result not only from their effect on ALP, and thus on phosphorylation/dephosphorylation pathways, but also from a direct effect of the drugs on the transporter itself. As a matter of fact, we have no direct evidence to support this hypothesis at present. However, it seems highly improbable that so many different compounds, belonging to different chemical classes, would all affect the ^{3}H-MPP^+ transporter.

Thus, the results are consistent with a hypothesis that uptake of MPP^+ is regulated by phosphorylation/dephosphorylation mechanisms, and with a direct/indirect involvement of ecto-ALP activity in this process. A physiological role of ALP in the modulation of organic cation transport in the intestinal epithelia is thus suggested.

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