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Uptake of ^{3}H-1-methyl-4-phenylpyridinium (^{3}H-MPP$^+$) by human intestinal Caco-2 cells is regulated by phosphorylation/dephosphorylation mechanisms

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Abstract

Several transmembrane transporters of organic compounds are regulated by phosphorylation/dephosphorylation mechanisms. The aim of this study was to investigate the possible regulation of the intestinal uptake of organic cations by these mechanisms. The intestinal apical uptake of 1-methyl-4-phenylpyridinium (MPP$^+$) was studied by incubating Caco-2 cells at 37° for 5 min with 200 nM ^{3}H-MPP$^+$. Uptake of ^{3}H-MPP$^+$ by Caco-2 cells was not affected by activators of protein kinase G, and was not affected or slightly reduced (by 15–20%) by activators of protein kinase A or protein kinase C. Uptake of ^{3}H-MPP$^+$ by Caco-2 cells was reduced in a concentration-dependent manner by non-selective phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine (IBMX), caffeine, teophylline). The IC_{50} of IBMX was found to be 119 μM (102–138; $n = 9$). Uptake of ^{3}H-MPP$^+$ by Caco-2 cells was not affected by inhibition of protein tyrosine kinase, but it was concentration-dependently reduced in the presence of inhibitors of mitogen-activated protein kinase. Uptake of ^{3}H-MPP$^+$ by Caco-2 cells was strongly reduced by Ca^{2+}/calmodulin-mediated pathway inhibitors, but it was not dependent on extracellular Ca^{2+}. Our results suggest that the intestinal apical uptake of MPP$^+$ is regulated by phosphorylation/dephosphorylation mechanisms, being most probably active in the dephosphorylated state. Moreover, uptake of ^{3}H-MPP$^+$ by Caco-2 cells and by the extraneuronal monoamine transporter (EMT) are regulated in a very similar manner, suggesting an important participation of EMT in the intestinal uptake of this compound.

Keywords: Apical uptake; Organic cations; Protein kinase C; Protein kinase A; Ca^{2+}/calmodulin-mediated pathways

1. Introduction

The primary function of the intestinal epithelium is to absorb small molecules that are produced from digestion of food. Additionally, the intestinal epithelium constitutes one of the major routes of entry of drugs into the blood circulation. Various nutrients (e.g. the vitamin thiamine) and drugs are organic cations. Indeed, the majority of therapeutically used drugs, belonging to a wide array of clinical classes (e.g. skeletal muscle relaxants, antiarrythmics, antihistamines), are organic cations. So, intestinal transporters for organic cations play an important role in promoting and/or limiting the absorption of these compounds.

Several transmembrane transporters of organic cations are known to be regulated by phosphorylation/dephosphorylation mechanisms. Those include P-glycoprotein (reviews by [1,2]), neuronal transporters for monoamines [3–5] and organic cation transporters at the renal, hepatic and intestinal level (e.g. [6–11]).

The characteristics of organic cation uptake at the intestinal level were recently described by our group [12,13]. Using Caco-2 cells, we verified that uptake of ^{3}H-MPP$^+$ at the apical level seems to involve two different transporters, belonging to the same family (the ASF family of transporters): the extraneuronal monoamine transporter (EMT) and the organic cation transporter 1 (OCT1). OCT1 and EMT

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Abbreviations: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; CaM, Ca^{2+}/calmodulin; IBMX, 3-isobutyl-1-methylxanthine; MAP kinase, mitogen-activated protein kinase; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}/diacylglycerol-dependent protein kinase; PTK, protein tyrosine kinase; PKG, cGMP-dependent protein kinase.

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were recently found to be regulated by phosphorylation/dephosphorylation mechanisms. However, distinct mechanisms seem to operate for these two transporters. OCT1 is regulated by PKA, PKC and PTK [14,15]. On the other hand, EMT does not appear to be regulated by PKA, PKC or PTK, but rather by CaM-mediated pathways [16,17].

So, the aim of this work was to investigate the possible regulation of the organic cation uptake at the intestinal level by phosphorylation/dephosphorylation mechanisms, and to compare regulation of this uptake with the regulation of EMT- and OCT1-mediated transport. For this purpose, a survey of the putative involvement of different intracellular regulatory pathways known to play a role in the regulation of other membrane transporters of organic compounds on the uptake of the organic cation ^{3}H-MPP$^+$ by Caco-2 cells was made.

2. Materials and methods

2.1. Cell culture

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC 37-HTB, Rockville, MD) and was used between passage number 30–41. The cells 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^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°), split 1:3, and subcultured in plastic culture dishes (21 cm^2; Ø 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; Ø 16 mm; Corning Costar). For 24 hr before the experiment, the cell medium was free of fetal bovine serum. Uptake studies were performed 2–4 days after the cells formed a monolayer, corresponding to 7–9 days after the initial seeding. Each square centimeter contained about 200–250 μg cell protein.

2.2. 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°; then the cell monolayers were pre-incubated for 60 min in Hanks' medium at 37°. Uptake was initiated by the addition of 0.3 mL medium at 37° containing 200 nM ^{3}H-MPP$^+$. After 5 min, incubation was stopped by placing the cells on ice and rinsing the cells 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° overnight. Radioactivity in the cells was measured by liquid scintillation counting.

2.2.1. Effect of drugs

Drugs to be tested were present during both the pre-incubation and incubation periods.

2.2.2. Effect of ionic composition of the external medium

To study the influence of external Ca^{2+} on the uptake of ^{3}H-MPP$^+$, cells were pre-incubated and incubated in medium with 1.2 mM $CaCl_2$ (control) or 0 mM $CaCl_2$.

2.3. Protein determination

The protein content of cell monolayers was determined as described by Bradford [18], with human serum albumin as standard.

2.4. Cell viability

Caco-2 cells were pre-incubated for 60 min at 37° and then incubated for further 5 min in the presence of 200 nM MPP$^+$. Subsequently, the cells were incubated at 37° for 2 min with Trypan Blue (0.16% w/v) prepared in Hanks' medium. Incubation was stopped by rinsing the cells with 0.5 mL ice-cold Hanks' medium, and the cells were examined using a microscope. When tested, compounds were present during the pre-incubation and incubation with MPP$^+$.

2.5. Calculations and statistics

For the analysis of the time course of ^{3}H-MPP$^+$ accumulation the parameters of Eq. (1) were fitted to the experimental data by a non-linear regression analysis, using a computer assisted method [19].

$$A(t) = \frac{k_{in}}{k_{out}}(1 - e^{-k_{out}t}) \quad (1)$$

where $A(t)$ represents the accumulation of ^{3}H-MPP$^+$ at time t, k_{in} and k_{out} are the rate constants for inward and outward transport, respectively, and t is the incubation time. A_{max} is defined as the accumulation at steady-state ($t \rightarrow \infty$). k_{in} is given in μL mg protein^{-1} min^{-1} and k_{out} in min^{-1}.

For the calculation of IC_{50}'s, the parameters of the Hill equation for multisite inhibition ([20], Eq. (VIII-9)) were fitted to the experimental data by a non-linear regression analysis, using a computer assisted method [19].

Arithmetic means are given with SEM and geometric means are given with 95% confidence limits. Statistical significance of the difference between various groups was evaluated by one-way 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 $P < 0.05$.

2.6. Materials

^{3}H-MPP$^+$ (*N*-[methyl-^{3}H]-4-phenylpyridinium acetate; specific activity 82 Ci mmol^{-1}) (New England Nuclear Chemicals); caffeine (BDH Laboratory Chemicals Ltd.); MPP$^+$ (Research Biochemicals International); Triton X-100 (Merck); 8-bromo cGMP, calmidazolium, chelerythrine chloride, dibutyryl cAMP sodium salt, forskolin, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), IBMX, KN-62 (1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine), PD 98059, PDBu (phorbol 12,13-dibutyrate), PDDc (phorbol 12,13-didecanoate), PMA (phorbol 12-myristate-13-acetate), poly-L-ornithine hydrobromide, Rp-cAMPS (Rp-adenosine 3′,5′-cyclic monophosphothioate) triethylamine, SB 203580, sodium nitroprussiate, staurosporine, teophylline, trifluoperazine dihydrochloride, Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), tyrphostin A1, tyrphostin A25 (Sigma).

When the drugs to be tested were dissolved in ethanol or DMSO, the final concentration of these solvents in the buffer was 1%. Controls for these drugs were run in the presence of the solvent.

3. Results

3.1. Time-course of ^{3}H-MPP$^+$ uptake by Caco-2 cells

In preliminary experiments, Caco-2 cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for various periods of time (Fig. 1). Analysis of the time course of accumulation revealed a k_{in} of 2.0 ± 0.3 μL mg protein^{-1} min^{-1}, a k_{out} of 0.06 ± 0.01 min^{-1} and an A_{max} of 6.7 ± 0.3 pmol mg protein^{-1} ($n = 36$). In other words, an amount of

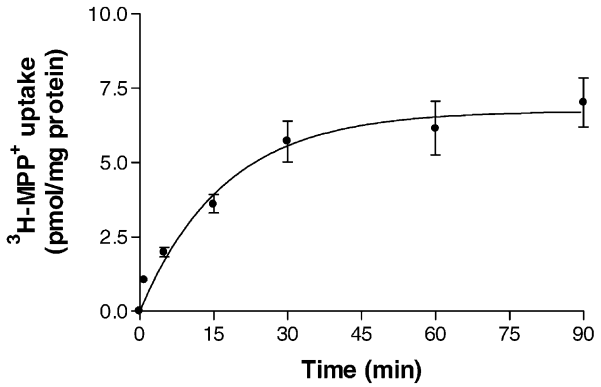

Fig. 1. Time-course of ^{3}H-MPP$^+$ uptake into Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$. Exponential functions were fitted to the experimental data. Shown are means ± SEM ($n = 6$).

Caco-2 cells corresponding to 1 mg of cell protein cleared 2 μL incubation medium of ^{3}H-MPP$^+$ per min, and simultaneously 6% of intracellular ^{3}H-MPP$^+$ left the cells per min. As shown in Fig. 1, uptake of ^{3}H-MPP$^+$ was linear with time for up to 5 min of incubation. These results are in agreement with previous experiments from this group [12,13]. So, in all the subsequent experiments, cells were incubated with ^{3}H-MPP$^+$ for 5 min, in order to determine initial rates of uptake.

3.2. Role of intracellular protein kinase-mediated pathways

3.2.1. PKA

Involvement of a PKA-mediated pathway in the regulation of ^{3}H-MPP$^+$ uptake by Caco-2 cells was tested by examining the effect of pre-treating the cells for 60 min with compounds that are known to increase intracellular cAMP levels (dibutyryl cAMP, forskolin and the non-selective PDE inhibitors caffeine, teophylline and IBMX) and thus activate PKA, on ^{3}H-MPP$^+$ uptake (Figs. 2 and 3).

Forskolin (up to 100 μM) had no effect on ^{3}H-MPP$^+$ uptake, but dibutyryl cAMP at the highest concentration used (2.5 mM) produced a 15% reduction in ^{3}H-MPP$^+$

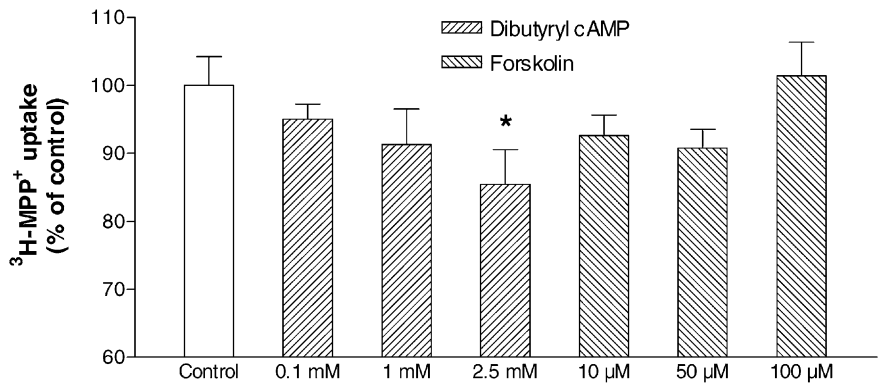

Fig. 2. Effect of PKA activators on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 9$) or presence of dibutyryl cAMP ($n = 7$) or forskolin ($n = 10$). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

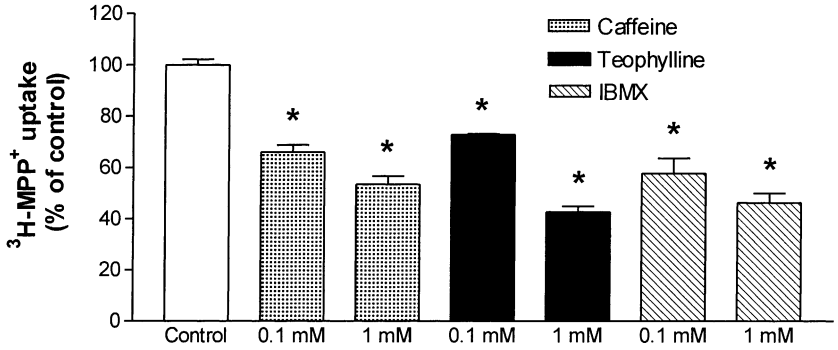

Fig. 3. Effect of PDE inhibitors on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 6$) or presence of caffeine ($n = 3$), teophylline ($n = 4$) or IBMX ($n = 8$). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

uptake (Fig. 2). Moreover, all the PDE inhibitors tested concentration-dependently inhibited ^{3}H-MPP$^+$ uptake (Fig. 3). For IBMX, its IC_{50} was determined and found to be 118.7 (102.0–138.2) μM ($r = 0.9999$) (Fig. 4).

3.2.2. PKG

The role of a PKG-mediated pathway in the regulation of ^{3}H-MPP$^+$ uptake by Caco-2 cells was tested by examining the effect of compounds known to increase intracellular cGMP levels (8-bromo cGMP and sodium nitroprussiate) [8] and thus activate PKG, on ^{3}H-MPP$^+$ uptake. As can be seen in Table 1 pre-treatment of the cells for 60 min with these compounds did not change ^{3}H-MPP$^+$ uptake.

3.2.3. PKC

The possible role of PKC in the regulation of ^{3}H-MPP$^+$ uptake by Caco-2 cells was tested by examining the effect of pre-treating the cells for 60 min with the PKC activators phorbol 12,13-dibutyrate (PDBu) and phorbol 12-myristate-13-acetate (PMA) [3,7,11]. Phorbol 12,13-didecanoate (PDDc) was used as a negative control for these drugs. As can be seen in Fig. 5, PMA did not affect ^{3}H-MPP$^+$ uptake, but PDBu (0.2 μM) caused a significant (±20%) inhibition in the uptake of this compound.

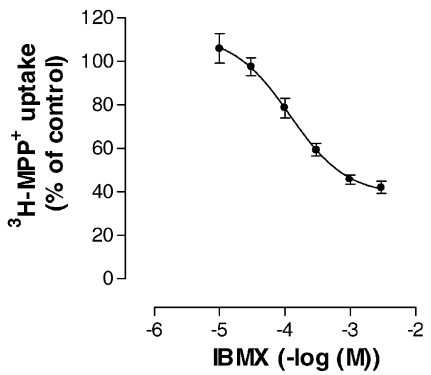

Fig. 4. Inhibitory effect of IBMX on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° for 5 min with 200 nM ^{3}H-MPP$^+$. Cells were pre-incubated for 60 min with various concentrations of the compound. Shown are arithmetic means ± SEM of the uptake in the presence of IBMX relative to the control ($n = 9$).

3.2.4. Relation between PKA- and PKC-mediated pathways

In these experiments, the effect of a simultaneous stimulation of PKA and PKC on ^{3}H-MPP$^+$ uptake by Caco-2 cells was investigated (Fig. 6). ^{3}H-MPP$^+$ uptake in the presence of both a PKA activator (dibutyryl cAMP or IBMX) and a PKC activator (PDBu) was reduced to the same extent as in the presence of only one of the compounds. Similarly, inhibition of ^{3}H-MPP$^+$ uptake in the presence of dibutyryl cAMP plus IBMX was quantitatively similar to that observed in the presence of either compound alone (Fig. 6). So, it seems that there is no summation of the inhibitory effects of the different drugs.

3.2.5. Effect of protein kinase inhibitors

In the next series of experiments, the effect of protein kinase inhibitors was studied (Figs. 7 and 8). Two non-selective protein kinase inhibitors were used: H-7 [21] and staurosporine [22]. Pre-incubation of the cells for 60 min with 1 μM staurosporine did not affect ^{3}H-MPP$^+$ uptake, but it completely abolished the inhibitory effect of PDBu upon ^{3}H-MPP$^+$ uptake. On the other hand, 100 μM (but not 10 μM) H-7 produced a 40% decrease in uptake, and in the presence of 100 μM of this compound an increase in the inhibition produced by either PDBu or dibutyryl cAMP was observed (Figs. 7 and 8).

Table 1
Effect of PKG stimulators on ^{3}H-MPP$^+$ uptake by Caco-2 cells

Compound	Concentration (mM)	^{3}H-MPP$^+$ uptake (% of control)	n
Control	–	100.0 ± 2.5	7
8-Bromo cGMP	0.5	108.7 ± 4.6	4
	1.5	108.6 ± 8.6	7
Control	–	100.0 ± 3.0	11
Sodium nitroprussiate	0.1	104.4 ± 2.8	7
	1	101.2 ± 2.0	3

Cells were pre-incubated for 60 min at 37° with compounds to be tested. The cells were subsequently incubated with ^{3}H-MPP$^+$ (200 nM) for 5 min. Shown are arithmetic means ± SEM.

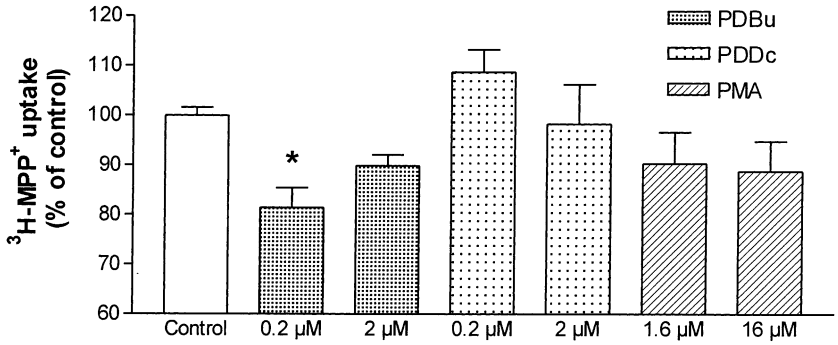

Fig. 5. Effect of PKC activators on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 15$) or presence of PDBu ($n = 12$), PDDc ($n = 8$) or PMA ($n = 10$). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

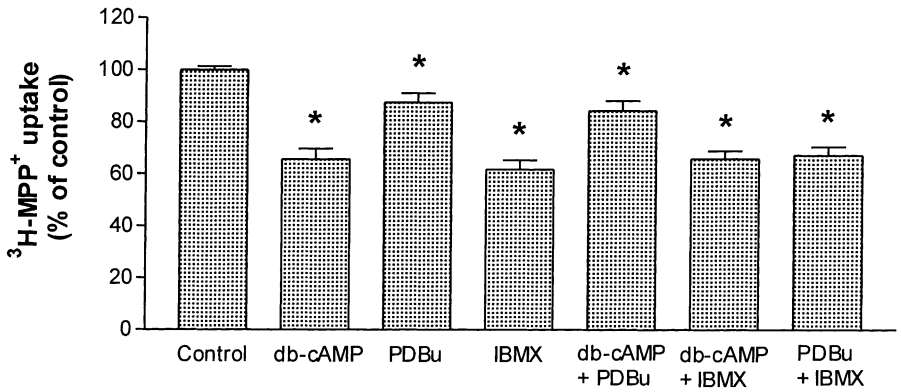

Fig. 6. Effect of a combination of PKA and PKC activators on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 11$–13) or presence of 2.5 mM dibutyryl-cAMP (db-cAMP; $n = 10$), 0.2 μM PDBu ($n = 13$), 0.3 mM IBMX ($n = 7$), 2.5 mM db-cAMP + 0.2 μM PDBu ($n = 7$), 2.5 mM db-cAMP + 0.3 mM IBMX ($n = 7$) or 0.2 μM PDBu + 0.3 mM IBMX ($n = 4$). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

The selective PKC inhibitor chelerythrine [23] decreased ^{3}H-MPP$^+$ uptake to about 70% of control, and the inhibitory effect of PDBu increased in the presence of this compound (Figs. 7 and 8). Rp-cAMPS, a cAMP analogue that acts as a competitive antagonist of cAMP-induced activation of PKA [24,25], induced a small (10%) increase in ^{3}H-MPP$^+$ uptake. However, it was not able to modify the inhibition of ^{3}H-MPP$^+$ uptake induced by the PKA activators dibutyryl cAMP or IBMX (Fig. 8).

3.2.6. PTK

The putative involvement of PTK in the regulation of ^{3}H-MPP$^+$ uptake by Caco-2 cells was studied by testing the effect of pre-treating the cells for 60 min with the PTK

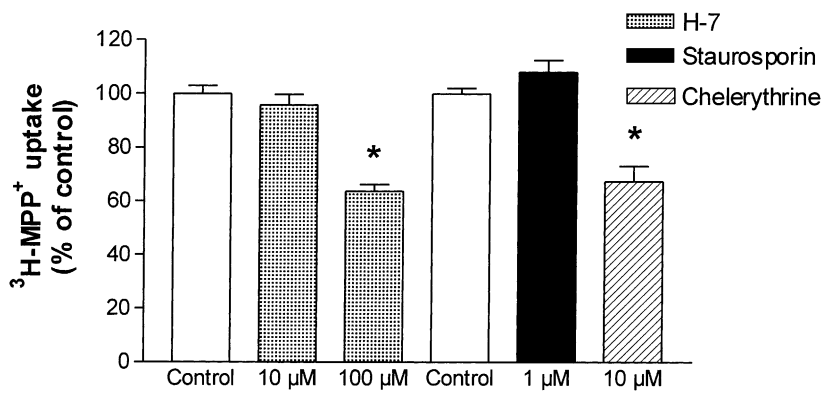

Fig. 7. Effect of protein kinase inhibitors on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 11$–23) or presence of H-7 ($n = 4$), staurosporin ($n = 14$) or chelerythrine ($n = 7$). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

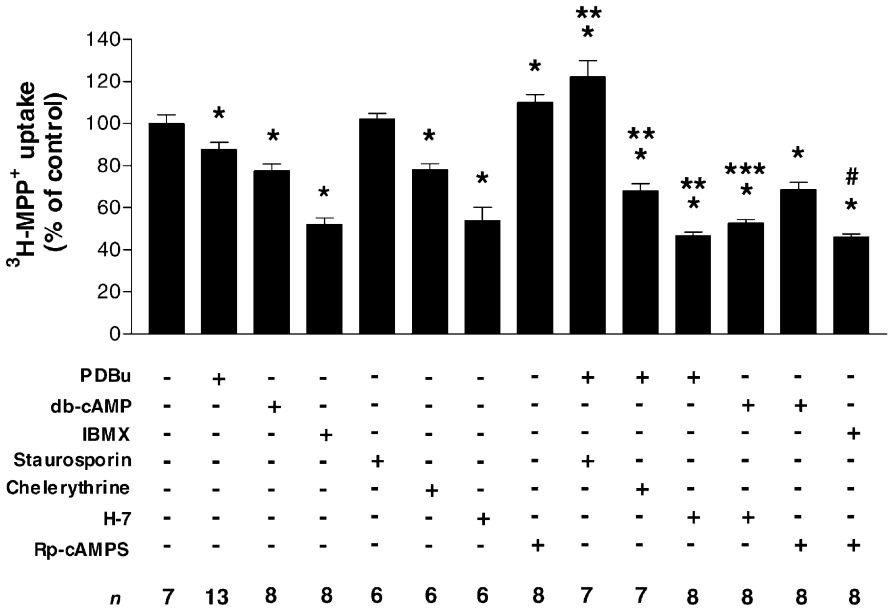

Fig. 8. Influence of protein kinase inhibitors on the effect of PKA and PKC activators upon ^{3}H-MPP$^+$ uptake by Caco-2 cells. After a pre-incubation of 60 min, cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. When present, inhibitors were added 30 min before the pre-incubation period. Drugs used were PDBu (0.2 μM), dibutyryl-cAMP (db-cAMP; 2.5 mM), IBMX (0.3 mM), staurosporine (1 μM), chelerythrine (10 μM), H-7 (100 μM) and Rp-cAMPS (100 μM). Shown are arithmetic means ± SEM. Significantly different from control (*), PDBu (**), db-cAMP (***) or IBMX (#) ($P < 0.05$).

inhibitor tyrphostin A25 [11], or with its negative control tyrphostin A1. Although the PTK inhibitor tyrphostin A25 (100 μM) inhibited ^{3}H-MPP$^+$ uptake (to 87 ± 5% of control), the same concentration of its negative control also reduced the uptake (to 74 ± 8% of control) ($n = 4$). These findings indicate that the inhibition caused by tyrphostin A25 is non-specific in nature.

3.2.7. MAP kinase

Involvement of MAP kinase (MAPK) was studied by testing the effect of specific inhibitors of MAPK kinase 1/2 (PD98059) and p38 MAPK (SB 203580) [26,27] on ^{3}H-MPP$^+$ uptake. As shown in Fig. 9, both compounds significantly and concentration-dependently reduced ^{3}H-MPP$^+$ uptake.

3.2.8. CaM-mediated pathways

Pre-treatment of the cells (for 60 min) with the calmodulin inhibitors calmidazolium (10–100 μM) and trifluoperazine (30–90 μM) or with the inhibitor of CaM-dependent protein kinase II, KN-62 (5–15 μM) [10,11], led to a significant and concentration-dependent inhibition of ^{3}H-MPP$^+$ uptake (Fig. 10).

The dependence of ^{3}H-MPP$^+$ uptake on extracellular Ca^{2+} was also studied, by examining the effect of the Ca^{2+} ionophore A 23187 and of Ca^{2+} omission from the extracellular medium. ^{3}H-MPP$^+$ uptake after pre-incubation of Caco-2 cells for 60 min in the absence or presence of 3 μM A 23187, or with 0 mM Ca^{2+}, was not different (1.6 ± 0.2, 1.5 ± 0.2 and 1.6 ± 0.2 pmol mg protein^{-1}, respectively; $n = 7$). These results suggest that ^{3}H-MPP$^+$

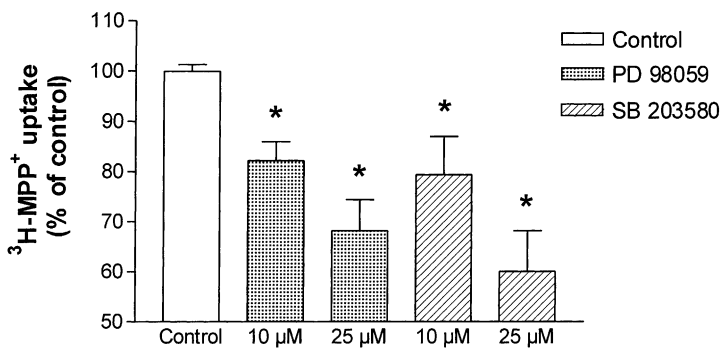

Fig. 9. Effect of the MAP kinase inhibitors PD 98058 and SB 203580 on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 23$) or presence of PD 98058 ($n = 7$–10) or SB 203580 ($n = 3$–10). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

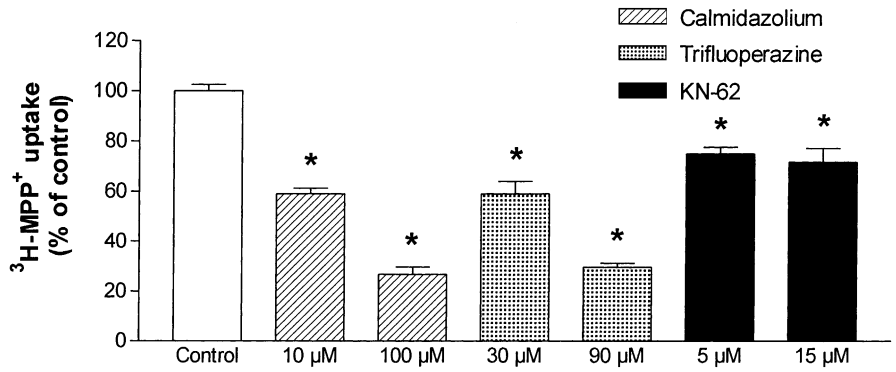

Fig. 10. Effect of inhibitors of CaM-mediated pathways on ^{3}H-MPP$^+$ uptake by Caco-2 cells. Cells were incubated at 37° with 200 nM ^{3}H-MPP$^+$ for 5 min. Cells were pre-incubated for 60 min in the absence (control; $n = 11$) or presence of calmidazolium ($n = 7$), trifluoperazine ($n = 7$) or KN-62 ($n = 7$–11). Shown are arithmetic means ± SEM. (*) $P < 0.05$, significantly different from control.

uptake by Caco-2 cells is not dependent on extracellular Ca^{2+}.

3.3. Effect of drugs on cell viability

Under control conditions, more than 95% of the cells excluded the dye (Trypan Blue). None of the compounds tested (dibutyryl cAMP 2.5 mM, PDBu 0.2 μM, PDDc 0.2 μM, H-7 100 μM, staurosporine 1 μM and chelerythrine 10 μM) significantly affected cellular viability.

4. Discussion

The aim of this study was to investigate the putative regulation of the intestinal transport of organic cations by phosphorylation/dephosphorylation mechanisms. MPP$^+$ was chosen as the model organic cation because it is a very good substrate for the apical transport in Caco-2 cells [12,13], and because it is not subjected to metabolism [28,29].

In the first series of experiments, we examined the role of PKA, PKG and PKC on ^{3}H-MPP$^+$ uptake. These intracellular regulatory pathways play an important role in the regulation of transmembrane transporters in intestinal and other epithelia (e.g. [30–35]), including transporters for organic cations [6–11].

The lack of effect of exposure of the cells to the cGMP analogue, 8-Br-cGMP, or to the guanylyl cyclase activator sodium nitroprussiate, showed that cGMP had no effect on the transport of ^{3}H-MPP$^+$, either by a direct effect or by stimulation of PKG.

Uptake of ^{3}H-MPP$^+$ by Caco-2 cells seems to be inhibited by increased intracellular cAMP levels, because it was inhibited by treatments that induce an increase in the intracellular concentration of cAMP (dibutyryl cAMP and phosphodiesterase inhibition). The mechanism involved in the effect of cAMP was investigated by examining the effect of the non-selective protein kinase inhibitor H-7, and of the selective PKA inhibitor Rp-cAMPS, on the effect of dibutyryl cAMP or IBMX on ^{3}H-MPP$^+$ uptake. H-7 caused a 40% inhibition of ^{3}H-MPP$^+$ uptake in Caco-2 cells, and so it was not possible to conclude on the influence of this compound on the effect of dibutyryl cAMP. On the other hand, Rp-cAMPS produced a small increase in uptake, but it did not reverse the inhibitory effect of dibutyryl cAMP or IBMX on ^{3}H-MPP$^+$ uptake.

The observation that uptake of ^{3}H-MPP$^+$ into Caco-2 cells was decreased in the presence of the phorbol ester PDBu suggests that activation of PKC inhibits the transport. The mechanism involved in the effect of PDBu was investigated by examining the effect of the non-selective protein kinase inhibitors, H-7 and staurosporine, on the effect of PDBu on ^{3}H-MPP$^+$ uptake. H-7 was not ideal in that it caused a 40% inhibition of ^{3}H-MPP$^+$ uptake in Caco-2 cells. Exposure of the cells to staurosporine had no effect on ^{3}H-MPP$^+$ uptake, and it completely abolished the inhibitory effect of PDBu on ^{3}H-MPP$^+$ uptake. This observation suggests that the effect of PDBu was mediated through activation of PKC. However, staurosporine inhibits not only PKC, but also other Ca^{2+}-dependent kinases at the concentration used (1 μM) [22]. So, the effect of a selective inhibitor of PKC, chelerythrine [23] was investigated. However, chelerythrine caused a 30% inhibition of ^{3}H-MPP$^+$ uptake in Caco-2 cells. Hence, it was not possible to conclude on the effect of this compound on the effect of PDBu.

In summary, we could not conclude on a direct effect of drugs on PKA or PKC because the protein kinase inhibitors that are available are also potent inhibitors of ^{3}H-MPP$^+$ uptake over the same concentration range.

We next examined the involvement of PTK in the regulation of ^{3}H-MPP$^+$ uptake, because this kinase has been shown to play a role in the regulation of some transmembrane transporters. Similarly to what happens in relation to thiamine and riboflavin transport by epithelial cells [10,11], a PTK-mediated pathway does not seem to be involved in the regulation of ^{3}H-MPP$^+$ transport by Caco-2 cells.

The observation that inhibition of the CaM-mediated pathway caused a pronounced and concentration-dependent

decrease in ^{3}H-MPP$^+$ uptake suggests that ^{3}H-MPP$^+$ uptake by Caco-2 cells is under the regulation of CaM-mediated pathways. This dependence has been discussed for several other transporters (e.g. [10,11,36,37]). Because removal of Ca^{2+} from the extracellular medium or inhibition of extracellular Ca^{2+} influx did not affect ^{3}H-MPP$^+$ uptake, this mechanism is most probably dependent on intracellular sources of Ca^{2+}.

The decrease in ^{3}H-MPP$^+$ transport found with the specific MAP kinase inhibitors suggest that MAP kinase activation stimulates ^{3}H-MPP$^+$ transport. MAP kinase is regarded as a major pathway for growth factor signalling from the cell surface to the nucleus [38] and MAP kinase activation has been noted in response to stimulation of many G-protein coupled receptors [39]. So, it is possible that activation/inhibition of MAP kinases is involved in the regulation of intestinal uptake of ^{3}H-MPP$^+$ by second messengers (e.g. Ca^{2+}) generated after activation of G-protein coupled receptors.

Recently, we verified that Caco-2 cells express mRNAs for EMT and OCT1, and functional studies indicated that uptake of ^{3}H-MPP$^+$ at the apical level of these cells seems to involve both transporters [12,13]. EMT and OCT1 are known to be regulated by phosphorylation/dephosphorylation mechanisms, but distinct mechanisms operate for these two transporters. OCT1 was found to be stimulated by PKA, PKC and PTK activation [14,15], and EMT does not appear to be regulated by PKA, PKC or PTK, but rather by CaM-mediated pathways [16,17]. So, it seemed interesting to compare the regulation of ^{3}H-MPP$^+$ transport in Caco-2 cells with the regulation of EMT- or OCT1-mediated transport. Analysis of our results show that regulation of ^{3}H-MPP$^+$ uptake by Caco-2 cells presents many similarities with regulation of EMT-mediated transport, but is very distinct from regulation of OCT1-mediated transport. Several facts support this conclusion: (1) OCT1-mediated transport is stimulated by activation of PKA and PKC, but apical uptake of ^{3}H-MPP$^+$ by Caco-2 cells and EMT-mediated transport were not affected or were even inhibited by activation of PKA or PKC; (2) apical uptake of ^{3}H-MPP$^+$ by Caco-2 cells and EMT-mediated transport were not affected by PKG and PTK modulators; (3) apical uptake of ^{3}H-MPP$^+$ by Caco-2 cells and EMT-mediated transport were inhibited by MAP kinase inhibitors; (4) apical uptake of ^{3}H-MPP$^+$ by Caco-2 cells and EMT-mediated transport were strongly inhibited by CaM pathway inhibitors, and they were both independent from extracellular Ca^{2+}. As a more general observation, the apical transport of ^{3}H-MPP$^+$ in Caco-2 cells is inhibited in the phosphorylated status, similarly to EMT, suggesting a preferential participation of this transporter in Caco-2 cells.

Finally, it is possible that the reported effects of drugs on ^{3}H-MPP$^+$ transport result not only from an effect on phosphorylation/dephosphorylation pathways, but also from a direct effect of the drugs on the transporter(s) itself. However, direct evidence to support the hypothesis that drugs affect the phosphorylation status of the carrier protein(s) (e.g. by using specific antibodies against EMT or OCT1) is not feasible at present.

In conclusion, we demonstrate that the apical uptake of the organic cation ^{3}H-MPP$^+$ in Caco-2 cells is regulated by phosphorylation/dephosphorylation mechanisms, and that CaM-mediated pathways play an important role in this modulation. Because treatments that induce an activation of PKA, PKC or MAP kinase produce an inhibition in ^{3}H-MPP$^+$ uptake, we suggest that ^{3}H-MPP$^+$ transporter(s) at the apical membrane of Caco-2 cells is(are) most likely active in the dephosphorylated state. Finally, uptake of ^{3}H-MPP$^+$ by Caco-2 cells and by EMT are regulated in a very similar manner, suggesting a more important participation of EMT than OCT1 in the intestinal uptake of this compound.

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