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UPTAKE OF QUATERNARY AMMONIUM COMPOUNDS INTO RAT INTESTINAL BRUSH BORDER MEMBRANE VESICLES

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Abstract—In order to elucidate the mechanism by which quaternary ammonium compounds are transported across the gastro-intestinal epithelium, transport of five quaternary ammonium compounds through rat intestinal brush border membrane vesicles was investigated. Transport across the intestinal brush border membrane is only observed when the organic cations possess a hydrophobic tail and their charge is somehow screened by the presence of a bulky group around the charged atom. Transport appeared to be a passive process. Uptake was stimulated by a transmembrane electrical potential difference, but not by an excess of anions like I^- or taurocholate. The implications for the *in vivo* absorption of quaternary ammonium compounds and for their use as probes for measuring the transmembrane electrical potential difference are briefly discussed.

Transport of cationic drugs through the intestinal epithelium is a poorly understood phenomenon. These compounds are absorbed to a varying extent and sometimes even excreted into the gastro-intestinal tract [1].

Since the major part of the plasma membrane of the epithelial cells of the intestine consists of lipids and since the presence of a charged group will in general largely decrease the lipid solubility and thus the membrane permeation, the prevailing mechanisms proposed for transport of organic ions through biological membranes are simple diffusion in the form of the uncharged and therefore lipid-soluble ion-pairs [2] or some kind of carrier mediated transport [3]. A third alternative for the intestinal absorption of organic cations, which in general has not been considered, is the paracellular pathway [4].

Carrier mediated transport of ionized organic drugs through biological membranes has been shown for a wide variety of compounds [3], e.g. choline [5]. In most cases, however, non-carrier mediated passive diffusion of charged compounds or ion-pairs could not be excluded. The importance of ion-pair formation in the membrane transport of charged drugs has often been suggested, but experimental evidence is generally lacking [6–9].

Membrane vesicle preparations offer a possible solution in elucidating the mechanism of transport of cationic drugs through the intestinal epithelium. In such systems many drawbacks of isolated organs or relatively intact tissue preparations such as interference by paracellular transport or intracellular metabolism and/or compartmentation are absent. Furthermore experiments can be carried out under conditions in which the composition of the medium at both sides of the membrane can be controlled. By using vesicle preparations derived from the mucosal side or from the serosal side of the intestinal epithelial cell membrane it is possible to differentiate between transport through the brush border membrane and the baso-lateral membrane.

The object of the present study is to use membrane vesicles as a system for the investigation and characterization of organic cation transport through the brush border membrane of rat small intestine. The results presented here demonstrate, that rat intestinal brush border membrane vesicles are permeable for organic cations that possess certain physicochemical properties. Their transport is passive, stimulated by the presence of a transmembrane electrical potential difference (inside negative) and not accelerated by the presence of an excess of anions like I^- or bile salts like taurocholate.

MATERIALS AND METHODS

Preparation of membrane vesicles. Brush border membrane vesicles were isolated from rat small intestine as described by Kessler *et al.* [5]. Vesicles were suspended in a buffer containing 100 mM sucrose plus 10 mM Hepes/KOH (pH=7.4) to give a protein concentration of 5–10 mg/ml and stored in liquid nitrogen. For each experiment vesicles were used that were frozen and thawed only once. Membrane vesicles stored in liquid nitrogen for up to three months retained the same transport activity as freshly prepared vesicles.

Transport assays. Assays were carried out at 25°. The reaction mixture (40 μl) consisted of 1 mg membrane protein per ml, 100 mM sucrose, 10 mM Hepes, radioactively labelled organic cation and 130 mM of an inorganic salt as indicated in the legends to the figures adjusted to pH=7.4 with KOH.

Abbreviations used: MDT, methyldeptropine; *p*CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, transmembrane electrical potential difference.

The uptake experiment was started by addition of the vesicle suspension and stopped by rapid filtration of 20 μl of the reaction mixture over cellulose acetate filters (Oxoid Nuflow N 25/45 UP, pore size 0.45 μm). The prewetted filters were immediately washed twice with 2 ml of ice-cold suspension buffer plus 0.1 M $NaNO_3$. For each experiment the amount of organic cation bound by the filters themselves was determined and subtracted from the amount when vesicles were present. Results are expressed as nmol organic cation taken up per mg of vesicle protein. Internal concentrations of solutes inside the membrane vesicles were calculated using 1.2 μl/mg membrane protein as the intravesicular volume [10, 11].

Generation of a valinomycin-potassium diffusion potential. A suspension of membrane vesicles in 100 mM sucrose plus 10 mM Hepes/KOH (pH=7.4) was incubated at room temperature during 5 min with 10 μM valinomycin. The vesicles were washed once and resuspended in the same buffer to a protein concentration of 4, 8, 13, 20 and 40 mg/ml. At zero time these suspensions were diluted to a final protein concentration of 1 mg/ml into a reaction-mixture (final volume 40 μl), which contained 100 mM sucrose, 10 mM Hepes and radioactively labelled organic cation, adjusted to pH=7.4 with NaOH. This dilution results to a theoretical transmembrane electrical potential difference of $\Delta\psi = RT/F^e$ log (dilution factor).

Estimation of the transmembrane electrical potential difference. The transmembrane electrical potential difference $\Delta\psi$ was calculated with the Nernst equation: $\Delta\psi = RT/F^e \log (C_{in}/C_{out})$, where: C_{in} = internal concentration organic cation; C_{out} = medium concentration organic cation; R, T and F have their usual meanings.

Protein determination. Protein was assayed by the Lowry method [12].

Materials. ^{14}C-labelled 3α(10,11-dihydro-5H-dibenzo-(*a*,*d*)-cyclohepten-5-yl)oxy-8-methyltropaniumiodide (methyldeptropine (sp. act. 1.21 μC/μmol) was purchased from the central laboratory of TNO(Delft, The Netherlands). ^{14}C-labelled *N*-methyltropaniumiodide (methyltropine) (spec. act. 1.21 μC/μmol) was synthesized according to [13]. ^{14}C-labelled *N*-methylatropineiodide (methylatropine) (sp. act. 6.98 μC/μmol) was a generous gift of Dr. A. M. Soeterboek. ^{14}C-labelled procainamideethobromide (sp. act. 4.15 μC/μmol) and ^{3}H-labelled *d*-tubocurarine chloride (sp. act. 35.4 μC/μmol) were obtained from New England Nuclear. Sodium taurocholate was obtained from Fluka A.G. (Buchs SG, Switzerland).

RESULTS AND DISCUSSION

Physico-chemical properties

The five tested monoquaternary ammonium compounds were methyldeptropine, methylatropine, methyltropine, procainamideethobromide and tubocurarine. Methyldeptropine and methylatropine are synthetic analogues of atropine and belong together with procainamideethobromide to the pharmacological class of anticholinergics [13–15]. Methyltropine is the product of hydrolysis of both methyldeptropine and methylatropine. These three compounds all contain the quaternary tropine part; only the chemical structure of the substituted alcohol group of the tropine moiety varies. Tubocurarine is a non-depolarising muscle relaxant with a completely different structure. The partition of these five organic cations between octanol and various aqueous phases has been determined as an indication for their lipophilicity (Table 1). Lipophilicity increases in the following sequence: methyltropine < procainamideethobromide < methylatropine < tubocurarine < methyldeptropine. The partition behaviour of the three structurally related compounds methyltropine, methylatropine and methyldeptropine differs considerably. Only methyldeptropine dissolves well in octanol. This observation shows that the side group of the tropine part determines to a large extent this partition behaviour.

Uptake of organic cations by rat intestinal brush border membrane vesicles

In experiments uptake could be observed of only methyldeptropine and tubocurarine. Tubocurarine was very slowly taken up (equilibrium was not reached even after 90 min, in contrast to the 10 min needed for methyldeptropine), so uptake studies in response to diffusion potentials, lasting maximally 10 min, could only be performed with methyldeptropine. The observation that the other quaternary ammonium compounds are not taken up, could be of importance for their in vivo absorption, since for instance methylatropine is absorbed when orally administered [14]. Since this absorption does not take place via the intestinal brush border membrane, there has to be a different route, e.g. the paracellular pathway [4].

Transport characteristics of methyldeptropine

No regional differences in the uptake pattern of methyldeptropine were found with brush border membrane preparations derived from the duodenal,

Table 1. Partition of various organic cations between octanol and various aqueous phases, determined by the rotating flask method

Compound	Per cent dissolved in octanol (pH=7.4)				
	H_2O	Krebs-NaOH	Krebs-bicarbonate	120 mM NaCl	120 mM NaI
Methyltropineiodide	0.1	–	–	–	0.6
Methylatropineiodide	0.8	1.6	1.6	2.5	7.1
Methyldeptropineiodide	24.5	88.5	90.8	88.4	100
d-Tubocurarinechloride	8.0	13.9	22.0	9.1	22.6
Procainamideethobromide	1.8	–	–	–	3.1

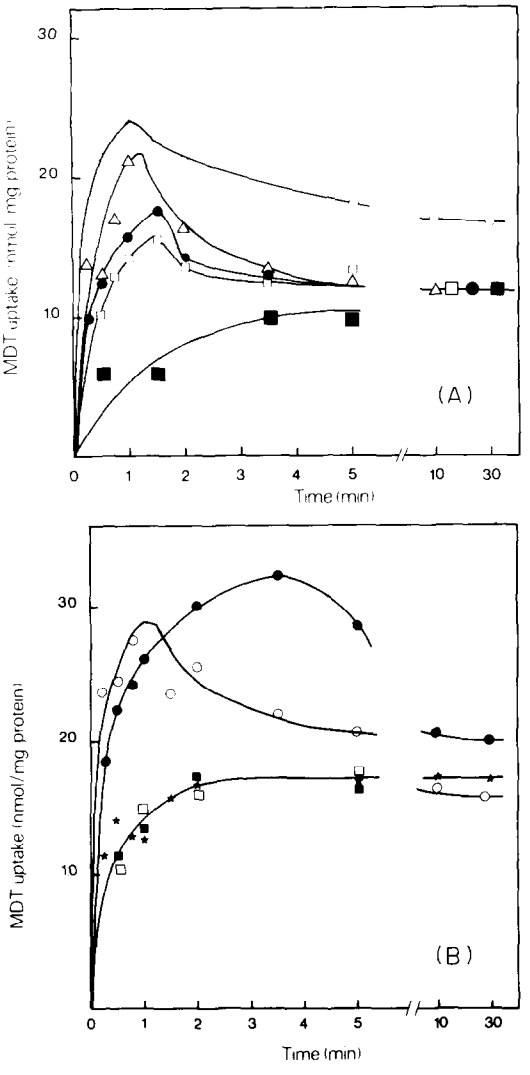

Fig. 1. Uptake of methyldeptropine by rat intestinal brush border membrane vesicles. (a) Uptake of methyldeptropine (275 μM) was measured in response to a 130 mM $NaNO_3$ gradient (Δ); 130 mM KNO_3 gradient (○); 130 mM $NaNO_3$ gradient after 2 min of pre-equilibration (□); a valinomycin-potassium diffusion potential (●); 130 mM $NaNO_3$ gradient plus 2.5 μM FCCP (■). (b) Uptake of methyldeptropine (275 μM) was measured in response to a 130 mM NaCl gradient (○); 130 mM choline chloride gradient (●); 130 mM Na_2SO_4 gradient (■); 130 mM $NaHCO_3$ gradient (□); no salts added (*).

jejunal and ileal part of the small intestine. Therefore all further studies were confined to membrane preparations derived from the total small intestine. The time course for methyldeptropine uptake in these membrane vesicles was measured under various experimental conditions (Fig. 1). A transient uptake is observed in the presence of a gradient of NO_3^- or Cl^-, but not in the presence of a gradient of SO_4^{2-} or HCO_3^-. Preequilibration for 2 min of the vesicles in the presence of $NaNO_3$ partially abolishes this transient uptake [Fig. 1(a)]. Na^+, K^+ or choline$^+$ affect the time course and the steady state of the uptake, but have no effect on the overshoot. Also in the presence of a valinomycin-potassium diffusion potential ($K^+_{inside} > K^+_{outside}$) an overshoot is observed [Fig. 1(a)]. In the presence of a $NaNO_3$ gradient plus 2.5 μM FCCP the overshoot phenomenon disappears [Fig. 1(a)].

The interpretation of the above results is as follows. Cl^- and NO_3^-, due to their higher permeability as compared to Na^+, K^+ or choline$^+$, are able to create for a short period an electrical transmembrane potential difference $\Delta\psi$ (inside negative), whereas SO_4^{2-} and HCO_3^- are not. The duration of this potential depends on the exact ratio between the permeabilities of the anion and the cation used for the creation of the $\Delta\psi$. Since choline$^+$ is less permeable than Na^+ or K^+, the $\Delta\psi$ will exist longer in the presence of choline$^+$ than in the presence of Na^+ or K^+. Preequilibration of the vesicles will of course abolish these diffusion potentials. These phenomena have been widely used in the investigation of the transport characteristics of sugars, amino acids and bile acids in intestinal brush border membrane preparations [16]. Also in the presence of a valinomycin-potassium diffusion potential a $\Delta\psi$ is created, due to the fact that valinomycin makes the membrane highly permeable for K^+, but not for its counter ion (OH^-) [17]. In the presence of the protonophore FCCP any existing $\Delta\psi$ will immediately be abolished, independent of the way it was created, due to a compensatory proton flux [17]. In bacterial vesicles it has been shown, that uptake of methyldeptropine is driven by a transmembrane electrical potential difference [13]. The results presented here for intestinal brush border membrane vesicles indicate a similar uptake mechanism. Na^+, K^+ or choline$^+$ modify the uptake as far as they influence the duration of the $\Delta\psi$. No specific dependence on a particular cation for the uptake of methyldeptropine was found. The differences in equilibrium uptake after 30 min are probably due to differences in binding.

Uptake of methyldeptropine into an osmotically active space

To distinguish between binding of methyldeptropine to the vesicle membrane and transport into the intravesicular space, uptake of methyldeptropine was measured as a function of the osmolarity of the incubation medium. Changes of the volume of the intravesicular space by increasing the osmolarity of the incubation medium has a significant effect on transport, while causing no change in binding. As shown in Fig. 2, equilibrium uptake of methyldeptropine (275 μM) measured after 10 min in the presence of 130 mM $NaNO_3$ was dependent on the osmolarity of the incubation medium. Extrapolation to infinite osmolarity reveals that the total binding (intra-plus extravesicular) under equilibrium conditions amounts to 0.8 nmol/mg protein.

Mechanism of methyldeptropine transport

The possible involvement of a carrier system was investigated by measuring the initial influx rate between 15 and 45 sec after the start of the incubation as a function of the methyldeptropine concentration in the presence of 130 mM $NaNO_3$ or 130 mM KNO_3, but in the absence of a gradient of these two salts. No differences were found between the initial influx

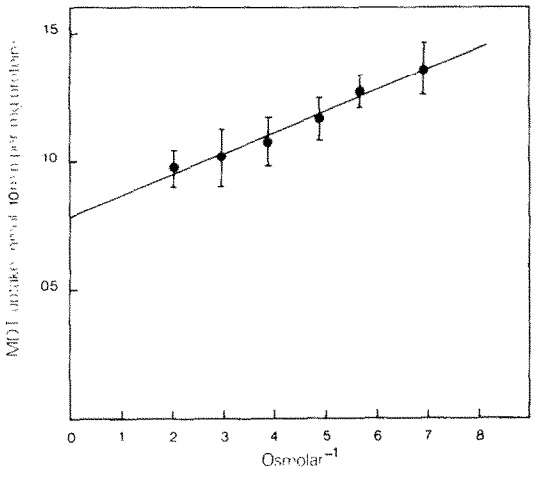

Fig. 2. Relation between medium osmolarity and methyldeptropine uptake. The uptake of methyldeptropine (275 μM) in the presence of 130 mM $NaNO_3$ was measured after 10 min of incubation. Medium osmolarity was manipulated with various concentrations of sucrose. Uptake was terminated using a medium to which sucrose had been added in order to obtain a corresponding osmolarity. Means of five experiments ± S.D. are given. Osmolarity refers to the delta osmolarity produced by sucrose.

rate in the presence of KNO_3 and $NaNO_3$ and no saturation was measured up to a concentration of 9 mM. These measurements were made in the absence of a salt gradient, since high influx rates of the positively charged methyldeptropine would lead to a rapid abolishment of the $\Delta\psi$. The measurements were made in the presence of valinomycin-plus K^+ (no gradient) to prevent the generation of a diffusion potential (inside positive) due to the influx of methyldeptropine.

Also the influence of the temperature on the uptake of methyldeptropine in the presence of a 130 mM $NaNO_3$ gradient was investigated. At 0° the same uptake pattern was observed as at 25°. This observation argues against a carrier-mediated transport mechanism for methyldeptropine.

The influence of the possible inhibitor choline, a quaternary ammonium compound for which a transport system in intestinal brush border membranes has been described [5], was investigated. However, choline [130 mM, see Fig. 1(b)] showed no inhibitory effect on the uptake of methyldeptropine.

Finally, the influence of two carrier-blocking agents, the thiol-reagents *p*CMB and NEM, was investigated (Fig. 3). This figure shows, that *p*CMB (1 mM) and NEM (0.5 mM) considerably increased the maximum uptake value for methyldeptropine. If transport of methyldeptropine was carrier-mediated, the uptake values would be largely decreased. The observed increase argues against a carrier-mediated transport system for this organic cation and must be due to a longer lasting and higher membrane potential. The fact that these two thiol-reagents block all kinds of leakage pathways for sodium, the major cation that determines the height and the duration of the electrical diffusion potential imposed to the membrane by the addition of $NaNO_3$, is most probably the cause of this phenomenon.

From these observations I conclude, that transport of methyldeptropine through the intestinal brush border membrane is not carrier-mediated.

Influence of an excess of anions

Ion-pair transport would be greatly increased in the presence of an excess of anions that form readily ion-pairs with organic cations. Two kinds of anions are claimed to be very potent in this respect, e.g. certain inorganic anions of which I^- was taken as a representative, and bile salts like taurocholate. However it was found that 1 mM I^- did not change the uptake pattern for methyldeptropine in the presence

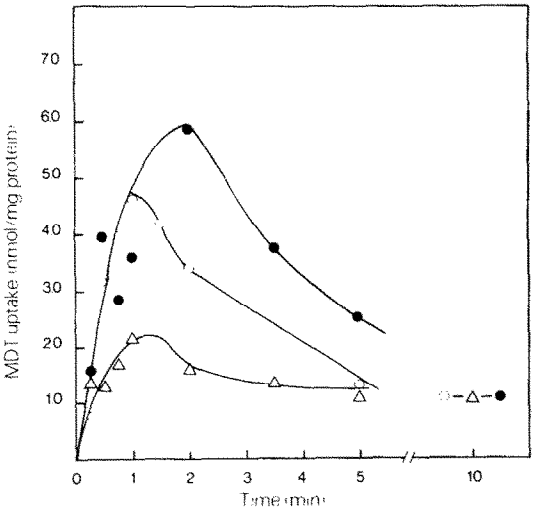

Fig. 3. Influence of carrier-blocking agents. Uptake of methyldeptropine (275 μM) was measured in the presence of a 130 mM $NaNO_3$ gradient plus 0.5 mM NEM (●) or 1.0 mM pCMB (○) and in the absence of a thiol reagent (△).

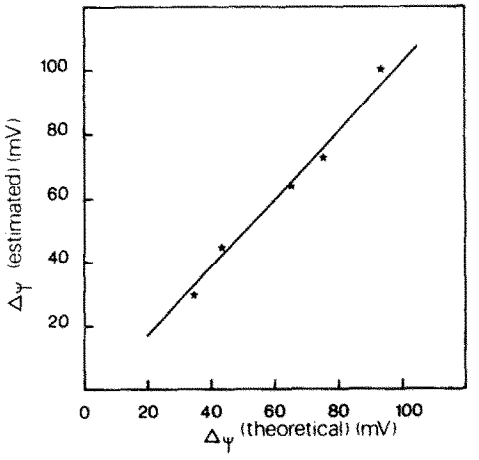

Fig. 4. Calibration of methyldeptropine uptake and the transmembrane electrical potential difference $\Delta\psi$. The valinomycin-potassium diffusion potentials were plotted as a function of the $\Delta\psi$ calculated with the Nernst-equation from the difference between the maximum uptake value of methyldeptropine and the equilibrium uptake value after 10 min. $\Delta\psi$ (theoretical) emerges from these valinomycin-potassium diffusion potentials calculated as described in Materials and Methods.

of a 130 mM $NaNO_3$ gradient. Moreover, under the same conditions no influence was found by the addition of 0.25 mM taurocholate, nor induced 1 mM I^- the uptake of the impermeable organic cation methylatropine. A positive effect of these anions on the methyldeptropine uptake would also be incompatible with the previous findings, since it was shown that the transport of methyldeptropine is accelerated by the presence of a transmembrane electrical potential difference (inside negative), which indicates that methyldeptropine is transported in the form of the positively loaded cation. Transport of neutral ion-pairs will not be influenced by the presence of a transmural electrical potential difference.

Estimation of the transmembrane electrical potential difference

The estimated $\Delta\psi$, calculated with the Nernst-equation from the difference between the maximum uptake value of methyldeptropine and the equilibrium uptake value after 10 min was plotted as a function of the theoretical valinomycin-potassium diffusion potentials (Fig. 4). A linear relationship between both values exists. This strongly indicates that the methyldeptropine uptake is a good indication of the $\Delta\psi$ under various experimental conditions. The maximum value of this $\Delta\psi$ in the presence of a 130 mM gradient of NO_3^- amounts to *ca.* 35 mV (inside negative); 2 min of preequilibration lowers this $\Delta\psi$ to *ca.* 20 mV. In the presence of *p*CMB and NEM the $\Delta\psi$ is much higher and amounts to 65 and 70 mV respectively, probably due to the fact that under these conditions all kinds of leakage pathways for sodium are blocked.

CONCLUSIONS

The partition behaviour of organic cations between octanol and water appears to be an indication for their permeability. Partition behaviour measures the potency of organic cations in forming ion-pairs. However it is shown in this study that ion-pair formation is not mandatory for transport of organic cations through the intestinal brush border membrane. If organic cations posess a large hydrophobic tail and their charge is somehow screened by the presence of a bulky group around the charged nitrogen atom, they can pass biological membranes per se. These requirements are only met by a very limited number of quaternary ammonium compounds in use to day as drugs. Besides, even if these compounds can pass the intestinal brush border membrane, the existence of an electrical transmural potential profile between the mucosal and the serosal side of the gastro-intestinal tract as proposed by Schultz [18] represents a significant barrier for their absorption. When offered from the mucosal side, they will be trapped into the intestinal epithelial cell layer; when offered from the serosal side they will be excreted due to the net electrical potential difference between serosa and mucosa.

If an organic cation can pass the intestinal brush border membrane sufficiently fast (e.g. methyldeptropine), it can be used to estimate the transmembrane electrical potential difference in intestinal brush border membrane vesicle preparations under different experimental conditions. Studies regarding the transport of organic cations through the basolateral membrane of the intestinal epithelium to complete this picture are in progress in our laboratory.

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