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UPTAKE OF QUATERNARY AMMONIUM COMPOUNDS INTO RAT LIVER PLASMA MEMBRANE VESICLES

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Abstract—In order to elucidate the mechanism by which quaternary ammonium compounds are transported across the rat hepatocyte plasma membrane, the transport of five quaternary ammonium compounds through rat hepatocyte plasma membrane vesicles was investigated. Transport is only observed when the organic cations possess a high lipophilicity. Uptake appeared to be a passive process and was not stimulated by a transmembrane electrical potential difference nor by the presence of an excess of anions like I^-. Taurocholate decreased the uptake. However in the presence of a catalytic amount of tetraphenylborate a transmembrane electrical potential difference (inside negative) stimulated the uptake.

Hepatic transport mechanisms for quaternary ammonium compounds have been the subject of numerous investigations in intact animals, isolated perfused livers and isolated hepatocytes [1]. From these studies it seems that the liver possesses uptake and secretory mechanisms for a wide variety of organic cations. One of the major problems is that most studies describe overall transport from plasma into bile and ignore the possibility that the mechanisms for uptake into the hepatocytes, intracellular transport and canalicular secretion could be fundamentally different.

Since important spots in these transport mechanisms presumably are located at the level of the hepatocyte plasma membrane, investigations with isolated plasma membrane preparations might reveal to what extent the observed phenomena are due to effects at the level of the plasma membrane. In isolated membrane vesicle preparations, 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 sinusoidal or the canalicular side of the hepatocyte plasma membrane, it is possible to differentiate between transport through the sinusoidal and the canalicular plasma membrane.

The object of the present study is to use plasma membrane vesicles as a system for the investigation and characterization of organic cation transport through the plasma membrane of the rat hepatocyte. The results presented here demonstrate that the hepatocyte plasma membrane is permeable for some organic cations. Their transport is passive and independent of the presence of a transmembrane electrical potential difference. Uptake was not influenced by the presence of an excess of anions like I^-, but decreased in the presence of bile salts like taurocholate. In the presence of catalytic amounts of TPB^-* a transmembrane electrical potential difference (inside negative) stimulated the uptake.

MATERIALS AND METHODS

Materials

^{14}C-Labelled 3α(10,11-dihydro-5H-dibenzo-(*a,d*)-cyclohepten - 5 - yl)oxy - 8 - methyltropaniumiodide (methyldeptropine, sp. act. 1.21 mCi/mmole) was purchased from the Central Laboratory of TNO (Delft, The Netherlands). ^{14}C-Labelled *N*-methyltropaniumiodide (methyltropine, sp. act. 1.21 mCi/mmole) was synthesized according to [9]. ^{14}C-Labelled *N*-methylatropineiodide (methylatropine, sp. act. 6.98 mCi/mmole) was a gift of Dr. A. M. Soeterboek (Eindhoven, The Netherlands). ^{14}C-Labelled procainamideethobromide (sp. act. 4.15 mCi/mmole) and ^{3}H-labelled *d*-tubocurarinechloride (sp. act. 35.4 mCi/mmole) were obtained from New England Nuclear (Boston, MA). Sodium taurocholate was obtained from Fluka A.G. (Buchs SG, Switzerland).

Preparation of membrane vesicles. Plasma membrane vesicles were isolated from rat liver as described by van Amelsvoort *et al.* [2]. Vesicles were suspended in homogenization buffer (0.25 M sucrose/0.2 mM $CaCl_2$/10 mM Hepes, with KOH adjusted to pH 7.5) to give a protein concentration of 5–10 mg/ml and stored in liquid nitrogen. For each experiment vesicle suspensions were used, which 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) contained 1 mg mem-

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

brane protein/ml, 0.25 M sucrose, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, radioactive labelled organic cation and 125 mM of an inorganic salt as indicated in the legends to the figures; the mixture was adjusted to pH 7.5 with KOH. The uptake experiment was started by the 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 homogenization buffer plus 0.1 M $NaNO_3$. Values were corrected for the radioactivity bound to the filters. Results are expressed as nmole organic cation taken up per mg of vesicle protein.

Control experiments. To detect if the presence of microsomal, mitochondrial and/or lysosomal material in the plasma membrane preparation might contribute to the observed transport phenomena of the quaternary ammonium compounds tested, rat liver homogenate was subjected to classical subfractionation according to the methods reported previously [3]. The following marker enzymes were determined: acid phosphatase (lysosomal) [4], malate dehydrogenase (mitochondrial) [5], glucose-6-phosphatase (microsomal) [6] and (Na^+,K^+)-ATPase (sinusoidal plasma membrane) [7], both in the obtained subfractions (mitochondrial, lysosomal and microsomal) and in the plasma membrane preparation. These fractions were also used for studying organic cation transport with the rapid filtration technique described above.

Protein determination. Protein was assayed by the Lowry method [8].

RESULTS AND DISCUSSIONS

Physico-chemical properties. The physico-chemical properties of the five tested monoquaternary ammonium compounds, methyltropine, methyldeptropine, methylatropine, procainamideethobromide and tubocurarine were described in a previous paper [10]. Lipophilicity increased in the following sequence: methyltropine < procainamideethobromide < methylatropine < tubocurarine < methyldeptropine.

Uptake of organic cations by rat liver plasma membrane vesicles. Uptake of methyldeptropine and tubocurarine could be observed only. Tubocurarine was very slowly taken up and equilibrium was not reached even after 90 min (in contrast to the 10 min needed for methyldeptropine). Therefore uptake studies in response to diffusion potentials, lasting maximally 10 min, could only be performed with methyldeptropine. This observation is similar to the results found in rat intestinal brush border membrane vesicles [10], in which uptake was also observed of methyldeptropine and tubocurarine only.

The time course for methyldeptropine uptake in the membrane vesicles was measured under various experimental conditions (Fig. 1). No significant difference in the uptake pattern is observed in the presence of Na^+, K^+ or choline$^+$ or in the absence or the presence of a gradient of NO_3^-. In the presence of 2.5 μM FCCP a small decrease in the steady state uptake value was observed. These findings indicate that transport of methyldeptropine in this plasma membrane preparation is not Na^+-dependent and that the presence of a transmembrane electrical potential difference, induced by a gradient of NO_3^- ions, has no influence on the uptake pattern for methyldeptropine.

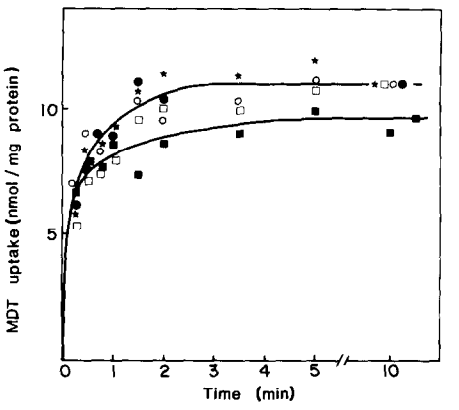

Fig. 1. Uptake of methyldeptropine by rat hepatocyte plasma membrane vesicles. Uptake of methyldeptropine (0.15 mM) was measured in response to a 125 mM $NaNO_3$ gradient (★), 125 mM KNO_3 gradient (●), 125 mM $NaNO_3$ no gradient (○), 125 mM choline chloride gradient (□), 125 mM $NaNO_3$ gradient plus 2.5 μM FCCP (■).

The plasma membrane fraction used was reported to be slightly contaminated with mitochondrial material, microsomes and lysosomes [2], which in principle might contribute to the observed effects. However no contamination with mitochondria could be found as indicated by the absence of activity of the mitochondrial marker enzyme malate dehydrogenase. The microsomal fraction appeared not to take up methyldeptropine after correction for cross-contamination with mitochondrial, lysosomal and plasma membraneous material. The lysosomal fraction did take up some methyldeptropine. However this uptake appeared to be dependent on a transmembrane electrical potential difference and was to a large extent diminished in the presence of 2.5 μM FCCP (in the presence of this protonophore an existing $\Delta\psi$ will immediately be abolished due to a compensatory proton flux) [11]. The small decrease measured in the plasma membrane preparation in the presence of 2.5 μM FCCP could be due to a small contamination of this preparation with lysosomal material. I conclude therefore that the observed methyldeptropine uptake is not due to uptake by these contaminations.

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, but not on binding. Equilibrium uptake of methyldeptropine (0.15 mM) measured after 10 min in the presence of 125 mM $NaNO_3$ appeared to be dependent on the osmolarity of the incubation medium (Fig. 2). Extrapolation to infinite osmolarity reveals, that the total binding (intra- and

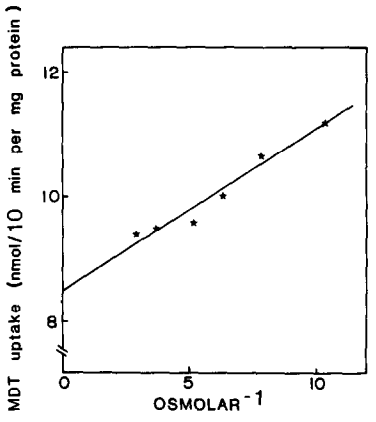

Fig. 2. Relation between medium osmolarity and methyldeptropine uptake. The uptake of methyldeptropine (0.15 mM) in the presence of 125 mM $NaNO_3$ was measured after 10 min of incubation. Medium osmolarity was manipulated with various concentrations of sucrose. Uptake was terminated using a medium to which sucrose had been added in order to obtain a corresponding osmolarity. Means of five experiments are given. Osmolarity refers to the change in osmolarity produced by sucrose.

extravesicular) under equilibrium conditions amounts to 8.5 nmole/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 125 mM $NaNO_3$ or KNO_3. No differences could be found between the initial influx rate in the presence of KNO_3 or $NaNO_3$ or in the presence or the absence of a gradient of these two salts. Moreover no saturation was observed at concentrations up to 9 mM.

Also the influence of the temperature on the uptake of methyldeptropine in the presence of a 125 mM $NaNO_3$ gradient was investigated. At 0° initially the uptake values are somewhat lower, but the same uptake level is reached after 5 min (Fig. 3).

The influence of the possible inhibitor choline, a quaternary ammonium compound, was investigated. However 125 mM choline (Fig. 1) showed no inhibitory effect on the uptake of methyldeptropine.

Finally the influence of a carrier-blocking agent, the thiol-reagent NEM, was investigated (Fig. 3). This figure shows that 0.5 mM NEM had no influence on the methyldeptropine uptake.

From these observations it can be concluded that transport of methyldeptropine through the rat hepatocyte plasma membrane is not carrier-mediated.

Influence of an excess of anions. The previous findings suggest, that transport of methyldeptropine through the rat hepatocyte plasma membrane occurs by a passive and electroneutral process, for instance as an ion-pair together with its own counterion (iodine) or an anion present in the reaction mixture (nitrate and/or chloride). 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 5 mM I^- did not change the uptake pattern for methyldeptropine in the presence of a 125 mM $NaNO_3$ gradient (Fig. 3), nor induced 5 mM I^- the uptake of the impermeable organic cation methylatropine. This negative result could be due to the high concentration of NO_3^- and/or Cl^- in the reaction mixture. As shown in a previous paper [10], these anions, if present in sufficiently high concentration, are capable of increasing the octanol–water partition coefficient for a number of quaternary ammonium compounds. Omittance of Cl^- and/or NO_3^- from the reaction mixture appeared to be impossible, since that would lead to high levels of binding of methyldeptropine to the vesicle preparation.

Taurocholate (5 mM, see Fig. 3) decreased the

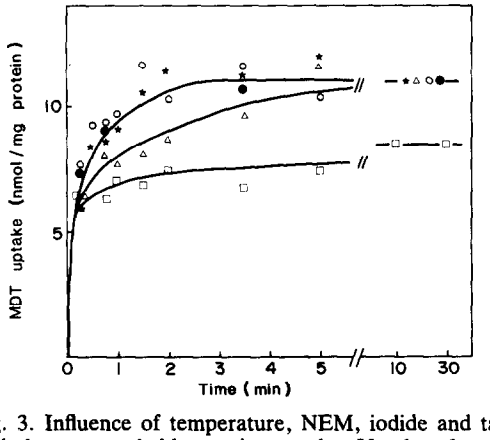

Fig. 3. Influence of temperature, NEM, iodide and taurocholate on methyldeptropine uptake. Uptake of methyldeptropine (0.15 mM) was measured in the presence of a 125 mM $NaNO_3$ gradient at 25° (★) or at 0° (△), and in the presence of a 125 mM $NaNO_3$ gradient plus 0.5 mM NEM (○) or 5 mM I^- (●) or 5 mM taurocholate (□).

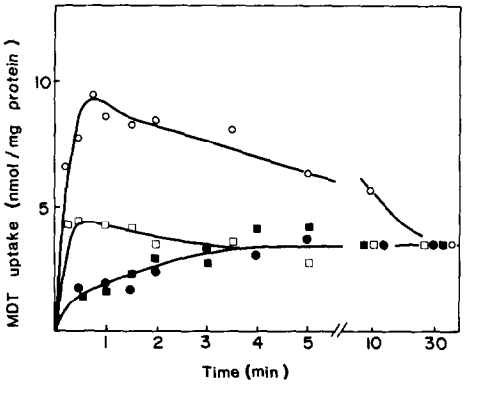

Fig. 4. Influence of TPB^- on methyldeptropine uptake. Uptake of methyldeptropine (0.15 mM) plus TPB^- (2 μM) was measured in response to a 125 mM $NaNO_3$ gradient (○), 125 mM $NaNO_3$ no gradient (●), 125 mM $NaNO_3$ gradient plus 2.5 μM FCCP (□), 125 mM $NaNO_3$ no gradient plus 2.5 μM FCCP (■).

uptake of methyldeptropine; the equilibrium uptake value was lowered from 11.0 to 8.5 nmole/mg protein, the same value as the total binding in this vesicle preparation. It seems therefore that 5 mM taurocholate solubilizes the vesicles. The same effect of taurocholate was found in isolated hepatocytes [12] where this bile salt decreased the uptake of a number of quaternary ammonium compounds in a non-specific way.

In addition the effect of a catalytic amount of TPB^- (2 μM) was investigated. To measure transport of some organic cations in response to a transmembrane electrical potential difference, trace amounts of this organic anion appear to be necessary in certain membrane vesicle preparations [13–15]. The possible role of this anion has been considered by Haydon and Hladky [16]. Figure 4 shows the uptake pattern of 150 μM methyldeptropine plus 2 μM TPB^- under various experimental conditions. TPB^- appears to have two effects: firstly it lowers the equilibrium uptake value considerably, but more importantly uptake of methyldeptropine now appears to be dependent on the presence of a transmembrane electrical potential difference. A transient uptake is observed in the presence of a gradient of NO_3^-. Pre-equilibration for 30 min of the vesicles in the presence of $NaNO_3$ or dissipation of the transmembrane electrical potential difference by the addition of 2.5 μM FCCP abolishes the transient uptake. The disappearance of this overshoot phenomenon in the presence of FCCP is not due to some other effect of FCCP (e.g. an influence on the binding of methyldeptropine to the vesicles or a solubilizing effect), since in the presence of $NaNO_3$ (no gradient) no difference is measured in the methyldeptropine uptake whether FCCP is present or absent (Fig. 4). 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 in the presence of 2 μM TPB^-. Equilibrium uptake of methyldeptropine (0.15 mM) measured after 10 min in the presence of 125 mM $NaNO_3$ plus 2 μM TPB^- appeared to be dependent on the osmolarity of the incubation medium (Fig. 5) and consequently methyldeptropine is also under these conditions taken up into an osmotically active space.

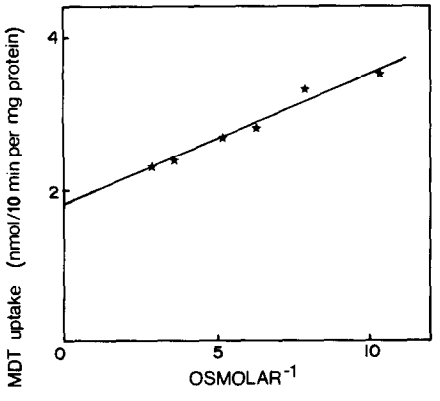

Fig. 5. Relation between medium osmolarity and methyldeptropine uptake in the presence of TPB^-. The uptake of methyldeptropine (0.15 mM) in the presence of 125 mM $NaNO_3$ plus 2 μM TPB^- was measured after 10 min of incubation. Medium osmolarity was manipulated with various concentrations of sucrose. Uptake was terminated using a medium to which sucrose had been added in order to obtain a corresponding osmolarity. Means of five experiments are given. Osmolarity refers to the change in osmolarity produced by sucrose.

While methyldeptropine equilibrates passively according to a transmembrane electrical potential difference without TPB^- in bacterial membrane vesicles [9] and in rat intestinal brush border membrane vesicles [10], in rat liver plasma membrane vesicles a catalytic amount of TPB^- has to be present. For transport of organic cations in their charged form through lipid bilayers derived from egg-lecithin the presence of trace amounts of TPB^- appeared also to be necessary [17].

In conclusion, the transport of quaternary ammonium compounds across the hepatocyte plasma membrane is only possible for a limited number of organic cations. A high lipophilicity seems to be an essential prerequisite. Transport appeared to occur passively and was not stimulated by a transmembrane electrical potential difference. However in the presence of a catalytic amount of TPB^- a transmembrane electrical potential difference (inside negative) stimulated the uptake. This indicates, that in the presence of TPB^- methyldeptropine can pass the hepatocyte plasma membrane in the form of the charged ion and equilibrates passively according to this $\Delta\psi$. In other membrane vesicle preparations it has been shown that methyldeptropine can be used to estimate transmembrane electrical potential differences [9, 10]. The results of this paper indicate that in the presence of TPB^- this compound could be used to estimate the transmembrane electrical potential difference in rat hepatocyte plasma membrane preparations under different experimental conditions.

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