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CHARACTERISTICS OF THE TRANSPORT OF THE QUATERNARY AMMONIUM 1-METHYL-4-PHENYLPYRIDINIUM BY CHROMAFFIN GRANULES

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Abstract—1-Methyl-4-phenylpyridinium (MPP^+), an active metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine which induces Parkinson's disease in man, is a substrate of the monoamine uptake system of chromaffin granules. It is accumulated without chemical modification by bovine chromaffin granule membrane vesicles in the presence of ATP. The transport is saturable and is characterized by a K_m value of 0.8 μM at pH 8.0, similar to that of serotonin (5-HT). Transport occurs through the monoamine transporter since it is competitively inhibited by 5-HT and since MPP^+ competitively inhibits [^{3}H]5-HT uptake. Moreover, [^{3}H]MPP^+ uptake is blocked by the monoamine transporter inhibitors tetrabenazine and reserpine. Finally, MPP^+ efficiently displaces [^{3}H]reserpine and [^{3}H]dihydrotetrabenazine from their binding sites on the transporter. In the pH range 6–8, the K_m for [^{3}H]MPP^+ uptake and the EC_{50} of MPP^+ for the displacement of [^{3}H]dihydrotetrabenazine decrease logarithmically with the pH. MPP^+ is the first quaternary ammonium salt shown to be a substrate of the monoamine transporter and it has the same pH-dependency as monoamines.

In chromaffin granules and in other monoaminergic vesicles, monoamine uptake is an ATP-dependent active transport which involves: (i) an ATP-dependent H^+-pump generating a proton electrochemical gradient (inside acidic and positively charged) [1–3], and (ii) a monoamine transporter, which utilizes the proton electrochemical gradient to accumulate monoamines [4–8]. The monoamine transporter is inhibited by tetrabenazine (TBZ)* and reserpine (RES) [9, 10], and the specific binding of [^{3}H]dihydrotetrabenazine ([^{3}H]TBZOH) [11] and of [^{3}H]RES [12, 13] to the transporter has been described.

The vesicular monoamine transporter has a broad specificity. In contrast with uptake processes at the plasma membrane level, the same system translocates 5-HT and the different catecholamines [14, 15]. Meta-iodobenzylguanidine (MIBG), a compound structurally different from catecholamines and which has been developed as an adrenal medulla imaging agent, is also a substrate of the vesicle transporter [16]. Moreover, the quaternary ammonium, 1-methyl-4-phenylpyridinium (MPP^+) has been recently shown to be another substrate of this transporter [17, 18]. MPP^+ is a metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [19]. This toxin, when administered to humans or to various mammals, is metabolized to MPP^+ by monoamine oxidase B in non-neuronal cells [19]. MPP^+ is then accumulated in dopaminergic neurons via the neuronal uptake system, thus causing neuronal death and appearance of Parkinsonian symptoms [20].

Because MPP^+ is the first quaternary ammonium salt shown to be a substrate of the vesicular monoamine transporter, the characteristics of MPP^+ uptake and the effect of this compound on the binding of the specific ligands [^{3}H]TBZOH and [^{3}H]RES were investigated in detail.

MATERIALS AND METHODS

Chemicals

[^{3}H]MPP^+ (85 Ci/mmol) and [^{3}H]RES (13 Ci/mmol) were purchased from New England Nuclear (Boston, MA); [^{3}H]TBZOH (15 Ci/mmol) was from CEA (Saclay, France); [^{3}H]5-HT (12.3 Ci/mmol) was obtained from Amersham International (U.K.). MPP^+ was purchased from Research Biochemicals Inc. (Natick, MA); RES and TBZ were from Fluka (Buchs, Switzerland) and 5-HT from Sigma (St Louis, MO). The radiochemical purity of [^{3}H]MPP^+ was assessed by HPLC using a NOVA PAK C-18 column (Waters, Milford, MA), and H_2O–PIC Reagent B-7 (Waters)–acetonitrile (72.5:2.5:25) as solvent. MPP^+ standards were detected spectrophotometrically by their absorbance at 286 nm; 97% of the applied radioactivity was found at the position of the MPP^+ standard.

* Abbreviations used: MPP^+, 1-methyl-4-phenylpyridinium; 5-HT, 5-hydroxytryptamine (serotonin); MIBG, *meta*-iodobenzylguanidine; TBZ, tetrabenazine; TBZOH, dihydrotetrabenazine (2-hydroxy-3-isobutyl-9, 10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo (*a*) quinolizine); [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine; RES, reserpine; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography.

Chromaffin granule membrane preparation

Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer. Membranes were frozen in liquid nitrogen and then stored at −80° [21, 22]. Protein was measured according to Bradford. with bovine serum albumin as a standard [23].

[^{3}H]MPP$^+$ and [^{3}H]5-HT uptake experiments

[^{3}H]MPP$^+$ uptake was initiated by addition of [^{3}H]MPP$^+$ (1.4–2.4 nM) to membranes (80 μg of protein/ml) preincubated at 30° in 0.3 M sucrose/40 mM HEPES–KOH pH 8.0 containing 2.5 mM ATP–1.3 mM $MgSO_4$. After incubation (5–10 min) at 30°, the medium was diluted 10 times in ice-cold 0.3 M sucrose buffered at the same pH, and was filtered through GF/C filters (Whatman). The filters were washed twice with 2 ml of the dilution medium and their radioactivity was measured by liquid scintillation in Aqualuma (Lumac, The Netherlands).

The same procedure was followed for [^{3}H]5-HT uptake experiments; membrane and [^{3}H]5-HT concentrations were 20 μg of protein/ml and 24 nM, respectively.

Binding of [^{3}H]RES and of [^{3}H]TBZOH

Binding of [^{3}H]RES. Membranes (2 μg of protein/ml) were preincubated for 5 min at 30° in a medium containing 0.3 M sucrose, 40 mM HEPES–KOH pH 8.0, 2.5 mM ATP, 1.3 mM $MgSO_4$ and various concentrations of MPP$^+$ (4.0 ml final volume). [^{3}H]RES (25 pM) was then added and the mixture was incubated for 2 hr at the same temperature. The incubation medium was filtered through Whatman GF/C filters preincubated with 10 μM RES. The filters were then washed twice with 2 ml of 0.3 M sucrose, 20 mM Tris–HCl pH 8.0 containing 10 μM RES, and their radioactivity was measured by liquid scintillation in Aqualuma. Specific binding was obtained by subtracting non-specific binding determined in assays containing 100 nM RES.

Binding of [^{3}H]TBZOH. Membranes (10 μg of protein/ml) were incubated for 1 hr at 30° with 0.45 nM [^{3}H]TBZOH and MPP$^+$ at the indicated concentration in 0.3 M sucrose–50 mM Tris–NaH_2PO_4 at the indicated pH. The washing medium contained 100 μM TBZ. Non-specific binding was determined in assays containing 1 μM TBZ.

MPP$^+$ octanol–buffer partition coefficient

[^{3}H]MPP$^+$ and MPP$^+$ at a final concentration of 1 mM were added to 1 ml of 10 mM KCl in 50 mM Tris–phosphate buffer at various pH. To this aqueous phase, 3 ml of octanol were added, the mixture was stirred for 30 sec and the two phases were separated by centrifugation for 10 min at 2000 rpm. The octanol–buffer partition coefficient was derived from measurements of the radioactivity of the two phases by liquid scintillation. In the same experiment, the partition coefficient was also determined spectrophotometrically: the octanol phase (2.4 ml) was re-extracted by 1.2 ml of water and the absorbance of the aqueous phases was measured at 286 nm. The two determinations gave consistent values.

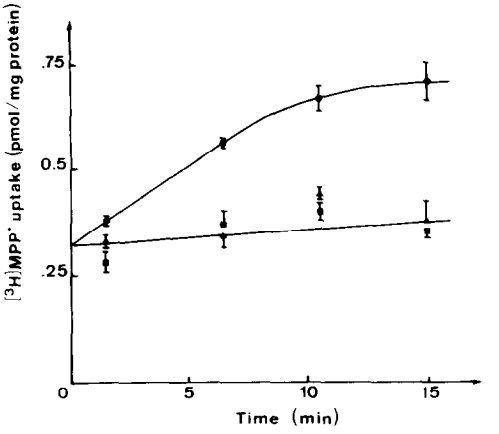

Fig. 1. [^{3}H]MPP$^+$ uptake by chromaffin granule membranes. Membranes were incubated with [^{3}H]MPP$^+$ and ATP–$MgSO_4$ in the absence (●) or in the presence of inhibitors: (▲) 1 μM TBZ; (■) 5 μM, CCCP. Aliquots were filtered at the indicated time. Controls performed in the absence of granule membranes indicated that the radioactivity observed at zero time was due to [^{3}H]MPP$^+$ binding to the filters. Points are the mean of two determinations.

RESULTS

[^{3}H]MPP$^+$ is taken up by chromaffin granule membrane vesicles

Since chromaffin granules contain high concentrations of catecholamines and of ATP, which may obscure uptake and binding data, membrane vesicles derived from purified chromaffin granules were used. In the presence of 2.5 mM ATP–1.3 mM $MgSO_4$, chromaffin granule membranes accumulated [^{3}H]MPP$^+$ in a time-dependent manner (Fig. 1). At concentrations of 50–80 μg/ml of membrane protein, uptake was linear for more than 5 min. The particulate radioactive material was released by an osmotic shock [17], and was thus considered to be transported inside the vesicles. The chemical nature of this material was investigated by HPLC. Vesicles loaded by incubation with ATP–$MgSO_4$ and [^{3}H]MPP$^+$ were centrifuged, extracted with acetonitrile, and the extract analyzed by HPLC (Fig. 2). The tritiated material co-migrated with a MPP$^+$ marker. In the presence of 2.5 μM TBZ, an inhibitor of vesicular monoamine uptake, the size of this peak was markedly decreased.

[^{3}H]MPP$^+$ uptake was not observed when ATP–$MgSO_4$ was omitted [17]. Uptake in the presence of ATP was inhibited by addition of 5 μM carbonylcyanide *m*-chlorophenylhydrazone, a proton ionophore (Fig. 1), thus suggesting that [^{3}H]MPP$^+$ transport was driven by the H^+ electrochemical gradient. The rate of [^{3}H]MPP$^+$ uptake was saturable with respect to substrate concentration and it followed Michaëlis kinetics (Fig. 3). At pH 8.0 and at 30°, K_m and V_{max} values were 0.80 ± 0.17 μM and 136 ± 28 pmol/min/mg of protein (±SE), respectively. Under the same conditions and with the same vesicle preparation, the kinetic parameters of [^{3}H]5-HT uptake were: K_m = 0.35 ± 0.05 μM and V_{max} = 210 ± 27 pmol/min/mg of protein, and those

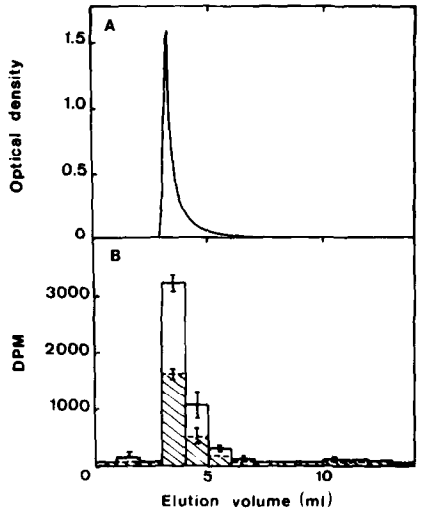

Fig. 2. HPLC analysis of the transported radioactive material. Chromaffin granule membranes (250 μg of protein/ml) were incubated with 8 nM [^{3}H]MPP$^+$ for 10 min at 30° in the absence (solid line) or in the presence (dotted line, hatched bars) of 2 μM TBZ. Samples (0.2 ml) were centrifuged for 5 min at 180,000 *g* and the pellets were resuspended in 50 μl of acetonitrile. The suspension was again centrifuged under the same conditions. Unlabelled MPP$^+$ (8 μl of a 10 mM solution) was added to 12 μl of the supernatant and the mixture was analyzed by HPLC as described in Materials and Methods. Absorbance at 286 nm was measured to detect MPP$^+$ elution (A). The radioactivity of 1 ml fractions was measured by scintillation counting (B).

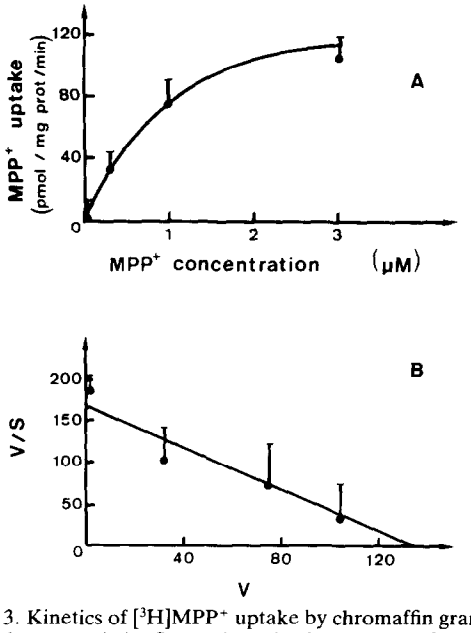

Fig. 3. Kinetics of [^{3}H]MPP$^+$ uptake by chromaffin granule membranes. (A) Saturation isotherm. Uptake was measured at 2, 4 and 6 min at pH 8.0. Non-specific uptake was measured in the presence of 1 μM TBZ and was subtracted. Each point is the mean of 3 determinations. (B) Woolf-Hofster plot of the data. The kinetic parameters, derived by regression analysis, were: $K_m = 0.80 \pm 0.17$ μM and $V_{max} = 136 \pm 28$ pmol/mg of protein/min (± SE). The mean values of K_m and V_{max} obtained in five similar experiments at the same pH were 0.71 ± 0.47 μM and 80 ± 35 pmol/mg of protein/min, respectively (± SE).

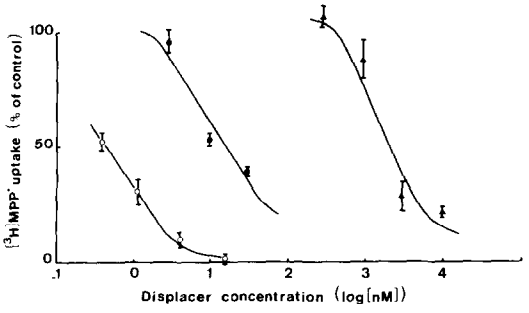

Fig. 4. Inhibition of [^{3}H]MPP$^+$ uptake by RES, TBZ and 5-HT. Chromaffin granule membranes (50 μg of protein/ml) were preincubated for 5 min with ATP–$MgSO_4$ and RES (○), TBZ (●) or 5-HT (▲) at the indicated concentration. [^{3}H]MPP$^+$ (2 nM) was then added and the mixture was incubated for 10 min at 30°. Non-specific uptake was measured in the presence of 1 μM TBZ. Each point is the mean of two determinations. Hill numbers derived from a Hill plot of the data were: RES, $n_H = 0.822 \pm 0.186$ ($r = 0.932$); TBZ, $n_H = 1.485 \pm 0.594$ ($r = 0.834$); 5-HT, $n_H = 1.216 \pm 0.263$ ($r = 0.928$).

of *l*-[^{3}H]noradrenaline were: $K_m = 2.5 \pm 0.2$ μM and $V_{max} = 212 \pm 16$ pmol/min/mg of protein.

Pharmacology of MPP$^+$ uptake

5-HT, a substrate of the monoamine transporter, inhibited ATP-dependent [^{3}H]MPP$^+$ uptake (Fig. 4). From the inhibition curve, a Hill number of 1.21 ± 0.26 ($r = 0.98$) was derived, which indicated an inhibition of the competitive type. The corresponding IC_{50} value, 1.8 μM, was of the same order of magnitude as the K_m for ATP-dependent [^{3}H]5-HT uptake under the same experimental conditions ($K_m = 0.35$ μM, Table 1). It may be noted that MPP$^+$ inhibited [^{3}H]5-HT uptake. In two separate experiments, IC_{50} values of 1.8 and 1.15 μM were measured, which have to be compared to a K_m of 0.8 μM for [^{3}H]MPP$^+$ uptake (Table 1).

[^{3}H]MPP$^+$ uptake was also inhibited by the monoamine transporter inhibitors TBZ and RES (Fig. 4 and Table 1). The corresponding IC_{50} values were similar to those observed for the inhibition of 5-HT uptake (Table 1). A further indication that the monoamine transporter was involved in MPP$^+$ uptake has been gained by the investigation of the effect of MPP$^+$ on [^{3}H]RES and [^{3}H]TBZOH binding. In the presence of ATP–$MgSO_4$, [^{3}H]RES binds to a high affinity site on chromaffin granule

Table 1. Inhibition of [^{3}H]MPP$^+$ and of [^{3}H]5-HT uptake

Inhibitor	Substrate [^{3}H]MPP$^+$ K_m or IC_{50} (μM)	Substrate [^{3}H]5-HT K_m or IC_{50} (μM)
MPP$^+$	0.8	1.5
5-HT	1.8	0.35
TBZ	15×10^{-3}	12×10^{-3}*
RES	0.5×10^{-3}	0.5×10^{-3}*

* B. Gasnier, unpublished data.

Table 2. Inhibition of [^{3}H]RES and [^{3}H]TBZOH binding to the monoamine transporter

Inhibitor	Ligand [^{3}H]RES	Ligand [^{3}H]TBZOH
	K_d or EC_{50} (μM)	
MPP$^+$	6	70
5-HT	2.3*	340†
TBZ	–	3×10^{-3}‡
RES	30×10^{-6}‡	—

* B. Gasnier, unpublished data.
† Ref. [11].
‡ F. Darchen, D. Scherman and J. P. Henry, unpublished data.

membranes (K_d = 30 pM, Darchen, Scherman and Henry, unpublished data), which has been shown to be located on the monoamine transporter. MPP$^+$ displaced [^{3}H]RES from this site. The displacement curve was characterized by an EC_{50} of 6 μM (data not shown), which may be compared to a value of 2.3 μM for [^{3}H]RES displacement by 5-HT (Table 2). MPP$^+$ also displaced [^{3}H]TBZOH from its binding site on the monoamine transporter (see Fig. 7). As was the case for monoamines [24], this effect does not require the presence of ATP. MPP$^+$ was more efficient than 5-HT in displacing [^{3}H]TBZOH since EC_{50} values at pH 7.5 were 70 μM and 340 μM, respectively.

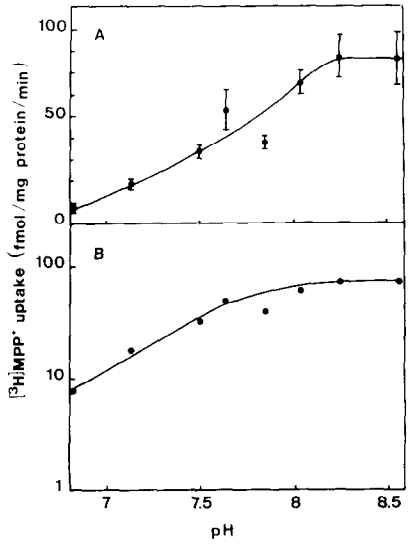

Fig. 5. Effect of the pH on [^{3}H]MPP$^+$ uptake. Uptake experiments were conducted as described in Materials and Methods, except for the pH of the incubation medium. The medium was buffered at the indicated pH value with 50 mM HEPES–KOH. The washing medium was buffered at pH 7.5. (A) Specific uptake as a function of the pH. Non-specific uptake, measured in the presence of 2 μM TBZ was subtracted. Each point is the mean of three determinations. (B) Logarithmic plot of the data.

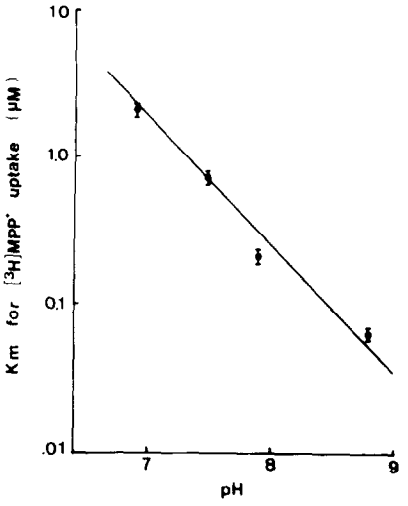

Fig. 6. Effect of the pH on the K_m for [^{3}H]MPP$^+$ uptake. Incubations were buffered at the indicated pH with 50 mM Tris–NaH_2PO_4 buffers. Membranes were incubated for 7 min at 30° with 1.9 nM [^{3}H]MPP$^+$ and various concentrations of unlabeled MPP$^+$. The wash medium was buffered at pH 6.6. K_m values were derived from Woolf–Hofster plots of v/s as a function of v. The regression coefficients of the various lines were: pH 6.9, $r = 0.94$; pH 7.5, $r = 0.81$; pH 7.9, $r = 0.96$; pH 8.8, $r = 1.0$.

Effect of pH on [^{3}H]MPP$^+$ uptake and on displacement of [^{3}H]TBZOH by MPP$^+$

At low [^{3}H]MPP$^+$ concentrations, uptake was pH-dependent (Fig. 5). In two independent experiments, specific uptake, defined as ATP-dependent TBZ-sensitive [^{3}H]MPP$^+$ uptake, was shown to increase with pH from 6.8 to 7.8 and to level off at pH values higher than 8.0. When the log of the rate of uptake was plotted as a function of the pH, a linear relationship characterized by a slope of +1.0 was observed in the first part of the curve (Fig. 5). Because of the low substrate concentration, this experiment suggested an effect of the pH on the Michaëlis constant K_m. This parameter was thus measured at four pH values ranging from 7 to 9; it decreased from 25 μM at pH 6.9 to 0.6 μM at pH 8.8. The log of K_m varied linearly with the pH, with a slope of −1.0 (Fig. 6). This result is similar to that previously reported for noradrenaline [25, 26].

The efficiency of MPP$^+$ in displacing [^{3}H]TBZOH from its site was also affected by the pH (Fig. 7). The EC_{50} value decreased from 200 μM at pH 7.0 to 5 μM at pH 8.75. As for the kinetic parameter K_m, the log of EC_{50} varied linearly with the pH, with a slope of −1.0 (Fig. 7, inset). Noradrenaline has been previously reported to displace [^{3}H]TBZOH in a similar pH-dependent manner [24].

Monoamines such as noradrenaline are characterized by a pH-dependent lipophilicity, resulting from their acido-basic properties. Such a dependency, which is important in consideration of the interpretation of the effect of pH, was not observed for MPP$^+$. The apparent octanol–buffer partition coefficient of MPP$^+$, which reflects its membrane–buffer partition coefficient, increased only slightly,

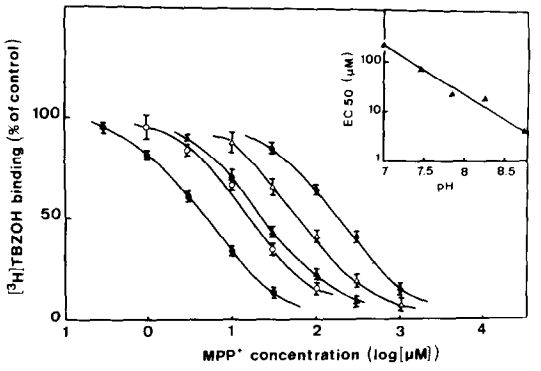

Fig. 7. Effect of the pH on the displacement of [^{3}H]TBZOH by MPP$^+$. pH values of the incubation media were: 6.95, (◆); 7.45, (△); 7.85; (▲); 8.25, (○) and 8.75 (●). Each point is the mean of two determinations. Hill numbers were: pH 6.95, $n_H = 0.974 \pm 0.047$ ($r = 0.994$); pH 7.45, $n_H = 0.95 \pm 0.06$ ($r = 0.985$); pH 7.85, $n_H = 0.933 \pm 0.043$ ($r = 0.992$); pH 8.25, $n_H = 0.921 \pm 0.057$ ($r = 0.986$); pH 8.75, $n_H = 1.042 \pm 0.101$ ($r = 0.974$). Inset: semi-log plot of EC_{50} for displacement of [^{3}H]TBZOH binding by MPP$^+$ at various pH.

from 0.013 at pH 6.6 to 0.017 at pH 8.65 (data not shown).

DISCUSSION

The present data support the contention that MPP$^+$ is a substrate of the vesicular monoamine transporter. MPP$^+$ and 5-HT compete for the same transporter, as shown by the fact that 5-HT inhibited [^{3}H]MPP$^+$ uptake and that MPP$^+$ inhibited [^{3}H]5-HT uptake. Moreover, RES and TBZ inhibited [^{3}H]MPP$^+$ and [^{3}H]5-HT uptake with similar efficiencies. Finally, MPP$^+$ had an affinity for [^{3}H]TBZOH and [^{3}H]RES binding sites comparable or higher than that described for 5-HT. MPP$^+$ is the first quaternary ammonium salt shown to be a substrate of the monoamine transporter. It may be noted that it is also a substrate of the noradrenaline and dopamine uptake systems of the plasma membrane of striatal and cortical neurons [20]. MIBG was also taken up by bovine adrenomedullary cells through the plasma membrane noradrenaline uptake system [27]. Thus, two different compounds structurally unrelated to monoamines, MIBG and MPP$^+$, are substrates of both vesicular and plasmalemmal monoamine uptake systems, a result which may indicate some relationship between the vesicular transporter and the different plasma membrane transporters, in spite of the well-known differences in the pharmacological profiles of these systems.

That MPP$^+$ is a substrate of the vesicular monoamine transporter is surprising since it has been proposed generally that the uncharged form, and not the cationic form, of monoamines was the substrate of the transporter [24–26, 28–30]. It is also surprising that the pH effects observed in the presence of substrates and previously attributed to variations of the concentration of amine neutral form [24–26] are also observed with the permanent cation MPP$^+$ (Figs. 5 and 7).

A possible interpretation of the results observed with MPP$^+$ is that the transported species is not the cation itself, but a related amine, formed in the presence of the chromaffin granule membrane preparation. However, HPLC analysis of the transported material indicated that the accumulated radioactive material was MPP$^+$ (Fig. 2). A hypothesis consistent with the data is the transport of a radical MPP$^\cdot$ obtained by one-electron reduction of MPP$^+$ in the presence of membranes and of endogenous reducing agents, followed by its re-oxidation by molecular oxygen in the vesicle matrix. The radical MPP$^\cdot$ is a tertiary amine, and such amines might be substrates of the transporter under their neutral form. Reduction of MPP$^+$ to MPP$^\cdot$ has been proposed to occur under physiological conditions [31]. Though this hypothesis cannot be completely excluded [32], it is unlikely since the reduction of MPP$^+$ is difficult [33] and is not observed in the presence of rat liver microsomes and NADPH [34]. Moreover, [^{3}H]MPP$^+$ uptake was neither inhibited by oxidizing agents such as ferricyanide or NAD$^+$ nor stimulated by reducing agents such as NADH, dithionite or ferrocyanide at 50 μM concentration (data not shown).

In view of the results obtained with MPP$^+$, the hypothesis of transport of the neutral form of monoamines deserves a critical examination. This hypothesis was consistent with the linear decrease with pH of the log of K_m for noradrenaline [25, 26]. This observation was interpreted as originating in the variation with pH of the concentration of the neutral form of the monoamines at pH values below their pK_a. Since the same observation was made with MPP$^+$ (Fig. 6), the hypothesis is untenable. An alternate possibility is that the limiting factor was the concentration of substrate in the lipidic phase, regardless of the ionization state of the monoamine, the charged and the neutral form both being substrates. The pH-dependency of noradrenaline uptake would thus have only reflected the larger solubility in the membrane phase of the neutral form of the monoamine [35]. Extending this type of hypothesis to MPP$^+$ would lead to the suggestion that the solubility of MPP$^+$ in the membrane phase is the limiting factor and that this solubility is controlled by the pH. Such a control might result from the formation of an ion pair between MPP$^+$ and hydroxyl ions, which would increase the solubility of MPP$^+$ in the membrane phase. However, this possibility appears unlikely since the octanol–buffer partition coefficient of MPP$^+$, which should reflect its membrane–buffer partition coefficient, seems to be pH-independent.

A third possibility is that the ionizable group responsible for the K_m effect on MPP$^+$ uptake is not borne by the substrate, but rather by the transporter itself, and that protonation of an acido-basic residue of the protein results in an inhibition of uptake. Thus, the direct relationship existing between the apparent K_m for the substrate and the H$^+$ concentration would indicate a competition between cationic substrates and protons. The linearity of this relationship in the 6–8 pH range would suggest that the ionizable group has a pK higher than 8. It may be noted that the protonation of an acido-basic resi-

due of the protein has already been proposed to account for the proton antiport associated with monoamine uptake [36] and for the functional asymmetry of the monoamine transporter [37].

However, the hypothesis of the transport of the neutral form of monoamines was consistent not only with the pH-dependency of the apparent K_m, but also with that of the inhibition constants K_i of the inhibitors TBZ and TBZOH [24, 28]. These tertiary amines have a rather low pK_a (6.0 for TBZ and 7.5 for TBZOH) and their activity was shown to be correlated with the concentration of their neutral form [28]. The affinity constant of the transporter for TBZOH did not show a logarithmic variation with pH in the 6–9 pH range, but levelled off at pH values higher than the pK_a of TBZOH [24]. Finally, the potency of noradrenaline in competitively displacing [^{3}H]TBZOH increased logarithmically with the pH [24] and this observation was attributed to the increase with pH of the concentration of noradrenaline neutral form. Since the latter effect is also observed with MPP^+, it should be interpreted in a different way. A possible hypothesis is the involvement of the ionizable group of the transporter responsible for the K_m effect. It may be suggested that this group is located at the substrate binding site or in its vicinity and that the inhibitor binding site overlays only partially the substrate binding site. Binding of inhibitors would be controlled by their solubility in the membrane phase. Since the neutral form of these inhibitors is by far the more soluble in this phase, its binding would be favoured. Such a neutral molecule would not experience the effect of the ionizable group of the protein, thus accounting for the pH-dependency of TBZOH binding and activity. On the other hand, substrates such as noradrenaline and MPP^+ would displace inhibitors, and their binding would be affected by the putative protein ionizable group. This group might be negatively charged and might bind the positively charged substrates by an ionic interaction. It might also be a protonated amine residue, which would exert a repulsive inhibitory effect towards cationic substrates.

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