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Characteristics of tetraethylammonium transport in rabbit renal plasma-membrane vesicles

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Transport of [^{14}C]tetraethylammonium (TEA), an organic cation, was studied in brush-border (BBMV) and basolateral (BLMV) membrane vesicles isolated from rabbit kidney cortex. In BBMV, the presence of an outwardly directed H^+ gradient induced a marked stimulation of TEA uptake against its concentration gradient (overshoot phenomenon), whereas a valinomycin-induced inside-negative potential had no effect on TEA uptake. In BLMV, TEA uptake was significantly stimulated by the presence of an outwardly directed H^+ gradient and by an inside-negative potential, but the effect of H^+ gradient was absent when the vesicles were chemically 'voltage clamped'. In BBMV, internal H^+ stimulated TEA uptake in a non-competitive manner by binding at a site with apparent pK_a of 6.87. External H^+ inhibited TEA uptake through a direct interaction with the putative H^+/organic-cation exchanger at a site with apparent pK_a of 6.78. Changing external pH while maintaining the pH gradient constant produced a result similar to that obtained by changing external pH alone. Increasing external H^+ showed a mixed-type inhibition of TEA uptake. These results suggest that in the rabbit TEA transport across the basolateral membranes is driven by an inside-negative potential and that transport across the brush-border membrane is driven by a H^+ gradient via an electroneutral H^+/TEA antiport system.

INTRODUCTION

Many organic cations are actively secreted by the proximal tubule of the mammalian kidney (Rennick, 1972, 1976). Secretion requires uptake from blood into the cell across the basolateral membrane of the proximal-tubule epithelium and subsequent exit into the urine across the brush-border membrane. However, the molecular mechanisms by which organic cations are transported through both membranes have not been completely elucidated.

Schäli *et al.* (1983) and Tarloff & Brand (1986) found that isolated rabbit proximal tubules can develop a tissue-to-medium concentration gradient for tetraethylammonium (TEA) far in excess of that expected to occur on the basis of a passive distribution. Schäli *et al.* (1983) found no evidence for a carrier-mediated lumen-to-bath flux, and concluded that the transport of TEA across the basolateral membrane was active, but that across the luminal membrane was passive. On the other hand, studies with brush-border membrane vesicles (BBMV) isolated from the kidney cortex of several species, including rabbit, have suggested that organic cations are actively transported by H^+/organic-cation exchange process across the brush-border membrane and that a H^+ gradient is necessary for driving the uphill organic-cation transport in intact tubules (Holohan & Ross, 1981; Takano *et al.*, 1984; Wright, 1985; Rafizadeh *et al.*, 1987). The H^+ gradient is created by the Na^+/H^+ antiport system known to be present in the apical membrane. Little is known, however, about interaction of H^+ with the H^+/organic-cation exchange system.

In this study, we examined the interaction of H^+ with the H^+/organic-cation (TEA) exchange system in rabbit BBMV. Our results show that in BBMV internal H^+ increases TEA uptake in a non-competitive fashion, and external H^+ produces a mixed-type inhibition.

MATERIALS AND METHODS

Isolation of plasma-membrane vesicles

BBMV and basolateral-membrane vesicles (BLMV) were isolated from the renal cortex of rabbits by Percoll-density-gradient centrifugation and Mg^{2+} precipitation. These methods are routinely used and extensively documented (Goldinger *et al.*, 1984).

The membrane vesicles obtained by the above method were comparable with those preparations reported in the literature. The vesicles were resuspended in vesicle buffer, and the final volume was adjusted to yield a protein content of the suspension of 6 mg/ml and stored at 0 °C until use. The vesicle buffer had an appropriate ion content for the experimental conditions, as given in the Figure legends. Before transport studies the membranes were preincubated at 37 °C for 30 min. A preliminary study showed that the rate of TEA uptake after preincubation at 37 °C for 30 min was not significantly different from that measured after preincubation at 4 °C for 24 h, as described by others (Wright *et al.*, 1983).

Transport studies

The uptake of [^{14}C]TEA was measured by a rapid filtration technique similar to that described by Berner & Kinne (1976). Briefly, the reaction was initiated by adding membrane vesicles to buffer (a 1 : 10 dilution of membrane vesicle suspension) containing radioactive substrate at 25 °C. The composition of the incubation medium was indicated in the Figure legends. At the stated times,

Abbreviations used: TEA, tetraethylammonium; BBMV, brush-border membrane vesicles; BLMV, basolateral membrane vesicles.

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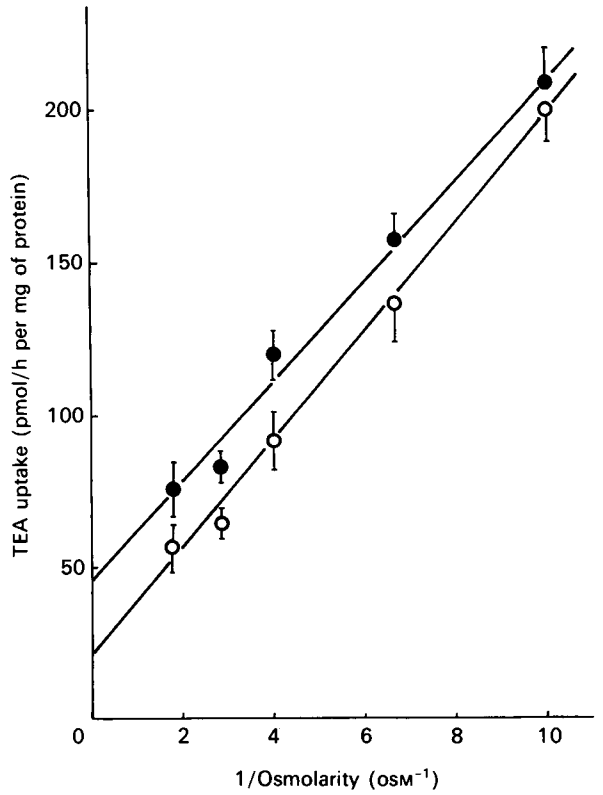

Fig. 1. Effect of osmolarity on TEA uptake by BBMV (○) and BLMV (●)

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl/10 mM-Hepes/Tris (pH 7.5) and incubated for 60 min in media containing 100 mM-mannitol, 100 mM-KCl, 50 μM-[^{14}C]TEA, 10 mM-Hepes/Tris (pH 7.5) and various concentrations of sucrose. Osmolarity refers to the osmolarity of impermeant solutes (mannitol and sucrose). Each point represents the mean ± S.E.M. for three determinations.

100 μl samples were taken and quickly filtered under vacuum through Millipore filters (HAWP; 0.45 μm pore size; 25 mm diameter). Filters were washed with 5 ml of ice-cold stop solution, containing 100 mM-mannitol, 100 mM-KCl and 10 mM-Hepes/Tris (pH 7.5). Scintillation fluid (Filter count, Packard) was added, and the amount of radioactivity taken up by vesicles was determined by liquid-scintillation spectrometry (Packard Tricarb 300C). No sample quenching was ever observed. Non-specific binding was determined by the same filtration procedure, except that vesicles were lysed with 0.1% deoxycholic acid. Radioactivity determined in this manner was assumed to be bound to the membrane or filter rather than taken up, and was subtracted from uptake data.

Protein was determined by the method of Bradford (1976), with γ-globulin as a standard. Data are reported as uptake per mg of vesicle protein and were analysed statistically by Student's t test. P values less than 0.05 were considered significant.

Materials

[^{14}C]TEA (3.7 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Valinomycin was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of the highest purity available.

RESULTS

Basic characteristics of TEA transport in BBMV and BLMV

Na^+–K^+-ATPase and alkaline phosphatase are well-established markers for BLMV and BBMV respectively. The purity of the prepared membrane vesicles was measured by comparing the enzyme activities of alkaline phosphatase and of Na^+–K^+-ATPase in the homogenate and the final vesicle preparation. The enrichment of Na^+–K^+-ATPase in BLMV and that of alkaline phosphatase in BBMV was 23- and 24-fold respectively. In Fig. 1 TEA uptake at equilibrium was measured with respect to the inverse of the osmolarity of the incubation medium. Increasing concentrations of an impermeant solute (sucrose) in the incubation medium decreased the TEA uptake in both BBMV and BLMV, indicating that TEA uptake represented transport into an intravesicular space. Extrapolating TEA uptake to infinite osmolarity suggests that binding comprised 11 and 22% of the non-corrected uptake value for BBMV and BLMV, respectively, under the incubation conditions normally used.

Fig. 2 shows the effect of a H^+ gradient on TEA transport in BBMV and BLMV. In BBMV the imposition of an outwardly directed H^+ gradient produced a marked stimulation and a transient overshoot of TEA uptake above the equilibrium value (Fig. 2*a*). An outwardly directed H^+ gradient also modestly increased TEA uptake in BLMV (Fig. 2*b*), but an overshoot phenomenon was not observed.

TEA, a quaternary ammonium compound, is posi-

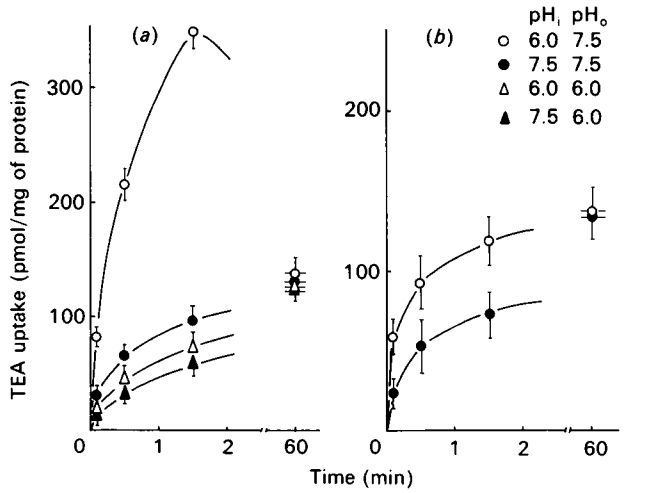

Fig. 2. Effect of H^+ gradient on TEA uptake by BBMV (*a*) and BLMV (*b*)

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris (pH 6.0) (○, △) or 10 mM-Hepes/Tris (pH 7.5) (●, ▲), and incubated in media containing 100 mM-mannitol, 100 mM-KCl, 50 μM-[^{14}C]TEA and either 10 mM-Mes/Tris (pH 6.0) (△, ▲) or 10 mM-Hepes/Tris (pH 7.5) (○, ●). Each point represents the mean ± S.E.M. for three determinations.

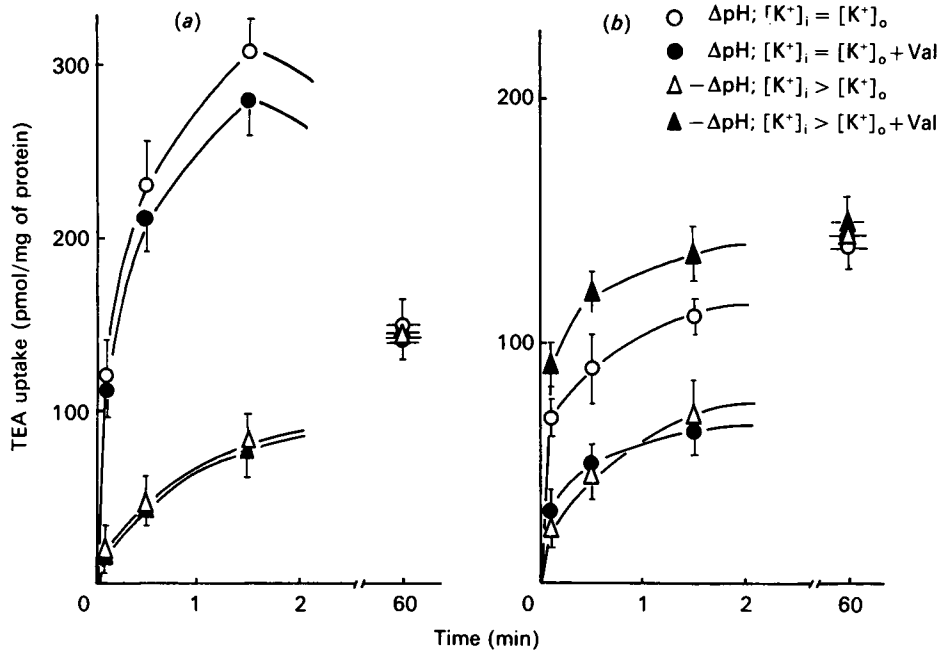

Fig. 3. Effect of valinomycin on TEA uptake by BBMV (*a*) and BLMV (*b*)

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris (pH 6.0) (○, ●) or 10 mM-Hepes/Tris (pH 7.5) (△, ▲), and incubated in media containing 100 mM-mannitol, 10 mM-Hepes/Tris (pH 7.5), 50 μM-[^{14}C]TEA and either 100 mM-KCl (○, ●) or 100 mM-NaCl (△, ▲) in the presence (●, ▲) or the absence (○, △) of valinomycin (Val; 8.7 μg/mg of protein). Each point represents the mean ± S.E.M. for three determinations.

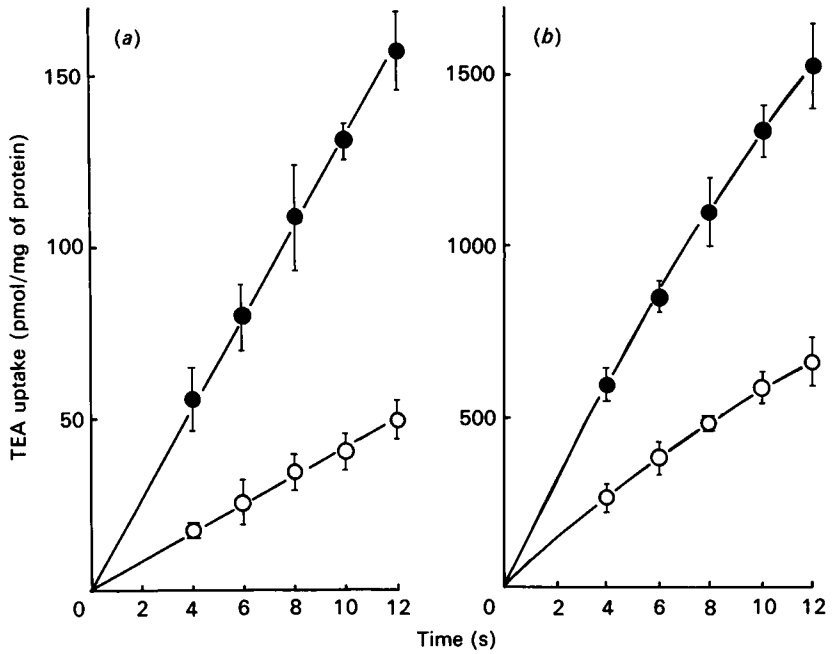

Fig. 4. Time course of uptake of (*a*) 50 μM- or (*b*) 1.8 mM-TEA into BBMV

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris (pH 6.0) (●) or 10 mM-Hepes/Tris (pH 7.5) (○), and incubated in media containing 100 mM-mannitol, 100 mM-KCl, [^{14}C]TEA and 10 mM-Hepes/Tris (pH 7.5). Each point represents the mean ± S.E.M. for four determinations. The line fitted to the TEA uptake was calculated for a quadratic equation (see the text).

tively charged in the range of physiological pH. Thus, it was decided to examine the influence of the electrical potential difference across BBMV and BLMV on the uptake of TEA, since there is a possibility that the stimulation of TEA uptake observed in the presence of pH gradient may be caused by an inside-negative potential secondary to the outward movement of H^+. To test this possibility, vesicles were chemically 'voltage clamped' by including valinomycin (8.7 μg/mg of protein) in the preparation with equal [K^+] (100 mM) inside

and outside the vesicles. Alternatively, the inside potential was made artificially negative by including valinomycin with an outwardly directed [K^+] gradient. As shown in Fig. 3, TEA uptake in BBMV, stimulated by a pH gradient, was not sensitive to the membrane potential. On the other hand, in the BLMV, enhanced TEA uptake appeared to be entirely due to an inside negativity produced by an outwardly directed H^+ or K^+ gradient, since, in the absence of a pH gradient or under voltage clamp, TEA uptake was minimal. The effect of membrane potential on TEA uptake was also examined by manipulating the electrical potential difference across the vesicle membrane by using gradients of permeant anions. Membrane vesicles are presumed to be relatively permeable to thiocyanate, but are relatively impermeable to gluconate (Wright, 1984). The inside-negative potential generated by an inwardly directed SCN^- gradient (100 mM-potassium gluconate inside and 100 mM-KSCN outside) increased TEA uptake in BLMV, but not in BBMV (results not shown). These results indicate that, in the rabbit brush-border membrane, H^+/organic-cation antiporter is present and this process is electroneutral. In contrast, in the basolateral membrane, TEA transport is at least partially driven by an inside-negative potential, and the increase in TEA uptake by the H^+ gradient (in-to-out) shown in Fig. 2(*b*) is not the result of the contamination of BBMV, but is from an inside-negative potential generated by the outward H^+ movement. Proton conductance in the basolateral membrane of kidney proximal tubule was suggested by some investigators (Burckhardt & Frömter, 1980; Sabolic & Burckhardt, 1983).

Effect of internal H^+ on TEA uptake in BBMV

We performed a kinetic analysis of the interaction between H^+ and TEA to define the effects of H^+ on H^+/TEA exchange. The initial rate of TEA transport in BBMV was measured in the presence and the absence of a pH gradient, and the results are shown in Fig. 4. The uptake of 50 μM-TEA was linear from 0 to 10 s in the presence and the absence of a pH gradient (Fig. 4*a*), and so the 10 s time point was used to estimate initial rates of TEA uptake. The uptake of 1.8 mM-TEA was not linear from 0 to 10 s (Fig. 4*b*), but slightly curvilinear, which may be described by the quadratic equation $U = a_0 + a_1 t + a_2 t^2$, where U is the uptake at time t, and a_0 is the estimate of initial rate (Jacquez, 1980). Since, however, the rate of TEA uptake at 10 s was only slightly different (18.2 and 16.3 % in the presence and the absence of a pH gradient, respectively) from the true initial rate estimated by using the above equation, we have used the uncorrected data as the initial rates of the uptake. The data under these circumstances are slight underestimates.

In Fig. 5, the initial rate of TEA uptake into BBMV as a function of different intravesicular pH values (pH_i = 5.5–8.0) at a fixed external pH of 8.0 was measured. TEA uptake was significantly increased by increasing internal H^+ concentrations. The Hill equation derived by Miller & Pollock (1987) was used for the data analysis: $v = V_{max.}/[10^{(pH \times h - pK_H)} + 1]$, where h has the same meaning as in the conventional Hill equation (Segel, 1975) and pK_H is the negative logarithm of the coefficient K_H. The data from pH 5.5 to 7.5 were corrected for the passive component of [^{14}C]TEA uptake measured in the presence of 25 mM unlabelled TEA. A non-linear-regression analysis of the corrected data produced values for h of 0.89, pK_H of 6.11 and $V_{max.}$ of 275 pmol/10 s per

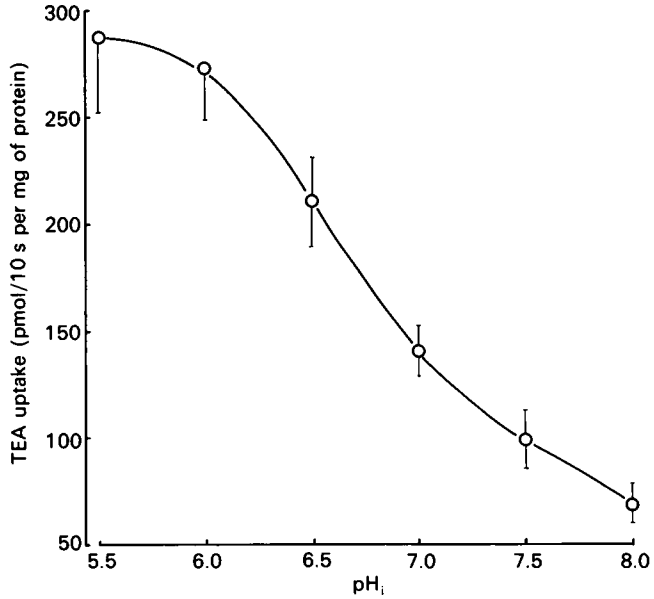

Fig. 5. Dependence of TEA uptake in rabbit BBMV on internal pH

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris or 10 mM-Hepes/Tris at different pH values, and incubated for 10 s in media containing 100 mM-mannitol, 100 mM-KCl, 50 μM-[^{14}C]TEA and 10 mM-Hepes/Tris (pH 8.0). Each value represents the mean ± S.E.M. for three determinations.

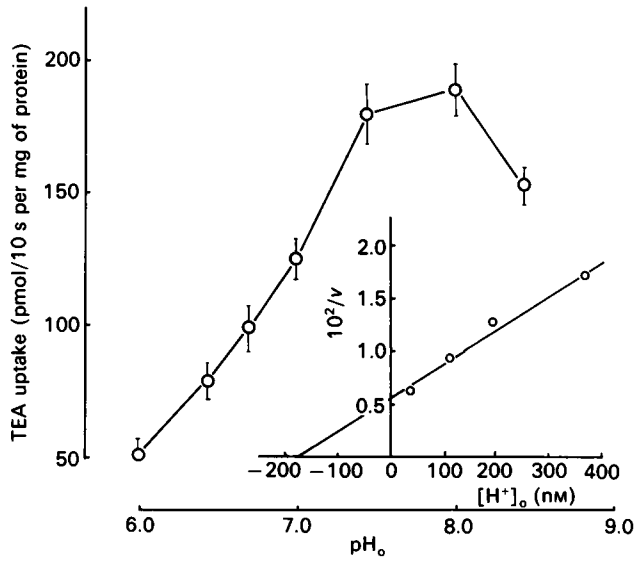

Fig. 6. Effect of external pH on TEA uptake in BBMV

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl/10 mM-Mes/Tris (pH 6.0), and incubated for 10 s in media containing 100 mM-mannitol, 100 mM-KCl, 50 μM-[^{14}C]TEA and either 10 mM-Mes/Tris or 10 mM-Hepes/Tris at different pH values. Each value represents the mean ± S.E.M. for three determinations. In the inset, the data from external pH (pH_o) 6.43 to 7.45 were plotted in the form of $1/v$ versus [H^+]$_o$ after correction for the passive component measured in the presence of 25 mM unlabelled TEA. The regression line was drawn by the least-squares method.

mg of protein. The value for pK_a^i (pH_i for $v = 0.5\ V_{max.}$) of 6.87 was calculated from values of pK_H and h by using the equation: $pK_a^i = pK_H/h$. These results suggest that the H^+/organic-cation exchanger contains only a single internal H^+-binding site, having an apparent pK_a of 6.87.

Effect of external H^+ on TEA influx in BBMV

The TEA uptake into BBMV with an internal pH of 6.0 was measured as a function of external pH. As shown in Fig. 6, the TEA uptake is biphasic, increasing with increasing external pH from 6.0 to 8.0, and then decreases. This result was similar to that reported in reconstituted phospholipid vesicles by Holohan *et al.* (1979). Although the value of $V_{max.}$ cannot be determined accurately since inhibition is observed at high external pH, a plot of the data from external pH 6.43 to 7.45 in the form $1/v$ versus external $[H^+]$ (Fig. 6 inset), after correction for the passive component of [^{14}C]TEA uptake (which was measured in the presence of 25 mM unlabelled TEA), yielded a straight line ($r = 0.989$), and the apparent K_H and $V_{max.}$ were 175 nM and 175 pmol/10 s per mg of protein respectively. The calculated Hill coefficient was 0.93, suggesting the presence of a single external H^+-binding site with an apparent pK_a of 6.78. The equilibrium uptake of TEA at 60 min was not affected in this range of external pH (results not shown).

In Fig. 7, the influence of external pH on TEA uptake with a constant pH gradient was measured in order to analyse the effect of external pH itself on the activity of the H^+/TEA antiport. Changing external pH with a constant pH gradient produced curves which have a pattern similar to Fig. 6. When TEA uptake was measured under these conditions in the presence of 50 mM-choline, under which circumstance carrier-mediated transport of TEA is presumed to be completely inhibited, any pH-dependency of TEA uptake was not observed. These results indicate that pH itself as well as the pH gradient affect carrier-mediated TEA transport in the brush-border membranes.

Kinetic analysis of the interaction of H^+ with H^+/organic-cation antiporter

To examine the nature of the stimulatory effect of internal H^+ in BBMV, the initial rate of TEA uptake into vesicles with internal pH 6.0 or 7.5 was determined as a function of external TEA concentration at a fixed external pH of 7.5. In both conditions, TEA uptake was saturable. The uptake rate was corrected for the non-saturable component by assuming that uptake observed at very high substrate concentrations is attributable entirely to diffusion. Uptake was measured at a TEA concentration of 25 mM (approx. 50 times the K_m), from which an apparent diffusion coefficient was calculated. The diffusive component of TEA was then subtracted from the total influx rate of TEA at lower substrate concentrations. Lineweaver–Burk plots of these data are illustrated in Fig. 8. The plots were fitted by the least-squares method. Increasing the intravesicular H^+ concentration from pH 7.5 to 6.0 significantly increased the apparent $V_{max.}$ (6.15 to 10.87 nmol of TEA/min per mg of protein) without a significant change in the apparent K_m (0.59 to 0.56 mM), consistent with a typical non-competitive interaction.

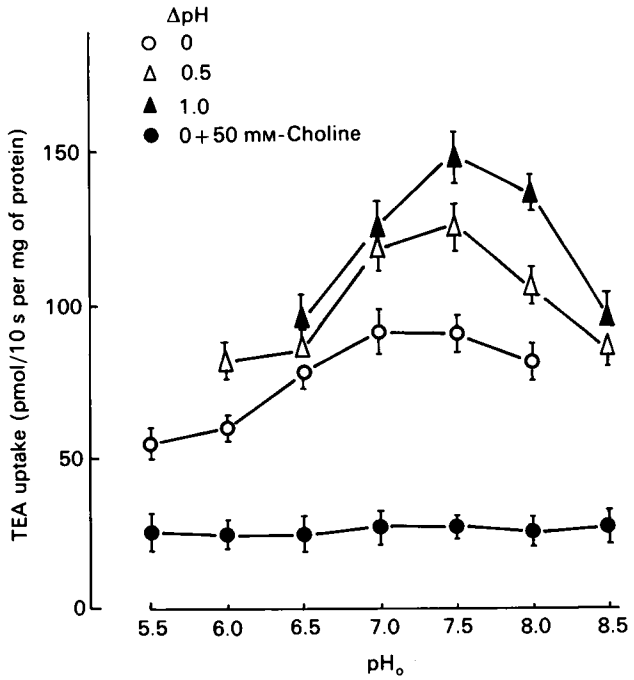

Fig. 7. Effect of external pH on TEA uptake by BBMV with a constant outward-directed pH gradient (ΔpH)

Membrane vesicles were suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris or 10 mM-Hepes/Tris at different pH values. They were incubated for 10 s in media containing 100 mM-mannitol, 100 mM-KCl, 50 μM-[^{14}C]TEA and either 10 mM-Mes/Tris or 10 mM-Hepes/Tris at different pH values. The ● symbols represent TEA uptake in the absence of a pH gradient and in the presence of 50 mM-choline instead of 100 mM-mannitol. Each value represents the mean ± S.E.M. for five determinations.

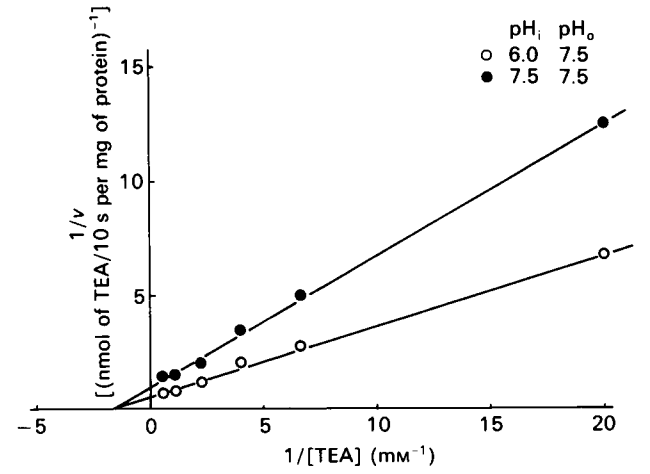

Fig. 8. Effect of internal pH on the kinetics of TEA uptake by BBMV

Membrane vesicles suspended in 100 mM-mannitol/100 mM-KCl in either 10 mM-Mes/Tris (pH 6.0) (○) or 10 mM-Hepes/Tris (pH 7.5) (●) were incubated for 10 s in media containing 100 mM-mannitol, 100 mM-KCl, 10 mM-Hepes/Tris (pH 7.5) and various concentrations of [^{14}C]TEA. The Figure shows a double-reciprocal plot of the data. The regression lines were drawn by the least-squares method. Each point represents the mean ± S.E.M. for four determinations.

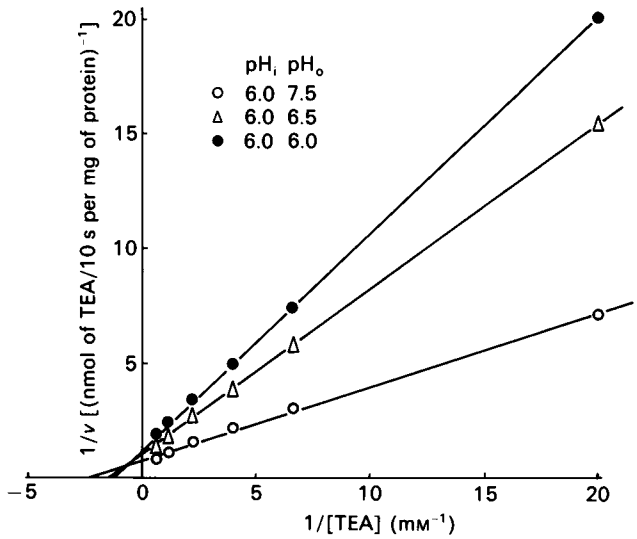

Fig. 9. Effect of external pH on the kinetics of TEA uptake by BBMV

Membrane vesicles suspended in 100 mM-mannitol/100 mM-KCl/10 mM-Mes/Tris (pH 6.0) were incubated for 10 s in media containing 100 mM-mannitol, 100 mM-KCl, either 10 mM-Mes/Tris (pH 6.0–6.5) or 10 mM-Hepes/Tris (pH 7.5), and various concentrations of [^{14}C]TEA. The Figure shows a double-reciprocal plot of the data. The regression lines were drawn by the least-squares method. Each point represents the mean ± S.E.M. for four determinations.

To examine the nature of the inhibitory effect of external H^+, the initial rate of TEA uptake into vesicles with an internal pH of 6.0 was determined as a function of an external TEA concentration at external pH 6.0, 6.5 and 7.5. The rate of uptake was corrected for the non-saturable component by the method described above, and the data were plotted in the form of a Lineweaver–Burk plot (Fig. 9). Raising the external pH increased the apparent $V_{max.}$ (5.13 at pH 6.0, 6.00 at pH 6.5 and 8.57 nmol of TEA/min per mg of protein at pH 7.5) and decreased apparent K_m (0.80 at pH 6.0, 0.72 at pH 6.5 and 0.43 mM at pH 7.5), indicating a mixed-type inhibition.

DISCUSSION

TEA transport in BBMV and BLMV

The mechanisms by which organic cations are actively secreted into the urine by the proximal-tubule epithelium have been the subject of considerable investigation (Rennick, 1981; Holohan & Ross, 1980). However, the individual roles of the apical and the peritubular membranes in the secretory process have not been totally elucidated.

Our results are consistent with the notion that TEA transport across the brush-border membrane is mediated by a H^+/TEA exchange mechanism (Fig. 1*a*), as previously suggested in dog (Holohan & Ross, 1981), rat (Takano *et al.*, 1984) and rabbit (Wright, 1985; Rafizadeh *et al.*, 1987) BBMV, and that transport through the basolateral membrane is mediated by facilitated diffusion which is stimulated by an intravesicular negative membrane potential (Figs. 2 and 3), as described previously in dog (McKinney & Kunnemann, 1985) and rat (Takano *et al.*, 1984) BLMV.

Although it has been reported that the H^+/organic-cation exchange process in the dog (Sokol *et al.*, 1985) and rat (Takano *et al.*, 1984) is electroneutral, the stoichiometry of H^+/organic-cation exchange in rabbit is controversial. Wright (1985) observed that an inside-positive potential in the absence of a pH gradient stimulated NMN transport, and suggested that NMN transport across rabbit BBMV is electrogenic and that the stoichiometry of the H^+/NMN antiporter is 2:1. However, procainamide (McKinney & Kunnemann, 1985) and TEA (Takano *et al.*, 1984) are transported by electroneutral exchange with protons. In the present study, the antiport of H^+/TEA was electroneutral, suggesting a stoichiometry of 1:1.

Interaction of H^+ with H^+/TEA exchange in BBMV

The effect of changes in internal and external pH on TEA transport was measured to define the interaction of H^+ with H^+/TEA exchange in BBMV. Increasing internal pH in the range 5.5–8.0 decreased TEA uptake (Fig. 5). The Hill coefficient of 0.89 was obtained from the data at pH 5.5–7.5. In a kinetic study, raising the internal H^+ concentration also increased apparent $V_{max.}$ without an effect on the apparent K_m for TEA (Fig. 8). These results suggest a single internal H^+-binding site on the H^+/TEA exchanger and that the binding of H^+ at this site does not affect the affinity of the external binding site for TEA.

Increasing external H^+ concentration in the pH range 6.0–8.0 with a constant internal pH 6.0 inhibited exchange of external TEA with internal H^+. A Dixon plot of these data ($1/v$ verses external [H^+]) was reasonably linear (Fig. 6), with a Hill coefficient of 0.93, implying that external protons inhibit TEA uptake by interacting at a single site. When the external pH was changed at a constant transmembrane pH gradient, TEA uptake was maximal at pH 7.5. Similar results were obtained in the absence of a pH gradient (Fig. 7). This result suggests that TEA transport across the luminal membrane is dependent on external H^+ concentration itself, as well as the magnitude of the pH gradient. In kinetic studies, increasing external H^+ decreases the $V_{max.}$ for exchange of external TEA with internal H^+ and increases the apparent K_m (Fig. 9). These results suggest single H^+- and TEA-binding sites, with each having a different locus on the H^+/TEA exchanger.

At high external pH (> 8.0) TEA uptake is inhibited (Fig. 6). This cannot be explained either by the availability of substrate, because TEA has a high pK_a, or by deterioration of vesicles, since the equilibrium uptake was not affected by this range of external pH. These results could be explained by the titration of an essential group in the TEA-binding site of the H^+/organic-cation exchanger.

In conclusion, TEA is transported across the basolateral membrane via a process stimulated by the intracellular negative potential. TEA transport across the brush-border membrane occurs via an electroneutral exchange of TEA for H^+ that has a stoichiometry of 1:1. Increasing the internal vesicular pH inhibits TEA uptake non-competitively. Decreasing the external pH increases the apparent K_m and decreases the apparent $V_{max.}$ of H^+/TEA exchange, indicating a mixed-type inhibition.

The results here are reminiscent of the exchange mechanism suggested for the Na^+:K^+ pump (Sachs,

1977), the adenine nucleotide exchange in mitochondrial inner membranes (Barbour & Chan, 1981) and the $Na^+:Ca^{2+}$ exchanger in mitochondria (Wingrove & Gunter, 1986). In such systems, the carrier (C)·substrate complex (in this case, C·H or C·TEA) can alternately translocate across the membrane; however, the triple complex (C·H·TEA) in this case cannot. Such a system predicts that H^+ and TEA should be mixed-type inhibitors, with linear Dixon plots, as observed in our experiments.

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