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Tetrapentylammonium (TPeA): slowly dissociating inhibitor of the renal peritubular organic cation transporter

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Abstract

The efflux of tetraethylammonium (TEA) from suspensions of rabbit renal proximal tubules is completely blocked by 500 μM tetrapentylammonium (TPeA) in the extracellular medium. The basis of this *trans*-inhibition of TEA transport by TPeA was examined in tubule suspensions. At TPeA concentrations < 10 μM, efflux of TEA was reduced by $\sim 50\%$, whereas at concentrations > 10 μM, TPeA reduced efflux an additional 50% to produce a near complete block of TEA efflux. Increasing concentrations of TPeA from 0–500 μM were found to produce a biphasic, concentration-dependent *trans*-inhibition of TEA efflux from tubule suspensions suggesting that TPeA may block efflux by binding to both a high and low affinity TPeA binding site. The *trans*-inhibition of TEA efflux by TPeA at low concentrations (< 10 μM) may result from a slow carrier turnover when TPeA is bound to the carrier site. To determine whether the inhibitory effectiveness of TPeA was also associated with its slow dissociation from the carrier site, the effect of a 10 s preincubation with 1 μM TPeA on TEA uptake was examined. The uptake of TEA by tubules preincubated for 10 s with TPeA was reduced by ~ 30–50% compared to control tubules not preincubated with TPeA. A 10 s preincubation with 150 μM unlabeled TEA had no effect on TEA uptake compared to control tubules not preincubated with TEA. When the 10 s preincubation with 1 μM TPeA was followed by a 10 min recovery period, TEA uptake returned to control levels, indicating that the prolonged inhibition was reversible. This prolonged inhibition of TEA uptake after a 10 s preincubation with 1 μM TPeA, as suspected, may arise from a slow dissociation of TPeA from the OC transporter following a rapid association to the binding site. TPeA inhibition of TEA uptake into tubules was competitive in nature with a K_i of 1 μM. The ability of TEA to compete with TPeA for binding to the carrier suggests that the binding of TPeA to the carrier can be displaced by large concentrations of TEA. These observations suggest that the interactions of TPeA, and perhaps similarly large hydrophobic OCs, with the OC transporter are complex.

Keywords: Proximal tubule; Tetrapentylammonium; Tetraethylammonium transport; Organic cation transport; (Rabbit kidney)

1. Introduction

The renal secretion of organic cations (OCs) involves their translocation from the blood into the cells of proximal tubules across the peritubular membrane and their subsequent exit across the apical membrane into the urine. The entrance of OCs into proximal cells across the peritubular membrane is believed to result from electrogenic, facilitated diffusion, driven by the transmembrane electrochemical gradient or, alternatively, electroneutral OC/OC exchange, whereas the exit step across the apical membrane involves the electroneutral exchange of an OC for H^+ [1]. Studies using intact perfused proximal tubules indicate that the apical transport step is probably the rate-limiting step in renal secretion of OCs [2]. Recent examination of the kinetics of peritubular OC transport in both single S2 segments and suspensions of renal proximal tubules are consistent with this conclusion: peritubular OC transport appears to have both a high affinity and high capacity for OCs, compared to the apical transport process [3].

The inhibitory interactions of OCs with the peritubular secretory pathway have received limited attention. Ullrich and his colleagues have examined the influence of molecular structure on the inhibition of peritubular transport of N^1-methylnicotinamide (NMN) in microperfused rat proximal tubules [4–7]. Although a general correlation was found between the degree of inhibition of NMN transport

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and the lipophilicity of the test agent, there were notable exceptions. For example, the long chain n-tetraalkylammonium (n-TAA) compounds did not follow this pattern. In contrast, our recent work examined the effect of n-TAA compounds on the transport of the prototypical OC tetraethylammonium (TEA) and observed an increase in inhibitory potency with increasing alkyl chain length [3]. Our study also noted that the relative rates of peritubular transport appeared to decrease with increasing alkyl chain length. The lower affinity n-tetraalkylammoniums, such as tetramethylammonium (TMA) and tetraethylammonium (TEA) at a concentration of 500 μM increased the efflux of [^{14}C]TEA from tubule suspensions, (i.e., *trans*-stimulated), which suggests that these agents are substrates for the OC transporter. However, a 500 μM concentration of tetrapentylammonium (TPeA), a high affinity inhibitor of TEA uptake ($K_{i,app}$ 0.8 μM), completely blocked tubular TEA efflux for as long as 5 min. The ability of TPeA to inhibit carrier-mediated efflux suggests that this compound binds to the OC transporter but that subsequent carrier turnover occurs very slowly, if at all. In the present study, the basis of this *trans*-inhibition of TEA transport by TPeA was examined further in suspensions of rabbit renal proximal tubules.

2. Materials and methods

2.1. Materials

[^{14}C]TEA (56 mCi/mmol) and [^{14}C]PAH (49.6 mCi/mmol) was purchased from New England Nuclear-Dupont (Boston, MA). [^{14}C]Glutarate (19 mCi/mmol) was purchased from ICN Biochemicals (Costa Mesa, CA). Unlabeled TEA and TPeA were purchased from Aldrich (Milwaukee, WI). The source of the remaining chemicals has been reported previously [8].

2.2. Isolation of tubule suspensions

Suspensions of rabbit renal proximal tubules were isolated and purified from New Zealand White rabbits by an enzymatic (collagenase) procedure based on the method of Vinay et al. [9] as modified by Groves et al. [8]. The tubule pellet was resuspended at a final protein concentration of 10 mg/ml or 1 mg/ml in an incubation medium containing (in mM): 1 alanine, 5 dextrose, 2 heptanoic acid, 4 lactate, 5 malate, 115 NaCl, 15 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 1 $CaCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.4, 295 mosmol/kg). Protein was measured using the Bio-Rad protein assay with a γ-globulin standard. For [^{14}C]PAH and [^{14}C]glutarate uptake studies, tubules were resuspended at 1 mg/ml in an incubation medium containing (in mM): 5 alanine, 0.9 glycine, 8.3 dextrose, 1.5 lactate, 1 malate, 10 sodium acetate, 1 sodium citrate, 110 NaCl, 2 NaH_2PO_4, 5 KCl, 1 $MgSO_4$, 1.8 $CaCl_2$ and 25 $NaHCO_3$ (pH 7.4, 295 mosmol/kg). This preparation results in an enriched population of renal proximal tubules with collapsed lumens as verified by the measurement of low brush border γ-GT levels ($< 20\%$ of total γ-GT) in tubule suspensions [10] and by microscopic visual inspection (using differential interference contrast microscopy; Groves and Wright, unpublished observations). Therefore, fluxes into or out of this preparation reflect transport events occurring at the peritubular membrane of proximal cells.

2.3. Measurement of [^{14}C]TEA uptake in tubule suspensions

Tubule suspensions (1 mg/ml) were preincubated in Erlenmeyer flasks for 15 min at 37°C and gassed with $95\%O_2/5\%CO_2$. [^{14}C]TEA (1.8 μM) was then added to the tubule suspension. At timed intervals from 1 to 30 min, 0.5 ml aliquots of the suspension were removed, added to tubes containing 5 ml of ice-cold 1 mM TPeA (dissolved in incubation buffer) to stop uptake, and the samples were centrifuged for ~ 25 s at $1480 \times g$ to pellet the tubules. The supernatant fraction was aspirated, and the pellet was rinsed a second time. The final pellet was dissolved in 1 M NaOH, and aliquots were taken for counting radioactivity.

To examine the kinetics of TEA uptake, tubule suspensions were preincubated as described above. An aliquot of tubule suspension (0.5 ml) was then transferred to a 15-ml tube containing 0.5 ml of incubation medium with 7.1 μM [^{14}C]TEA and increasing concentrations of unlabeled TEA. After 30 s uptake was terminated by the addition of 5 ml ice-cold incubation buffer containing 1 mM TPeA. The tubules were pelleted and prepared for counting radioactivity as described. To examine the effect of TPeA on the kinetics of TEA uptake, measurements of [^{14}C]TEA uptake with increasing concentrations of unlabeled TEA were performed as described in the presence of 2 μM unlabeled TPeA.

2.4. Measurement of TEA efflux

For efflux studies, tubules were preloaded with 1.8 μM [^{14}C]TEA for 30 min. Control uptake was measured by adding 0.5 ml aliquots to 5 ml of ice-cold 1 mM TPeA. Efflux was measured by adding 0.5 ml aliquots to 4.5 ml of incubation buffer containing TPeA at concentrations ranging from 0 to 500 μM (consult the figure legends for experimental details) after which the incubation was continued for 1 to 5 min. The efflux reaction was terminated by the addition of 5 ml ice-cold 1 mM TPeA and tubule pellets were prepared for counting radioactivity as described above. The efflux of TEA from preloaded tubules represents flux across the peritubular membrane since the flux across the luminal membrane is believed to represent an insignificant portion of the total TEA efflux from tubules.

2.5. Inhibition of TEA uptake after a brief exposure to TPeA or TEA

TPeA (1 μM, dissolved in incubation buffer), TEA (150 μM) or incubation buffer alone was added to tubule suspensions. After 10 s, tubules were diluted 10-fold with incubation buffer containing [^{14}C]TEA (1.8 μM) alone or [^{14}C]TEA with 0.1 μM TPeA or 15 μM TEA (control). At timed intervals, uptake was measured as described above. The control condition consisted of uptake measured in the presence of 0.1 μM TPeA or 15 μM TEA in tubules which were not preincubated with these OCs.

For recovery experiments, TPeA (1 μM) or incubation buffer alone was added to suspensions. After 10 s, tubules were diluted 10-fold with incubation buffer alone or 0.1 μM TPeA (control), and the incubation continued for 10 min. [^{14}C]TEA (1.8 μM) was then added to suspensions and uptake measured as described above.

2.6. Measurement of [^{14}C]PAH and [^{14}C]glutarate uptake in tubule suspensions

Tubule suspensions (1 mg/ml) were preincubated in Erlenmeyer flasks for 15 min at 37°C and gassed with 95%O_2/5%CO_2. An aliquot of tubule suspension (0.5 ml) was then transferred to a 15-ml tube containing 0.5 ml of incubation medium with [^{14}C]PAH (8.1 μM) or [^{14}C]glutarate (21.1 μM) and increasing concentrations of unlabeled TPeA. After 30 s uptake was terminated by the addition of 5 ml ice-cold Hepes-buffered incubation medium (Hepes buffer markedly decreases the uptake of PAH and glutarate in tubule suspensions; Groves, unpublished observations). The tubules were pelleted and prepared for counting radioactivity as described previously.

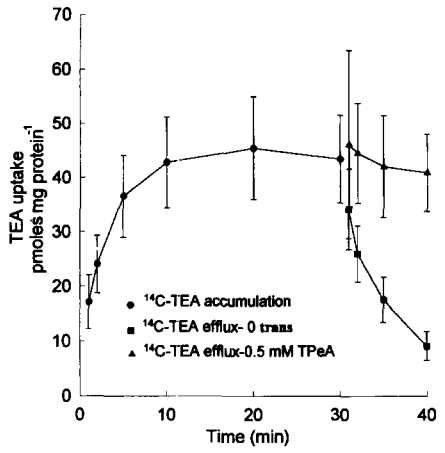

Fig. 1. Time-dependent accumulation and efflux of TEA from suspensions of rabbit renal proximal tubules. The bath concentration of [^{14}C]TEA during the uptake period was 1.8 μM. The efflux of [^{14}C]TEA was determined in the absence and presence of 500 μM *trans*-TPeA. Each point is the mean ± S.E. of triplicate measurements from three separate tubule preparations.

3. Results and discussion

The cumulative uptake of 1.8 μM [^{14}C]TEA by tubule suspensions increased with time and approached a steady state level after 10 min (Fig. 1). The efflux of [^{14}C]TEA from preloaded tubules was a time-dependent, first order reaction with a rate constant of 0.11 min^{-1} (Fig. 1). This rate of efflux was similar to that observed from single proximal S2 segments with oil-filled lumens (rate constant of 0.10 min^{-1}; [11]), which supports our contention that fluxes measured in the tubule suspension represent peritubular events. Our previous report [3] noted that the presence in the external medium of 500 μM TPeA inhibited efflux of TEA from preloaded tubules. This observation is confirmed and extended by the results presented in Fig. 1. The presence in the external medium of 500 μM TPeA effectively blocked the efflux of TEA for 5 min.

The ability of 500 μM TPeA to block the efflux of TEA suggests that carrier turnover in the presence of this high affinity inhibitor occurs slowly, if at all [1]. The $K_{i,app}$ for inhibition of TEA transport by TPeA is < 1 μM [3]. Thus, if TPeA inhibits TEA efflux through its interaction at the carrier-site, this effect should be noted at much lower concentrations than 500 μM. Indeed, with a $K_{i,app}$ of 0.8 μM, an external concentration of 10 μM would be expected to occupy > 90% of the carriers and, therefore, reduce efflux to a similar extent. To test this hypothesis, the effect of lower concentrations of TPeA on TEA efflux was examined. Increasing the external concentration of TPeA from 0 to 10 μM did affect the loss of TEA from preloaded tubules, reducing the 5 min efflux by approx. 50% (Fig. 2). Significantly, this degree of inhibition was substantially less than the complete block of efflux observed in studies employing 500 μM TPeA (e.g., Fig. 1). The fact that efflux of TEA from tubules can be completely eliminated implies that diffusive loss of TEA from tubules is virtually zero. Therefore, the efflux of TEA noted in the presence of a near-saturating concentration must be occurring via the peritubular OC transporter, despite the likelihood that the external aspect of the carriers is bound to TPeA. These data suggest that a TPeA-carrier complex must turnover, albeit at a rate that is slower than an unoccupied carrier. In other words, the complete block of TEA efflux observed in the presence of 500 μM external TPeA does not arise as a consequence of the binding of TPeA to the transport receptor and the subsequent production of a non-translocatable complex.

When the external TPeA concentration was increased from 10 μM to 500 μM, [^{14}C]TEA efflux was reduced by

[1] In this context, carrier 'turnover' refers to the suite of events encompassing the binding of substrate to the carrier, the subsequent translocation across/through the membrane of the substrate-carrier complex, dissociation of substrate from the carrier, and reorientation of the carrier to the initial aspect of the membrane.

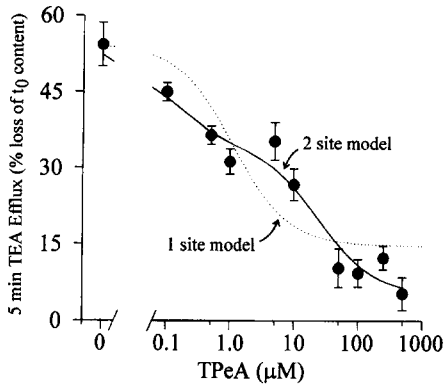

Fig. 2. Inhibition of tubular TEA efflux by increasing concentrations of TPeA. Tubule suspensions were preloaded with [^{14}C]TEA (1.8 μM) for 30 min. Aliquots of [^{14}C]TEA-loaded tubules were transferred to a bath containing the indicated concentration of TPeA or incubation buffer alone and 5 min efflux was then determined. Efflux is expressed as the percent loss of [^{14}C]TEA from the tubules relative to tubule content at time zero. Each point is the mean ± S.E. of triplicate measurements from three or four separate experiments. The lines were fit using a non-linear regression algorithm (Sigmaplot, Jandel Scientific) to either a one-site (dashed line) or two site (solid line) model of TPeA interaction with the TEA transporter (see text).

an additional 50% relative to the control level of efflux thereby achieving a near-complete inhibition of TEA efflux (Fig. 2). Thus, over the full concentration range studied, external TPeA was found to produce a biphasic, concentration-dependent *trans*-inhibition of TEA efflux from tubule suspensions. These observations suggest that at low (< 10 μM) concentrations, TPeA interacts specifically at the TEA transport site, whereas at high (> 10 μM) concentrations TPeA produces a separate, presumably allosteric inhibition of OC transport. The kinetics of the inhibition of TEA efflux by TPeA with the OC transporter also support the existence of a two site model for TPeA inhibition. The change in TEA efflux as a function of TPeA concentration was adequately described by a two site kinetic model of the form:

$$E_i = \left(\frac{E_i^{max^1}[\text{TPeA}]}{K_{50}^1 + [\text{TPeA}]}\right) + \left(\frac{E_i^{max^2}[\text{TPeA}]}{K_{50}^2 + [\text{TPeA}]}\right) \quad (1)$$

where E_i is the inhibition of TEA efflux caused by an external concentration of [TPeA]; K_{50} is the concentration of TPeA resulting in 50% inhibition of efflux by each of the superscripted processes (i.e., process 1 and 2); and the E_i^{max} parameters represent the decrease in total efflux contributed by each process. The solid line describing the relationship presented in Fig. 2 is the best fit to the data according to this model. The dotted line represents the best fit assuming a one site model. The systematic deviation of the one site model from the measured values for E_i argue for the presence of at least two sites. The values for K_{50}^1 and K_{50}^2 were 0.1 and 24 μM, respectively. The values for E_i^{max1} and E_i^{max2} were 19.8% and 29.3%, respectively

An alternative explanation for the failure of 10 μM TPeA to produce a complete block of TEA efflux involves the possibility that, from this concentration, TPeA binds to the TEA transport site with a comparatively slow on rate; i.e., if most of the transporters were to remain unoccupied by TPeA during the time course of the efflux period, then efflux of TEA could proceed at a sufficient rate to 'mask' that fact that 10 μM TPeA may eventually occupy > 90% of the transport sites producing a non-translocatable substrate-carrier complex. To determine whether the association of TPeA with the TEA transporter is particularly slow, 30 s TEA uptakes were measured in tubules which were preincubated for 30 s with increasing concentrations of TPeA. If association of the transporter with TPeA is slow, then preexposure of this ligand to the transport site should produce a 'left-shift' (i.e., decrease) of the apparent inhibitory constant for TPeA. This was not the case. The apparent K_i (i.e., concentration required to reduce TEA uptake by 50%) was 1.1 μM for control tubules vs. 1.0 μM for tubules preexposed to TPeA. This result argues against the likelihood that the failure of 10 μM TPeA to block efflux of TEA was a consequence of the slow binding of this inhibitor to the transporter.

Separate experiments addressed the issue of whether the inhibitory effects of TPeA on peritubular transport were specific for the OC transport pathway. Glutarate and *p*-aminohippurate (PAH) are accumulated by proximal cells by separate processes which appear to be unrelated to the transport pathway for OCs. Glutarate uptake involves a Na-dependent cotransport process specific for dicarboxylates [12,13], and PAH uptake involves the carrier-mediated exchange of PAH for dicarboxylates [13–15]. Although 10 μM TPeA had no effect on the uptake of [^{14}C]glutarate, 500 μM TPeA did result in a 19% (±7%; $P < 0.05$) reduction in glutarate uptake. Uptake of PAH was also inhibited by external TPeA, although the same degree of inhibition (17% ± 6% and 20% ± 7%) was noted at 10 and 500 μM TPeA, respectively. Several other studies have noted cross inhibitory interactions between transport pathways for organic cations and organic anions, most notably the inhibition of OC transport by OA inhibitors including probenecid [16–18]. The authors of these studies suggested that these interactions arise from the competitive interaction of the molecules at the transport receptors. However, in the light of the present results showing a secondary, possibly allosteric inhibitory interaction of TPeA with the OC transporter, the inhibition by TPeA of peritubular PAH and glutarate uptake may be the result of a rather non-specific interaction of this lipophilic cation with peritubular membrane proteins.

One means by which the binding of TPeA to the OC transporter could result in a reduction in net carrier turnover is if the dissociation (rather than association) of TPeA from the carrier were comparatively slow. If this were so, then a TPeA-carrier complex could be unavailable for uptake of another substrate for a protracted period of time. This hypothesis was tested by measuring the uptake of

TEA in tubule suspensions (10 mg/ml) which were preincubated for 10 s with 1 μM TPeA and then diluted 10-fold to a final TPeA concentration of 0.1 μM (Fig. 3). When tubules are exposed simultaneously to 1 μM TPeA and [^{14}C]TEA, uptake of TEA is reduced by ~50% [3]. However, exposure to 0.1 μM TPeA, a concentration well below the $K_{i,app}$ for TPeA, during uptake should reduce transport by < 10%. Following dilution, both sets of tubules were exposed to the same buffer conditions: radiolabeled TEA and a 0.1 μM concentration of TPeA. However, uptake into the tubules that were preexposed for 10 s to 1 μM TPeA was reduced by ~30%–50% compared to control uptake. This observation is consistent with the contention that, once bound, dissociation of TPeA from the peritubular OC transporter is a relatively slow process. The apparent affinity of the OC transporter for TEA itself is much lower than for TPeA; the K_t for TEA transport is 130 μM, compared to a $K_{i,app}$ for TPeA of 0.8 μM [3]. Thus, dissociation of TEA from the carrier might be expected to be more rapid than dissociation of TPeA. If so, preexposure of tubules to a comparably large concentration of unlabeled TEA (i.e., 150 μM) should have no effect on the subsequent uptake of [^{14}C]TEA by the diluted tubule suspension. Indeed, as shown in Fig. 3b, this was the case. The interaction of TPeA with the transporter was reversible, as might be expected from the conclusion drawn earlier that TPeA does appear to be transported by the OC transporter. The uptake of [^{14}C]TEA returned to control levels in tubule suspensions which were preincubated with 1 μM TPeA for 10 s, diluted to 0.1 μM and allowed to recover for 10 min prior to measuring uptake (Fig. 3c).

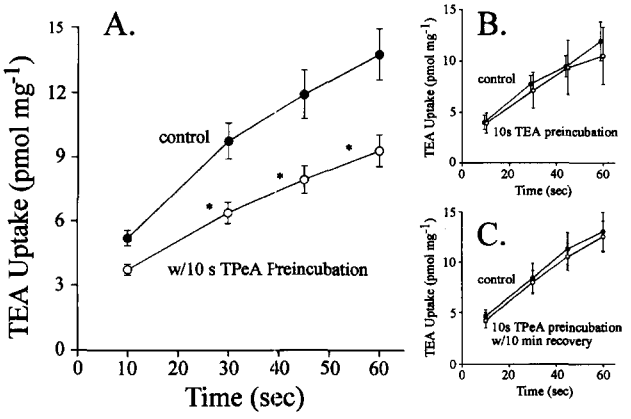

Fig. 3. (A) Effect of a brief exposure (10 s) to 1 μm TPeA on TEA uptake. Tubule suspensions (10 mg/ml) were incubated with 1 μM TPeA or incubation buffer (control) for 10 s then diluted 10-fold with [^{14}C]TEA (1.8 μM) or [^{14}C]TEA and 0.1 μM TPeA. The time course of [^{14}C]TEA uptake was then measured for each group of tubules. Each point is the mean ± S.E. of triplicate measurements from four separate experiments. (B) Effect of a brief exposure (10 s) to 150 μM TEA on TEA uptake. Tubule suspensions (10 mg/ml) were incubated with 150 μM TEA or incubation buffer (control) for 10 s then diluted 10-fold with [^{14}C]TEA (1.8 μM) or [^{14}C]TEA and 15 μM TEA. Each point is the mean ± S.E. of triplicate measurements from three separate experiments. (C) Recovery of TEA uptake following prolonged inhibition by TPeA. Tubule suspensions (10 mg/ml) were incubated with 1 μM TPeA or incubation buffer (control) for 10 s, then diluted 10-fold with incubation buffer only. After 10 min, [^{14}C]TEA (1.8 μM) or [^{14}C]TEA + 0.1 μM TPeA was added and 30 s uptake measured. Each point is the mean ± S.E. of triplicate measurements from four separate experiments. Values with an asterisk are significantly different than controls ($P < 0.05$).

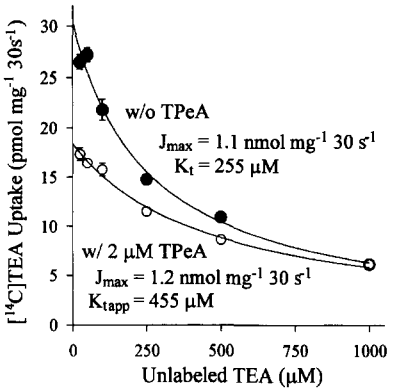

Fig. 4. Effect of TPeA (2 μM) on the kinetics of TEA transport in tubule suspensions. The uptake of [^{14}C]TEA (7.1 μM) was measured in the presence of increasing concentrations of unlabeled TEA in the presence and absence of 2 μM TPeA. Each point is the mean ± S.E. of triplicate measurements from six separate experiments.

This slow dissociation of TPeA from the OC transporter suggests that once bound TPeA may effectively decrease the number of sites available for the transport of TEA. Kinetically, this event might be expected to be manifest as a noncompetitive or mixed type inhibition of TEA transport. To test this hypothesis, the effect of a 2 μM concentration of TPeA on the kinetics (J_{max} and K_t) of TEA transport was examined in tubule suspensions (Fig. 4). In contrast to our expectations, TPeA increased the K_t for TEA transport from 170 μM to 560 μM but had no effect on J_{max}, indicating that TPeA is a competitive inhibitor of TEA uptake. The calculated K_i for TPeA was 0.9 μM, in close agreement with the $K_{i,app}$ of 0.8 μM for TPeA determined previously [3]. As noted above, the observed slow dissociation of TPeA from the OC transporter led to the expectation that inhibition by TPeA would reduce apparent J_{max}. The reason for this lack of TPeA effect on J_{max} is unclear. However, the apparent rapid association and subsequent slow dissociation of TPeA with the transporter was observed in tubules preincubated with TPeA alone. The results shown in Fig. 4 suggest that large concentrations of TEA are capable of displacing TPeA from the binding site(s) it occupies, thereby permitting TEA to compete effectively for transport.

Collectively, these observations show that the interactions of TPeA, and perhaps similarly large hydrophobic OCs, with the OC transport system are complex. TPeA appears to inhibit peritubular OC transport in rabbit renal proximal tubules through interaction with a high affinity and a low affinity binding site. At the high affinity site, TPeA appears to bind rapidly but is slow to dissociate, producing a prolonged inhibition of uptake. This effect

may be overcome by increasing the concentration of TEA. These interactions may be related to either the comparatively large size and/or hydrophobic nature of TPeA. A number of reports have documented significant, and sometimes deleterious, interactions of cationic drugs at the site of renal secretion (e.g., [19–22]). Some of these interacting agents (e.g., cimetidine) have been found to interact with renal OC transporters with very high affinity (e.g., [23]). The present results suggest that the nature of such drug interactions may be more complex than previously assumed.

Acknowledgements

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