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Effect of Probenecid on Tetraethylammonium (TEA) Transport Across Basolateral Membrane of Rabbit Proximal Tubule

Tae Ryong Choi and Yong Keun Kim

Department of Physiology, College of Medicine, Pusan National University

=ABSTRACT=

The effect of probenecid on the transport of tetraethylammonium (TEA) was studied in renal cortical slices and isolated membrane vesicles to investigate the interaction of organic anion with the organic cation transport system in proximal tubule. Probenecid reversibly inhibited TEA uptake by renal cortical slices in a dose-dependent manner over the concentration range of 1 and 5 mM. The efflux of TEA was not affected by the presence of 3 mM probenecid. Kinetic analysis indicated that probenecid decreased Vmax without significant change in Km. Probenecid inhibited significantly tissue oxygen consumption at concentrations of 3 and 5 mM. However, probenecid did not significantly reduce TEA uptake in brush border and basolateral membrane vesicles prepared from renal cortex even at a concentration as high as 10 mM.

These results indicate that probenecid reduces TEA uptake in cortical slices by inhibiting tissue metabolism rather than by an interaction with the organic cation transporter.

Key Words: Probenecid, TEA transport, Basolateral membrane, Rabbit proximal tubule

INTRODUCTION

Many organic cations are actively secreted by renal proximal tubule of the mammalian kidney (Rennick, 1981; Rennick & Farah, 1956). This 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. Studies with membrane vesicles isolated from the kidney cortex have consistently identified that organic cations are actively transported by a H^+/organic cation exchange process across the brush border membrane (Holohan & Ross, 1981; Jung et al, 1989; Takano et al, 1984; Wright et al, 1985). Transport of organic cation across the basolateral membrane has also been demonstrated to be an active process in studies with intact proximal tubule (Schäli et al, 1983; Tarloff & Brand, 1986), although studies with basolateral membrane vesicles suggested it a facilitated diffusion process (Ross & Holohan, 1983).

Numerous studies have demonstrated that the organic cation transport system is specific to organic cations and is clearly different from the organic anion transport system (Besseghir, 1989; Kinsella et al, 1979; Rennick, 1981). However, several *in vivo* and *in vitro* studies showed that probenecid, the classical competitive inhibitor of organic anion transport, also inhibits the renal transport of cimetidine, an organic cation (Cacini et al, 1982; McKinney et al, 1981). Hsyu et al (1988) also

demonstrated that probenecid inhibits N'-methylnicotinamide (NMN) uptake in a competitive fashion in renal brush-border membrane vesicles, suggesting that it interacts with the organic cation transport system.

This study was thus carried out to clarify if probenecid interacts with the organic cation transport system in basolateral membrane of proximal tubule. The effect of probenecid on TEA uptake was determined in slices and membrane vesicles prepared from rabbit kidney cortex.

MATERIALS AND METHODS

TEA uptake by cortical slices

Male New Zealand white rabbits weighing 1.5~2 kg were sacrificed and the kidneys were rapidly removed. The kidneys were immediately perfused through the renal artery with an ice-cold isotonic saline solution containing 140 mM NaCl, 10 mM KCl and 1.5 mM $CaCl_2$ to remove as much blood as possible. Thin (0.4~0.5 mm thick) slices of renal cortex were prepared using a Stadie-Riggs microtome and were stored in an ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose and 20 mM Tris/HCl (pH 7.4).

TEA uptake in renal cortical slices was determined as previously described (Kim et al., 1988). Approximately 50 mg (wet wt.) of slices were transferred into a 20 ml beaker containing 4 ml of modified Cross-Taggart medium, and incubated with 10 μM ^{14}C-TEA. The incubation was carried out for 60 min in a Dubnoff metabolic shaker at 25°C under a 100% oxygen atmosphere. At the end of incubation, the slices were quickly removed from the beaker, blotted, weighed and solubilized in 1 N NaOH. Aliquots of the incubation medium and the solubilized tissue were pipetted into a scintillation vial containing Aquasol (New England Nuclear) and the radioactivity was determined using a liquid scintillation counter (Packard Tricarb 300C). PAH uptake by renal slices was expressed as the slice to medium (S/M) ratio: the concentration of the compound in the tissue (mole/g wet tissue) divided by that in the medium (mole/ml medium).

In the kinetic analyses of active TEA uptake, the total and passive uptakes of TEA were measured separately, and the difference was taken as an active uptake (Kim et al. ,1988). For the determination of total uptake, slices were preincubated for 60 min under a 100% oxygen atmosphere and then transferred to the same medium containing various concentrations of TEA for a 10-min incubation. The passive uptake was measured similarly but under a 100% nitrogen atmosphere in a medium containing 10 mM choline chloride.

The efflux of TEA from renal cortical slices was determined by modification of the technique of Farah et al.(1959). Slices were first loaded with TEA by incubating them in a medium containing 50 μM ^{14}C-TEA for 60 min after which they were rinsed for 20 sec in a TEA-free medium in order to remove TEA adhering to the tissue surface. The slices were then transferred at 1-min intervals through a series of 30 beakers containing a TEA-free modified Cross-Taggart medium at 25°C. The experiment was performed in a Dubnoff metabolic shaker with a hinged plexiglas cover and 100% oxygen atmosphere. The quantity of TEA collected from each beaker as well as the amount of the compound remaining in the tissue was used to construct the efflux curve and to calculate the rate constant.

Oxygen consumption measurement

The oxygen consumption of renal slices was measured with an oxygen monitor (Yellow springs Instrument Co., model 53). Approximately 50 mg of slices were incubated in a reaction vessel containing 4 ml of the modified Cross-Taggart medium saturated with oxygen at 25°C. Decrease in PO_2 in the medium was measured using a Clark electrode for 15 min, and the rate of oxygen consumption was calculated. Oxygen consumption was measured in the absence (total consumption) and presence (ouabain-insensitive component) of 1 mM ouabain,

and the difference was taken as ouabain-sensitive component.

Membrane vesicle preparation

Brush border (BBMV) and basolateral (BLMV) membrane vesicles were simultaneously isolated from rabbit kidney cortex by Percoll-density gradient centrifugation and Mg^{2+}-precipitation as described previously (Kim et al,1993).

The vesicles were suspended in the vesicle buffer, adjusted to yield a protein concentration of 6 mg/ml and stored at −70°C until use. The composition of vesicle buffer was 100 mM mannitol, 100 mM KCl and 20 mM Mes/Tris (pH 6.0) for BBMV and 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.5) for BLMV. Prior to transport studies vesicles were preincubated at 37°C for 30 min to effectively load with appropriate buffer (Jung et al. 1989).

The purity of vesicle preparations was evaluated by comparing marker enzyme activities in initial homogenates and final membrane vesicle preparations. Na^+-K^+-ATPase and alkaline phosphatase are well established marker enzymes for BLMV and BBMV, respectively. The enrichment of Na^+-K^+-ATPase in BLMV and that of alkaline phosphatase in BBMV was 13.85 and 10.33, respectively.

TEA uptake by membrane vesicles

TEA uptake by vesicles was measured by a rapid filtration technique. Briefly, the reaction was initiated by adding membrane vesicles to the incubation medium (a 1 : 10 dilution of membrane vesicle suspension) containing [^{14}C]TEA at 25°C. The composition of the incubation medium was 100 mM mannitol, 100 KCl and 20 mM Hepes/Tris (pH 7.4) for BBMV, and 100 mM mannitol, 100 mM NaCl, 5 μM valinomycin and 20 mM Hepes/Tris (pH 7.5) for BLMV. At 10 sec of the incubation, 100 μl aliquots were taken and quickly filtered under vacuum through Millipore filters (HAWP, 0.45 μm pore size), which were soaked overnight in distilled water. The filters were then washed with 5 ml of ice-cold stop solution containing 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.4), and dissolved in 1.0 ml of methoxyethanol. After addition of 10 ml of scintillation cocktail, the amount of radioactivity taken up by vesicles was determined by liquid scintillation spectrometry (Packard Tricarb 300C). Nonspecific binding of substrate to vesicle membrane was determined by the same filtration procedure after vesicles were incubated in distilled water containing 0.1% deoxycholate and radioactive substrate.

Enzyme assays

Na-K-ATPase activity was measured by the method of Jörgensen and Skou(1971) and alkaline phosphatase activity by the method of Linhardt and Walter (1963). Protein was determined according to Bradford(1976), using g-globulin as a standard.

Statistical analysis

All experiments were performed on more than three separate membrane preparations. Student's *t*-test was used in statistical evaluation of the data and p values less than 0.05 were considered significant.

RESULTS

Effect of probenecid on TEA uptake in slices

Fig. 1. shows the time course of TEA uptake by renal cortical slices in the presence and absence of 3 mM probenecid. Probenecid significantly inhibited TEA uptake. To examine the concentration dependence for the inhibitory effect of probenecid, TEA uptake was measured in the presence of various concentrations of probenecid. The results are shown in Fig. 2. Relative S/M ratios of TEA at the end of 60 min incubation were plotted against probenecid concentration in the medium. Probenecid significantly inhibited TEA uptake at concentrations higher than 1 mM.

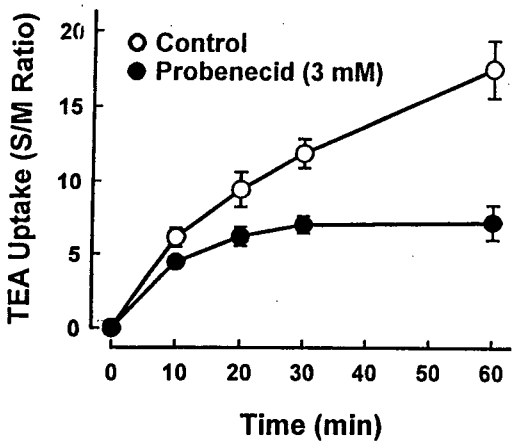

Fig. 1. Time course of TEA uptake by renal cortical slices in the presence and absence of 3 mM probenecid. Slices were incubated for various time periods at 25°C in a medium containing 10 μM ^{14}C-TEA under 100% oxygen atmosphere. Each point represents the mean ±SE of three experiments.

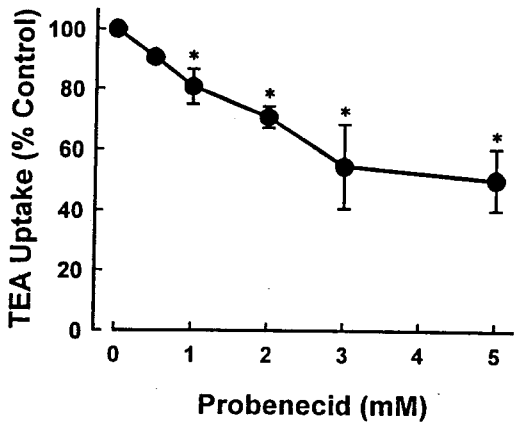

*Fig. 2. Effect of probenecid on TEA uptake by renal cortical slices. Slices were incubated for 60 min at 25°C in a medium containing 10 μM ^{14}C-TEA under 100% oxygen atmosphere. Each point represents the mean ±SE of four experiments. *P>0.05 compared with the control.*

Reversibility of probenecid inhibition in slices

To determine whether the effect of probenecid on TEA uptake was reversible, the slices were treated with probenecid of 1 and 2 mM concentrations for 30 min and then incubated with TEA in the presence and absence of probenecid. As shown in Fig. 3, when probenecid-treated slices were incubated in the absence of probenecid, TEA uptake was not significantly different from the control. These results indicate that probenecid inhibits reversibly TEA uptake.

Effect of probenecid on TEA efflux in slices

The efflux rate constant was determined in the presence and absence of 3 mM probenecid in the medium. In all paired experiments, the efflux rate constant was not altered by the presence of probenecid (0.028±0.03 vs. 0.027±0.003 per min). This indicates that the inhibitory effect of probenecid on the steady-state accumulation of TEA (Fig. 1) is due primarily to an inhibition of TEA influx from the medium into the cell across the basolateral mem-

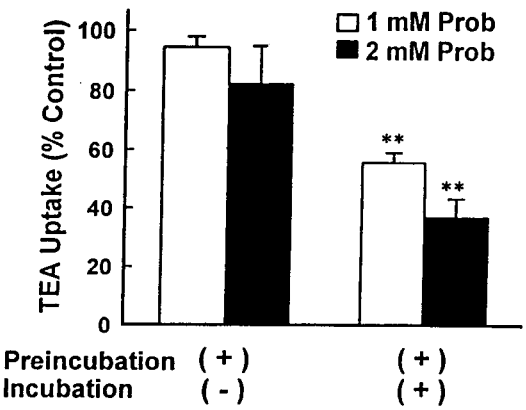

*Fig. 3. Reversibility of the inhibitory effect of probenecid on TEA uptake. Slices were preincubated for 30 min with probenecid and then incubated with substrate for 60 min in the presence(+) and absence(-) of probenecid. Data are expressed as percentages of control uptake measured in the absence of probenecid in both preincubated and incubated media. Each point represents the mean ±SE of four experiments. **P<0.01 compared with the control.*

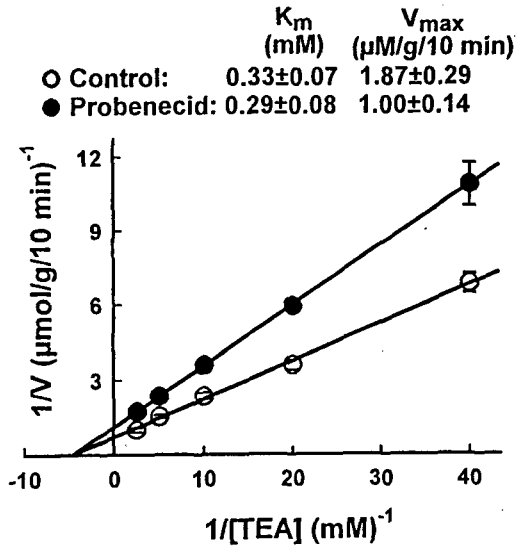

Fig. 4. Lineweaver-Burk plots of initial rate of active TEA accumulation into renal cortical slices in the presence and absence of 3 mM probenecid. TEA uptake was determined during a 10-min incubation in a medium containing various concentrations of PAH and was corrected for passive accumulation which was determined in the presence of 10 mM choline chloride under 100 % N_2 atmosphere. Each point represents the mean ± SE of four experiments.

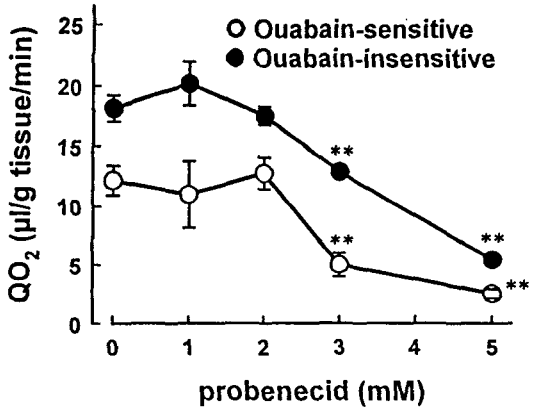

*Fig. 5. Effect of probenecid on oxygen consumption in renal cortical slices. Oxygen consumption was measured in the presence (ouabain-insensitive) and absence (total) of 1 mM ouabain, and the difference was taken as ouabain-sensitive component. Each point represents the mean ± SE of five experiments. **P<0.01 compared with the control.*

Table 1. Effect of probenecid on microsomal Na^+-K^+-ATPase activity

Probenecid (mM)	Na^+-K^+-ATPase Activity (μmole Pi/mg protein.hr)
0 (Control)	43.04±0.62
2	46.84±0.27
5	44.30±0.18

Data are the mean±SE of three determinations.

brane.

Effect of probenecid on kinetics of TEA uptake in slices

In order to determine the effect of probenecid on the kinetic parameters of TEA accumulation, we measured the uptake from media containing varying concentrations of TEA in the presence and absence of 3 mM probenecid. Lineweaver-Burk plots of the experimental data are shown in Fig. 4. Apparently, probenecid decreased the Vmax (from 1.87±0.29 to 1.0±0.14 μmole/g/10 min, P<0.05) without a significant change in Km (0.33±0.07 vs. 0.29±0.08 mM). Thus, it appears that probenecid is acting as a noncompetitive inhibitor.

Effect of probenecid on Na^+-K^+-ATPase activity and oxygen consumption in slices

Since active accumulation of TEA across the basolateral membrane is dependent in some manner on the Na^+-K^+-ATPase activity (Tarloff & Brand, 1986), it was examined whether the inhibition of TEA uptake was resulted from an impairment of Na^+-K^+-ATPase activity or the cell metabolism. As shown in Table 1, probenecid did not alter the enzyme activity at 2 and 5 mM, in which TEA uptake was significantly inhibited. However, pro-

Table 2. Effect of probenecid on TEA uptake in BBMV and BLMV

Probenecid (mM)	Initial Uptake (pmole/mg protein/10 sec)	Equilibrium Uptake (pmole/mg protein/60 min)
	BBMV	
0 (Control)	1272.30±61.48	739.65±36.42
1	1140.45±97.62	660.63±43.91
5	1056.93±69.18	715.96±28.45
10	1086.33±77.48	683.04±25.55
	BLMV	
0 (Control)	804.97±48.34	895.59±73.32
5	829.56±42.03	953.65±79.59
10	950.15±57.95	868.53±47.64

Membrane vesicles were preloaded with 100 mM mannitol, 100 mM KCl and 20 mM Mes/Tris (pH 6.0) [or 20 mM Hepes/Tris (pH 7.5) for BLMV]. Uptake of 50 μM ^{14}C-TEA was measured for 10 sec and 60 min in a buffer containing 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.5) for BBMV and 100 mM mannitol, 100 mM NaCl, 5 μM valinomycin and 20 mM Hepes/Tris (pH 7.5) for BLMV in the presence or absence of various concentrations of probenecid. Data are the mean±SE of four determinations.

benecid inhibited significantly both ouabain-sensitive and -insensitive oxygen consumption at 3 and 5 mM (Fig. 5).

Effect of probenecid on TEA uptake in plasma membrane vesicles

Table 2 summarizes the effect of probenecid on TEA uptake in brush border (BBMV) and basolateral (BLMV) membrane vesicles. Since it has been demonstrated that TEA uptake is driven by a H^+/TEA exchange process in BBMV and by a potential-dependent process in BLMV (Jung et al, 1989; Kim et al., 1992, 1993), TEA uptake was measured in the presence of an outwardly directed H^+ gradient in BBMV and in the presence of inside-negative potential in BLMV. Probenecid did not significantly reduce the initial (10 sec) uptake of TEA even at concentration as high as 10 mM in both membranes. The equilibrium value measured after 60 min incubation was also unaltered by probenecid.

DISCUSSION

Although probenecid has been used as the prototypic inhibitor of organic anion transport, this compound also inhibits the transport of organic cations such as cimetidine and NMN (Cacini et al, 1982; McKinney et al, 1981; Hsyu et al, 1988). However, it is unclear whether the observed effect of probenecid is due to a direct action on the organic cation transport system.

The present study demonstrated that probenecid inhibited significantly TEA uptake by renal cortical slices at concentrations higher than 1 mM. The kinetic analysis showed that probenecid inhibited TEA influx in a noncompetitive manner, in which it significantly decreased Vmax while it had no effect on Km for TEA. These results suggest that the inhibitory effect of probenecid is not due to mutual interaction between probenecid and TEA on the organic cation carrier.

In the present study, probenecid inhibited also significantly tissue oxygen consumption in cortical slices, in agreement with the report by Pakarinen & Runeberg (1969) who observed that probenecid inhibits the accumulation of dicarboxylates and oxygen consumption in kidney cortical slices. Since the transport of TEA uptake across the basolateral membrane of the renal proximal tubule is affected by the transmembrane potential generated at the expense of metabolic energy (Rennick & Farah, 1956; Nechay & Pardee, 1965), it would be reduced by an agent which impairs the cellular metabolism or the membrane potential. Thus, the results of the present study suggest that the inhibition of TEA uptake by probenecid was not due to a direct effect of this compound on the transport system but to changes in cellular metabolism. From slice experiments, however, it is difficult to distinguish whether

the inhibitory effect of probenecid is due to an effect on cellular metabolism or to a direct interaction of probenecid with the transport system. Thus, studies with membrane vesicles were carried out since the influence of cellular metabolism on transport process is excluded in the isolated vesicle system. Probenecid did not significantly inhibit the uptake of TEA in BBMV and BLMV even at concentrations as high as 10 mM (Table 2).

Contrary to what observed in this study, Hsyu et al (1988) found that probenecid inhibits competitively NMN transport in rabbit renal BBMV and concluded that the inhibition is due to a mechanism involved in the interaction of organic anions with the organic cation transporter. The reason for this discrepancy is not clear at present. However, this may be due to differences in the transport system for NMN and TEA. Renal slice accumulation of NMN is different from that of TEA in rabbits, the former being transported by a passive mechanism while the latter by an active process (Berndt, 1981). It has been demonstrated that renal brush border membranes also possess multiple carrier systems for organic cations (Miyamoto et al, 1989).

In this study, probenecid reduced slightly TEA uptake in BBMV, although not significant. TEA uptake by slices was also inhibited by 1 and 2 mM probenecid which did not affect the oxygen consumption. These results may be due to nonspecific membrane effect of probenecid rather than its interaction with the organic cation transport system, although the precise mechanism remains obscure. Nonspecific effects of probenecid on membrane transport systems has been demonstrated by several workers. Kippen et al (1979) reported that probenecid inhibits significantly glucose uptake by renal BBMV. Ullrich et al (1984) also demonstrated that this compound inhibits markedly succinate transport in intact proximal tubule.

In conclusion, the present study demonstrated that probenecid inhibits significantly TEA uptake in renal cortical slices but not in plasma membrane vesicles. The inhibitory effect may result from an influence on cellular processes such as metabolism rather than a direct interaction with the organic cation transporter.

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