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Mechanisms of interactions between organic anions and the organic cation transporter in renal brush border membrane vesicles

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Although data in the literature generally suggest that organic anions do not inhibit the renal transport of organic cations and vice versa [1], there are also studies suggesting that such interactions may occur [2–6]. In particular, a number of studies have demonstrated that probenecid, an organic anion, inhibits the renal transport of cimetidine, an organic cation, in isolated renal cortical preparations [2–5]. Recently, we carried out experiments to determine whether the observed interaction between cimetidine and probenecid was unique to these two compounds or whether organic anions may generally inhibit organic cation transport [5]. We also ascertained if the interactions between anions and cations occurred at the brush border membrane, the site of active organic cation transport in the proximal tubule. Our data suggested that both probenecid and furosemide inhibited the uptake of the model organic cation, N^1-methylnicotinamide (NMN*), in brush border membrane vesicles prepared from rabbit renal cortex. The interaction between probenecid and NMN was competitive. Since probenecid is structurally dissimilar from cimetidine, at least in terms of charge, we speculated that the interaction may involve hydrophobic displacement. The purpose of this study was to examine further the nature of the interactions between organic anions and organic cations.

Methods

Brush border membrane vesicles were prepared from rabbit renal cortex by a modification of the divalent cation precipitation procedure developed by Booth and Kenny [7]. The modified method has been described in detail previously [8]. The transport of [^{3}H]NMN (sp. act. 2.8 to 3.0 Ci/mmol, ICN, Irvine, CA) was studied in brush border membrane vesicles in which an outwardly directed proton gradient was imposed. Briefly, brush border membranes were resuspended in a buffer containing 10 mM HEPES and 150 mM KCl at pH 6.0 and adjusted to protein concentrations of 15–20 mg/mL. Vesicles (10 μl) were incubated with 20 or 40 μL (pH 7.4) buffer (10 mM HEPES, 150 mM KCl adjusted to pH 7.4 with KOH) containing [^{3}H]NMN (2.0 μM) alone or [^{3}H]NMN (2.0 μM) together with various unlabeled compounds. Incubations were carried out at 25° for 8 sec and 120 min. Michaelis–Menten studies examining the mechanism of interaction of furosemide with the organic cation transport system were carried out by determining the 8-sec uptake of NMN (0.25, 0.5, 1.0, 2.5, 5.0, 10 and 25 mM as 2.0 μM [^{3}H]NMN plus unlabeled NMN) in the presence and absence of 20 mM furosemide. Incubations were stopped by rapid filtering of the incubates over 0.3-μm cellulose nitrate filters (Millipore Corp., Bedford, MA) which had been placed on a vacuum filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). The amount of radioactivity associated with the filters was determined by liquid scintillation counting. [^{3}H]NMN binds to vesicles only minimally [8], and no correction was made for this vesicle-associated radioactivity. In each experiment, three replicate determinations were made to generate a single point. Experiments in separate brush border membrane vesicle preparations were used to generate mean values. The data are reported as means ± SE. In the studies in which the effect of furosemide on the transport of NMN was evaluated, Lineweaver–Burk plots of the data obtained in each individual experiment were constructed to obtain the apparent K_m and V_{max}.

Results and Discussion

Figure 1 depicts the effects of probenecid and the three congeners, *p*-(monopropylsulfamyl)benzoic acid (probenecid monopropyl), *p*-sulfamyl benzoic acid (probenecid depropyl) and *p*-(dibutylsulfamyl)benzoic acid (probenecid dibutyl), on the uptake of NMN determined at 8 sec. Probenecid (both 3 and 10 mM), probenecid monopropyl (10 mM) and probenecid dibutyl (3 mM) significantly reduced the initial uptake of NMN (probenecid 10 mM, 0.19 ± 0.03 pmol/mg; probenecid 3 mM, 0.25 ± 0.08 pmol/mg; probenecid monopropyl 10 mM, 0.40 ± 0.04 pmol/mg; and probenecid dibutyl 3 mM, 0.41 ± 0.09 pmol/mg) versus control (0.87 ± 0.14 pmol/mg, $P < 0.05$). The 8-sec uptake of NMN in the presence of probenecid depropyl (10 mM) was not significantly different (0.69 ± 0.09 pmol/mg) from the control. None of the compounds inhibited the uptake of NMN at 2 hr suggesting that neither vesicle integrity nor volume was altered by the compounds.

The effect of furosemide (20 mM) on the initial uptake of NMN is depicted in a Lineweaver–Burk plot shown in Fig. 2. As shown, furosemide appeared to interact competitively with NMN. The apparent K_m of NMN was

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NMN, N^1-methylnicotinamide; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; and DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene.

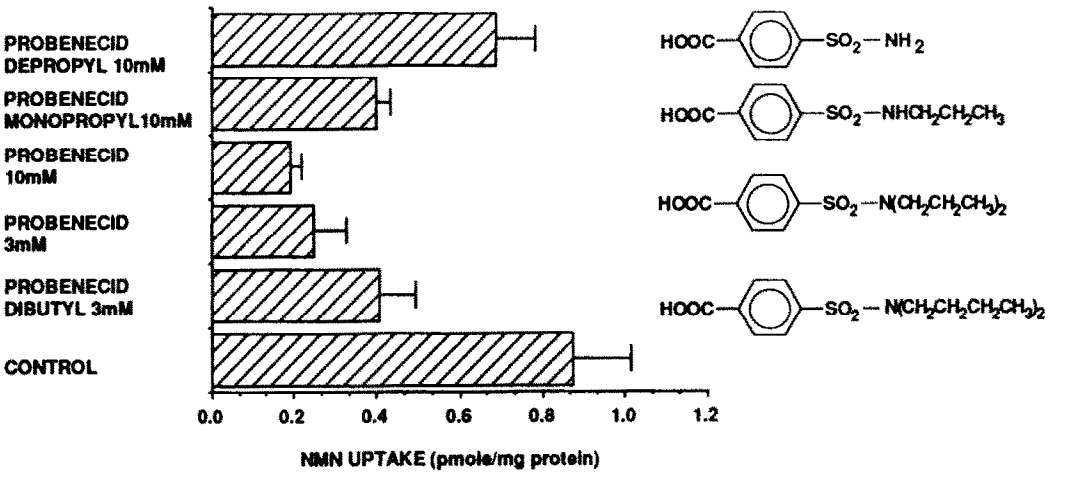

Fig. 1. Uptake of NMN alone and in the presence of 3 mM probenecid dibutyl, 3 mM probenencid, 10 mM probenecid, 10 mM probenecid monopropyl or 10 mM probenecid depropyl. Uptake in renal brush border membrane vesicles was determined at 8 sec. Data are the means ± SE of values from seven to eight separate experiments except for 3 mM probenecid and 3 mM probenecid dibutyl which are from three to four separate experiments.

2.0 ± 0.46 nM and was increased to 11.8 ± 5.8 mM in the presence of furosemide ($P < 0.05$). The V_{max} of NMN was 148 ± 31 pmol·mg^{-1}·sec^{-1} and was not increased significantly by furosemide (236 ± 72 pmol·mg^{-1}·sec^{-1}). These data suggest that the interaction of furosemide with NMN is competitive.

The effects of various organic anions on the uptake of NMN at 8 sec are shown in Table 1. The organic anions tested included two dicarboxylic acids (oxalate and succinate), 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS) and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS), *p*-aminohippurate, and the acidic amino acid glutamate. None of these anions significantly affected the uptake of NMN.

The observation that the interaction between furosemide and NMN was competitive is consistent with our previous data demonstrating that probenecid competitively inhibits NMN uptake in brush border membrane vesicles [5]. A characteristic of competitive inhibition is that the substrate and inhibitor are mutually exclusive; however, a number of molecular models are consistent with competitive inhibition [9]. Because of the obvious differences in the charge between organic anions and organic cations, we postulated in our previous study that NMN and probenecid do not bind to the same site [5]. Rather, we suggested that the two compounds may share a common hydrophobic binding site, but that the charged portions of the molecules bind to distinct sites.

In this study, we tested the hypothesis that probenencid inhibits NMN transport by hydrophobic displacement. We used two congeners of probenecid, probenecid monopropyl and probenecid depropyl, which differ from probenecid in terms of the hydrocarbon chains attached to the nitrogen (Fig. 1). Since the hydrophobicity of probenecid can be attributed primarily to the hydrocarbon chains, varying these side chains afforded an opportunity to study the effect of changing hydrophobicity on organic cation transport. Probenecid, which has two *n*-propyl side chains, was the most hydrophobic followed by probenecid monopropyl and probenecid depropyl respectively. Although the congeners have an additional acidic group (as compared to probenecid), the pK_a of this group would be expected to be about

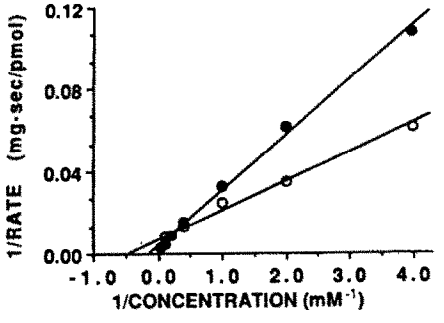

Fig. 2. Reciprocal of uptake of NMN at 8 sec as a function of the reciprocal of concentration. Data were obtained in the presence (●) and in the absence (○) of 20 mM furosemide. Each point is the mean of data from three separate experiments.

Table 1. Effects of various organic anions on the uptake of NMN in renal brush border membrane vesicles

Organic anion*	Uptake of NMN† (pmol/mg protein)
Control	0.85 ± 0.14
SITS	0.67 ± 0.09
DIDS	0.83 ± 0.02
Oxalate	0.78 ± 0.10
Succinate	1.16 ± 0.17
Glutamate	0.94 ± 0.32
p-Aminohippurate	0.68 ± 0.03

* The concentration of each organic anion was 10 mM except for SITS and DIDS in which a concentration of 1 mM was used. Abbreviations: SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; and DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene.

† Values are the means ± SE of data from three separate experiments in which the uptake of [^{3}H]NMN was determined at 8 sec.

9–10, the proton would not be removed in the physiologic pH range, and the group would be largely in the unionized state. Hence, the major difference among the three compounds is in their differing hydrophobicities. Of the three compounds, the most hydrophobic, probenecid, produced the greatest reduction in the initial uptake of NMN followed by the two less hydrophobic congeners. These data are consistent with our hypothesis that NMN and organic anions such as probenecid may share a common hydrophobic site [5] and suggest that the potency of the substituted sulfamylbenzoates in inhibiting organic cation transport is related to hydrophobicity. The dibutyl congener of probenecid [*p*-(dibutylsulfamyl)benzoic acid], at 3 mM, also produced a significant reduction in the initial uptake of NMN ($P < 0.05$), although it was not significantly more potent than 3 mM probenecid. Previously, other investigators have determined that increasing hydrophobicity of substituted sulfamylbenzoates is associated with increasing potencies of the compounds in inhibiting *p*-aminohippuric acid uptake in isolated kidney slices [10, 11].

Hydrophobic displacement of organic cations by substituted sulfamyl benzoates may not be specific for this series of anions, but may be a general mechanism for the interactions between organic anions and the organic cation transporter. By calculating the octanol/water partition coefficient (log P), the degree of hydrophobicity of compounds may be estimated [12]. For example, of the organic anions studied here, those that significantly reduced the uptake of NMN (furosemide, probenecid, probenecid monopropyl and probenecid dibutyl) had a calculated log $P > 1.00$, whereas those organic anions that did not inhibit organic cation transport (Table 1 and probenecid depropyl) had a calculated log $P < 0.50$. Therefore, the general hydrophobicity of a compound may play a role in the ability of an organic anion to inhibit organic cation transport.

The implications of organic anion–cation interactions to drug therapy are of interest. Recently, we observed that the renal clearance of cimetidine in humans is reduced by about 20% following chronic administration of probenecid [6]. Although the reduction was not significant to the therapeutic use of cimetidine, the study demonstrated that hydrophobic organic anions may inhibit the renal transport of organic cations in humans.

In summary, this study demonstrated that organic anions such as probenecid and furosemide inhibited organic cation transport in brush border membrane vesicles prepared from rabbit renal cortex. Like probenecid, furosemide appeared to be a competitive inhibitor of NMN transport. The inhibitory potency of probenecid was related to its hydrophobicity.

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