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Acridine orange transport in canine renal brush-border membrane vesicles

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Organic cations are actively secreted by the mammalian kidney [1]. The locus of these transport systems is the proximal tubule. The mechanism of transport across the brush-border membrane is through an organic cation/H^+ antiporter [2]. However, such a mechanism is indistinguishable kinetically and energetically from an organic cation/OH^- symporter. Evidence from several species (rat, rabbit and dog) suggests that this transporter is electroneutral [3–6]. The molecular mechanism of transport may involve a disulfide/sulfhydryl exchange since an essential requirement for disulfide and sulfhydryl groups exists [7]. The H^+ binding site contains essential carboxylate groups [8], whereas the substrate binding site has essential tyrosyl groups [9]. In addition, histidyl groups have been found to be important for transport [10].

Acridine orange, a weak base, has been used to fluorimetrically measure pH changes in brush-border membrane vesicles (BBMV) [11]. Hitherto, its distribution across the plasma membrane had been believed to be solely due to ionic diffusion or nonmediated means. In a previous study, we first observed that acridine orange had an effect on the organic cation/H^+ antiporter in BBMV [12]. The purpose of this report is to clarify this interaction by examining the effect of acridine orange on the transport of N^1-methylnicotinamide (NMN), a prototypic organic cation, in canine renal BBMV. The results demonstrate that acridine orange was transported across the brush-border membrane via the organic cation transporter.

Methods

These studies employed BBMV isolated from the outer cortex of canine kidneys by a divalent cation precipitation [13]. The purified membranes (3.6 to 9.2 mg protein/mL) were suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 mM K^+ gluconate, 200 mM mannitol, pH 7.5, and were frozen at −70° until used. The pH was adjusted using KOH. All the experiments were done by examining 50 μM [^{3}H]N^1-methylnicotinamide (17.3 Ci/mmol) or 50 μM [^{3}H]*p*-aminohippurate (162 mCi/mmol) transport over a given time period at 37°. The pH of all reaction solutions was 7.5. The assay was initiated by diluting the BBMV 10-fold with the reaction solution (see Figs. 1 and 2). A 100-fold dilution was employed in Figs. 3 and 4 to minimize the carry-over of acridine orange. The details of the experimental procedure have been reported previously [12, 14]. The conditions are outlined in the figure legends. All data are presented as means ± SE. Absence of a standard error bar denotes inclusion within the symbol. Each value was obtained using three to four different membrane preparations performed in quadruplicate. Statistical analysis was performed using ANOVA with testing of the means by the Fisher's test. The radioactive chemicals were purchased from Amersham; all other chemicals came from Sigma.

Results

A concentration–response curve for acridine orange was determined (Fig. 1) and compared to that of verapamil, a competitive inhibitor of the organic cation transport system [15]. The IC_{50} values for these compounds were calculated to be 5.0 and 50 μM respectively. The specificity of acridine orange inhibition was determined by examining what effect, if any, it had on the transport of the prototypic organic anion, *p*-aminohippurate (PAH) (Fig. 2). Acridine orange (20 μM) did not affect PAH transport. The probenecid-inhibitable transport was the same in the presence and absence of acridine orange. This same concentration inhibited NMN transport by greater than 80% (Fig. 1). In

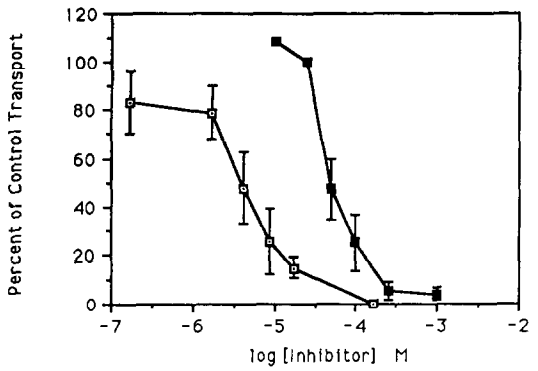

Fig. 1. Concentration–response curve for the effect of acridine orange on NMN influx. The concentration dependence of acridine orange (□) and verapamil (■) on the 15-sec transport of NMN was determined. Control NMN transport was 348 ± 26 pmoles/min/mg protein. Values are means ± SE, N = 3.

addition, acridine orange did not alter the equilibrium distribution of NMN or PAH.

If acridine orange were a substrate for the organic cation transporter, then it should accelerate the countermovement of [^{3}H]NMN giving rise to *trans* stimulation [16]. As shown (Fig. 3), both NMN and acridine orange produced *trans* stimulation of NMN influx. The time–course of the acridine orange *trans* effect on NMN influx was examined (Fig. 4). An overshoot, indicative of concentrative transport over the equilibrium value, was obtained. It is possible that the coupling of the acridine orange and NMN gradients could be, in part, an indirect one. Acridine orange could exchange for a proton which in turn exchanges for NMN. Such a possibility was tested by examining the effect of acridine loading on NMN uptake at the peak of the overshoot (Fig. 4) in the presence of a protonophore (50 μg/mg protein gramicidin D) and a voltage clamp (3.0 μg/mg protein valinomycin). Under these conditions, the uptake of NMN was 158 ± 12% of the equilibrium. This value did not differ from the uptake in the absence of the protonophore, indicating that the coupling of NMN to acridine orange is a direct one ($P > 0.05$). For this to occur, both NMN and

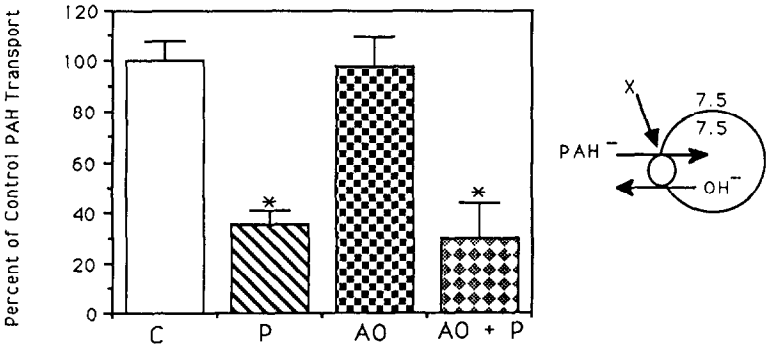

Fig. 2. Specificity of acridine orange: effect on PAH. Abbreviations: C, control PAH transport; P, 0.5 mM probenecid; and AO, 20 μM acridine orange. PAH transport was examined for 15 sec. Control PAH transport was 749 ± 58 pmol/min/mg protein. Key: (*) $P < 0.05$ vs control. Values are means ± SE, N = 3.

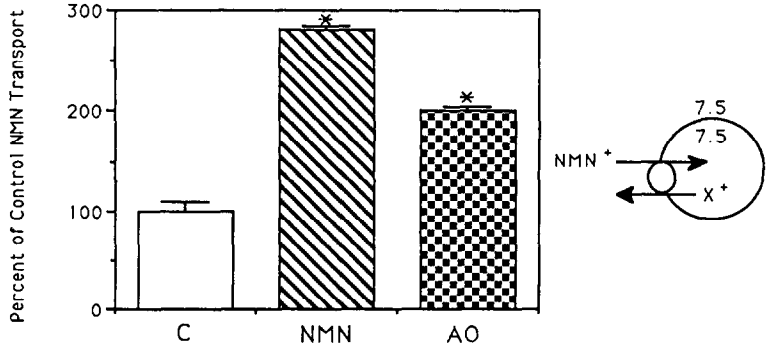

Fig. 3. *Trans* effect of acridine orange on NMN influx. BBMV were incubated with acridine orange (AO, 4 μM) or N^1-methylnicotinamide (NMN, 1 mM) for 30 min prior to assaying for transport (15 sec). Control NMN transport was 714 ± 121 pmol/min/mg protein. Key: (*) $P < 0.05$ vs control. Values are means ± SE, N = 4.

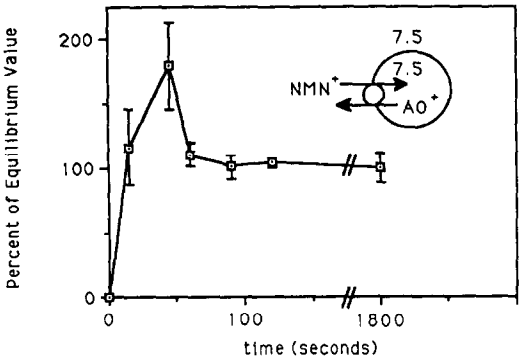

Fig. 4. Time–course of acridine orange *trans* effect on NMN influx. BBMV were incubated with acridine orange (4 μM) for 30 min prior to assaying for transport. The equilibrium uptake of NMN was 255 ± 31 pmol/mg protein. Values are means ± SE, N = 3.

acridine orange must interact at the same site. Therefore, this finding is most consistent with acridine orange being a competitive inhibitor of NMN. Assuming a K_m value for NMN of 94 μM under non-pH driven conditions ($pH_i = pH_o = 7.5$) [12], the apparent K_i values for the two competitive inhibitors, acridine orange and verapamil were calculated according to Cheng and Prusoff [17] and determined to be 3.3 and 33 μM respectively.

Discussion

The results demonstrate that acridine orange was transported across the renal brush-border membrane via the organic cation/H^+ antiporter. Acridine orange was a higher affinity substrate than verapamil. In addition, its concentration–response curve was more shallow than that of verapamil. At higher concentrations acridine orange is capable of forming dimers [18]. It is possible that dimer formation results in altered affinities for the transporter and thereby produces a shallower concentration–response curve. The effect was specific since acridine orange did not inhibit the transport of the organic anion, PAH. Acridine orange also produced *trans* stimulation with an overshoot of NMN transport, an important criterion for ascertaining a common transport system. It is believed that acridine orange directly exchanges with NMN to produce the overshoot since this effect was seen under both voltage and pH clamped conditions.

Previously, we [12] and others [19] employed acridine orange to study H^+ transport by the organic cation/H^+ antiporter. Since acridine orange was a putative substrate for this transport system, NMN concentrations were used which would saturate the transporter to minimize interactions of the dye with the transporter. We had inadvertently misquoted Hsyu and Giacomini [19] regarding the effect of NMN and tetraethylammonium on acridine orange fluorescence [12]. They did not study the interaction of acridine orange on organic cation transport as we had done.

In conclusion, acridine orange is a substrate for the canine renal brush-border organic cation transporter. This compound must be employed prudently when studying pH changes since its movement across the brush-border membrane is dictated by both a nonmediated (ionic diffusion) and a mediated (organic cation/H^+ antiporter) mechanism.

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