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Proton-coupled organic cation transport in renal brush-border membrane vesicles

Paul P. Sokol, Peter D. Holohan, Steven M. Grass and Charles R. Ross

Department of Pharmacology, State University of New York, Health Science Center at Syracuse, Syracuse, NY (U.S.A.)

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We had previously proposed that organic cations are transported across the brush-border membrane in the canine kidney by a H^+ exchange (or antiport) system (Holohan, P.D. and Ross, C.R. (1981) J. Pharmacol. Exp. Ther. 216, 294–298). In the present report, we demonstrate that in brush-border membrane vesicles the transport of organic cations is chemically coupled to the countertransport of protons, by showing that the uphill or concentrative transport of a prototypic organic cation, N^1-methylnicotinamide (NMN), is chemically coupled to the flow of protons down their chemical gradient. In a reciprocal manner, the concentrative transport of protons is coupled to the counterflow of organic cations down their concentration gradient. The transport of organic cations is monitored by measuring [^{3}H]NMN while the transport of protons is monitored by measuring changes in acridine orange absorbance. The functional significance of the coupling is that a proton gradient lowers the K_m and increases the V_{max} for NMN transport.

Introduction

The kidney maintains a homeostatic environment by removing various ingested chemicals, toxins and metabolic products from the body by filtration and/or secretion. Secretion is mediated by organic cation or organic anion transport systems that are located in the plasma membranes of the proximal tubule (for reviews see Refs. 1–4]. This report deals with the transport of organic cations across the brush-border membrane. We proposed that organic cations accumulate in the tubular fluid against their electrochemical gradient by an organic cation/H^+ antiporter [5] that operates via an electroneutral exchange mechanism [6]. Such a mechanism is indistinguishable kinetically and energetically from an organic cation/OH^- symporter. This is an example of a secondary active transport system whose energy is primarily derived from a proton gradient generated by the Na^+/H^+ antiporter [7,8].

The molecular mechanism of transport may involve a disulfide/sulfhydryl exchange since both disulfide and sulfhydryl groups are essential for transport, and the reactivity of these groups is affected by the presence of organic cationic substrates [9]. Additionally, organic cation transport is sensitive to modification by N,N'-dicyclohexylcarbodiimide (DCCD), although the presence of an organic cation does not protect against inhibition [10]. Presumably the DCCD modifies an essential carboxylate. The transport mechanism

Abbreviations: NMN, N^1-methylnicotinamide; DCCD, N,N'-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BBMV, brush-border membrane vesicles; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Correspondence: P.D. Holohan, Department of Pharmacology, State University of New York, Health Science Center at Syracuse, 750 E. Adams Street, Syracuse, NY 13210, U.S.A.

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therefore, would require communication between these distinct sites, i.e. cooperativity, if indeed the translocation of an organic cationic substrate were directly coupled to the countermovement of a proton.

The objective of the present study was to rigorously test the hypothesis that the transport of organic cations is coupled to the countermovement of H^+. We provide data affirming our original conclusion by showing that the concentrative transport of a prototypic organic cation, N^1-methylnicotinamide (NMN), is chemically coupled to the movement of protons down their concentration gradient, and conversely that the concentrative transport of protons is coupled to movement of NMN molecules down their concentration gradient. Functionally, a pH gradient increases the affinity and the turnover of the transporter.

Methods

Membrane preparation

Brush-border membrane vesicles (BBMV) were isolated from canine kidney cortex using a cation precipitation method [11]. The purified membranes (5.0–11.3 mg protein/ml) were suspended in an appropriate buffer and stored at −70 °C until used [10]. Details regarding the buffers employed are outlined in the figure legends. KOH was employed to titrate the Hepes. Protein concentration was assayed using bovine serum albumin as a standard [12].

Transport measurements

1. NMN influx studies. NMN transport was assayed using a rapid filtration technique [6,13]. BBMV were allowed to preequilibrate at 25 °C for 15 min. The reaction was started by diluting the BBMV (10 μl) 10-fold with the reaction solution containing [^{3}H]NMN. The reaction was terminated by adding 3 ml of ice-cold buffer to the reaction vessel at times specified in the figures. The quench solution was identical to the reaction solution. The reaction vessel was rinsed twice with 3 ml ice-cold buffer and the contents poured onto prewetted 0.3 μm PHWP Millipore filters where the solution was removed under vacuum (500 Torr). The filters were rinsed with an additional 3 ml of ice-cold buffer and transferred to liquid scintillation vials. 10 ml of scintillation fluid (Filtron X, National Diagnostics, Inc., Somerville, NJ) were added and the amount of radioactivity taken up by the vesicles was determined by standard liquid scintillation techniques using an external standard to correct for quench. Corrections for the amount of radioactivity bound to the filters were determined by running filter controls (reaction solution without BBMV) concurrently with each experiment.

The specifically mediated transport of 50 μM NMN was determined by assessing transport in the presence of 10-fold excess (0.50 mM) of mepiperphenidol (Darstine), a competitive blocker [14–16]. In the absence of a H^+ gradient ($pH_{in} = pH_{out} = 7.5$) the specifically mediated NMN transport was 260 ± 30 (S.E.) pmol/min per mg of protein. This value was calculated from multiple 15-s uptakes using six different membrane preparations ($n = 28$). In these experiments mepiperphenidol blocked 85 ± 2% of the total NMN transport and hence 15% or less of the total transport was non-carrier mediated.

2. NMN efflux studies. BBMV were preequilibrated with 0.5 mM [^{3}H]NMN for 30 min at 25 °C. The reaction was started by diluting BBMV (10 μl) 10-fold with buffer and terminated at the appropriate time interval as described above.

3. H^+ transport. The method used was that of Burnham et al. [17]. Changes in intravesicular pH were monitored by acridine orange absorbance at 492 nm using 546 nm as the reference wavelength. The measurements were made with an Aminco DW-2 spectrophotometer in the dual beam mode. The experiments consisted of diluting brush-border membrane vesicles in an excess of a medium containing 50 mM NMNCl, 115 mM KCl, 2.5 mM Hepes/*N*-methyl-D-glucamine chloride (pH 7), followed by two wash cycles (centrifugation at 20000 × *g* for 20 min, discarding the supernatant and resuspending the pellet in the above medium). Acridine orange was added to a final concentration of 20 μM and the suspension was left at room temperature for two additional hours for complete equilibration of the intra- and extravesicular compartments. Where indicated (see legends) ionophores were added from a stock ethanol solution to the membrane suspensions and equilibrated for 30 min prior to use. The experiments were ini-

tiated by transferring 10 μl of the suspensions (9–13 mg protein/ml) to 2.5 ml of an appropriate medium in a stirred cuvette, thermostatically maintained at 23°C. When desired, a gradient of NMN (in to out) was created by replacing NMNCl in the cuvette medium with *N*-methyl-D-glucamine chloride. Polyacrylic cuvettes were used to minimize dye binding.

4. Transport studies in presence of various reagents. The concentrations of all reagents employed were normalized per mg of protein in the reaction vessel. Stock solutions of gramicidin D, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and nigericin were prepared fresh daily in ethanol. After a 30 min preequilibration at 25°C, the transport assays were performed as outlined above. Controls containing an equivalent amount of ethanol as that used to dissolve the various reagents were tested for the effect of ethanol itself on transport. The final ethanol concentration was always less than 5%, a concentration that has no effect on NMN transport, consistent with our previous results [6]. A pH gradient was created in the nigericin experiments by preequilibrating BBMV (10 mM Hepes, 150 mM potassium gluconate (pH 7.5)) with [^{3}H]NMN and diluting them 10-fold into buffer free of K^+ (10 mM Hepes, 300 mM mannitol (pH 7.5)) containing [^{3}H]NMN of the same concentration and specific activity. The control was performed under the same conditions in the absence of nigericin and was used to correct, if necessary, for any changes in [^{3}H]NMN distribution not resulting from the nigericin generated H^+ gradient. Additional controls included diluting the BBMV into 10 mM Hepes, 50 mM potassium gluconate, 200 mM mannitol (pH 7.5).

The data are presented as means ± S.E. unless otherwise noted. Linear regression was used to analyze the data presented in Fig. 7 [18].

Materials

N^1-[^{3}H]Methylnicotinamide chloride (1.7 Ci/mmol) was obtained from ICN (Irvine, CA). The other chemical reagents were purchased from the following sources: acridine orange, *N*-methyl-D-glucamine chloride, unlabeled N^1-methylnicotinamide chloride, gramicidin D, carbonyl cyanide *m*-chlorophenylhydrazone, nigericin, from Sigma Chemical Co. (St. Louis, MO). Mepiperphenidol, Darstine®, 1-(3-hydroxy-5-methyl-4-phenylhexyl)-1-methylpiperidinium bromide, was purchased from Merck Sharp & Dohme (West Point, PA).

Results

Experiments were designed to demonstrate the chemical coupling between the gradients of NMN and H^+.

1. Gradient-coupled NMN transport

Previously, we and others [5,19,20] had employed ionophores to provide suggestive evidence that organic cation transport is directly coupled to the countermovement of protons. We reinvestigated the coupling phenomena for two reasons: first, to firmly establish the appropriate concentrations of ionophores that can be used in these experiments: and second, to conclusively document that the two gradients (NMN and H^+) are coupled together via the organic cation/H^+ antiporter.

Effect of ionophores

Earlier, we found that valinomycin, a K^+-selective ionophore, had a deleterious effect on NMN transport in canine BBMV [6]. Similar findings were reported by Wright [19] and Rafizadeh et al. [20] who studied rabbit BBMV. Therefore, we examined each ionophore over a concentration range in order to determine if they caused nonspecific effects on NMN transport. The experiments were carried out under nongradient-stimulated conditions (i.e., absence of a pH gradient). As shown (Fig. 1), high concentrations of CCCP, gramicidin D and nigericin inhibited NMN transport, although their dose-response curves differed. For example at 1 mM, nigericin inhibited transport by 60%, CCCP by 30% and gramicidin was noninhibitory. Since these experiments were conducted under nongradient-stimulated conditions, the inhibition observed presumably was due to some alteration of the lipid bulk phase. Once the appropriate concentrations of the ionophores were determined, we employed them to couple or uncouple the NMN/H^+ antiporter.

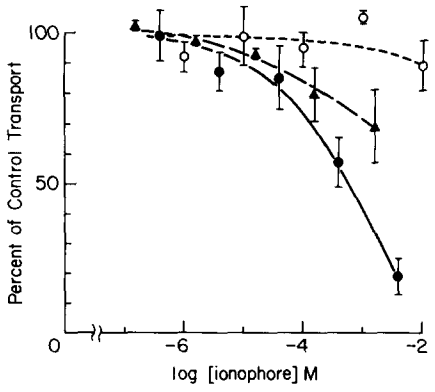

Fig. 1. Effect of ionophores on NMN transport. Non-specific effects of ionophores were ascertained by analyzing NMN transport under nongradient-stimulated conditions. Brush-border membrane vesicles in 10 mM Hepes, 50 mM potassium gluconate, 200 mM mannitol (pH 7.5) were preequilibrated for 30 min with various concentrations of nigericin (●), CCCP (▲), or gramicidin D (○) followed by initiation of transport by adding 90 μl of an identical medium containing 50 μM [^{3}H]NMN to 10 μl of the appropriate membrane suspension. Transport was allowed to proceed for 15 s. Each symbol is the mean ± S.E. of 4–8 determinations conducted with two or three different membrane preparations.

(a) Dissipation of H^+ gradient. The effect of dissipation of the H^+ gradient on NMN transport was examined using CCCP and gramicidin D.

(i) CCCP. It has been shown that CCCP is an effective uncoupler of H^+ gradient-stimulated processes [21,22]. The effect of 14 μM CCCP on H^+-stimulated NMN influx is shown (Fig. 2). In aggrement with our previous findings [5,6], a H^+ gradient (acidic inside) drives the concentrative transport of [^{3}H]NMN into BBMV. In the presence of CCCP, uphill transport was not observed and the time course of NMN influx was comparable to that seen in the absence of a pH gradient.

(ii) Gramicidin D. The effect of the H^+ ionophore gramicidin D [23] on NMN influx was examined (Fig. 3). As above, uphill transport was observed in the presence of a pH gradient (acidic inside) but not when gramicidin (190 μM) was added. Under the latter condition, influx was similar to that observed in the absence of a pH gradient. The effect of gramicidin D on [^{3}H]NMN efflux was also examined (Fig. 4). A pH gradient (inside alkaline) stimulated efflux compared to the control (absence of gradient) and the stimulation was abolished when gramicidin was present.

(b) Generation of H^+ gradient. Nigericin mediates the electroneutral exchange of potassium ions for hydrogen ions [23]. BBMV were first equilibrated in a K^+ medium (10 mM Hepes, 150 mM potassium gluconate (pH 7.5) containing 50 μM [^{3}H]NMN. Next, the BBMV were diluted 10-fold into a K^+-free medium (10 mM Hepes, 300 mM mannitol (pH 7.5)) containing 50 μM [^{3}H]NMN of the same specific activity. By this maneuver, the NMN concentration and specific activity remain at equilibrium, but a K^+ gradient is created (in to out). The BBMV contain nigericin

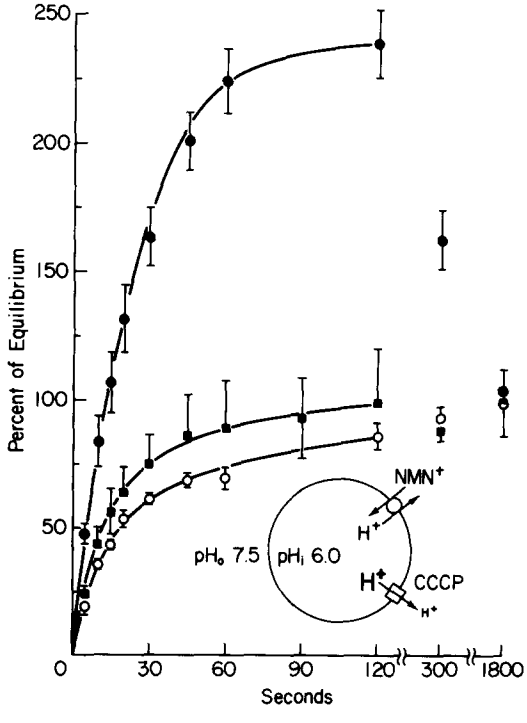

Fig. 2. Effect of CCCP on H^+ gradient-stimulated NMN influx. A pH gradient was created by adding 10 μl of brush-border membrane vesicles in 10 mM Hepes, 50 mM potassium gluconate, 200 mM mannitol (pH 6.0) to 90 μl of a medium of the same composition but at pH 7.5 and containing 50 μM [^{3}H]NMN. At selected time intervals, transport of [^{3}H]NMN was determined (see Methods). One portion of the membrane suspension was preequilibrated with 14 μM CCCP (■) and another with an equal amount of ethanol (●) for 30 min prior to initiating transport (see Methods). Transport in the absence of a pH gradient was determined at pH 7.5 (○). The data are expressed as percent of the equilibrium value (1800 s). Each symbol is the mean ± S.E. of four experiments conducted in quadruplicate using four separate membrane preparations. Inset: A representation of the experimental conditions.

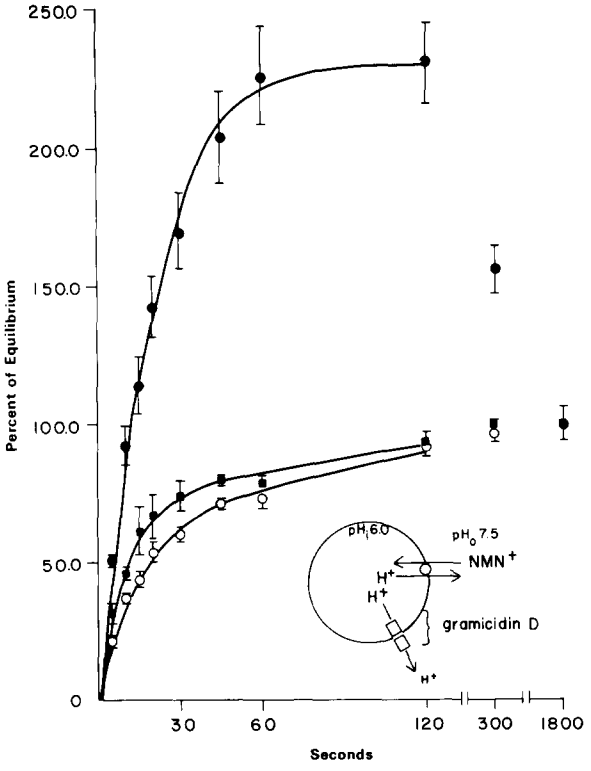

Fig. 3. Effect of gramicidin D on H^+ gradient-stimulated NMN influx. A pH gradient was created as described in legend to Fig. 2. A portion of the brush-border membrane vesicles were pretreated with either 190 μM gramicidin D (■) or ethanol control (●) for 30 min prior to initiating the influx of 50 μM [^{3}H]NMN. Transport in the absence of a pH gradient (○) was determined at $pH_i = pH_o = 7.5$. The data are expressed as percent of the equilibrium value (1800 s). Each symbol is mean ± S.E. of three experiments conducted in quadruplicate using three different membrane preparations. Inset: A representation of the experimental conditions.

(360 nM) which will electroneutrally exchange potassium ions for protons until the K^+ gradient is dissipated, but will in effect generate a H^+ gradient (acidic inside). If NMN transport is coupled to H^+ antiport, these maneuvers should perturb the NMN equilibrium position and drive [^{3}H]NMN into the vesicle until the H^+ gradient is dissipated. Eventually, the system should relax toward equilibrium. The response of NMN to a H^+ gradient generated in situ is shown (Fig. 5). NMN was transiently accumulated intravesicularly.

2. Gradient coupled H^+ transport

The interpretation of the data presented thus far is that the uphill or concentrative transport of NMN is coupled to the movement of H^+ down its concentration gradient. To unequivocally demonstrate the chemical coupling between NMN and H^+ gradients, the effect of an NMN gradient on H^+ transport was examined.

The acidification of BBMV was studied by following the change in acridine orange absorbance (see Methods). Acridine orange, a weak base, was found to be a substrate for the organic cation transport system. This conclusion is based upon the dye's capability to *cis* inhibit and *trans* stimulate NMN transport. It is important to note, however, that 20 μM acridine orange had a negligible effect on the transport of 10 mM NMN; the *cis* inhibition accounted for less than 10%. Therefore, in all the following experiments, NMN con-

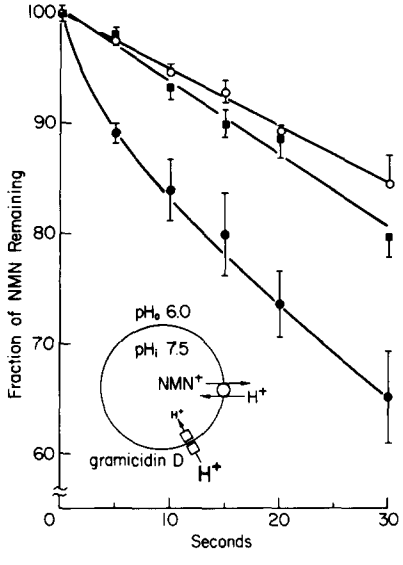

Fig. 4. Effect of gramicidin D on H^+ gradient-stimulated NMN efflux. Brush-border membrane vesicles in a medium consisting of 10 mM Hepes, 50 mM potassium gluconate, 200 mM mannitol (pH 7.5) and containing 50 μM [^{3}H]NMN were preequilibrated for 30 min at 25 °C with 190 μM gramicidin D (■) or with ethanol (●) or without any additions (○) and the efflux of 0.5 mM [^{3}H]NMN initiated by diluting the suspension (10 μl) 10-fold into a medium of the same composition at pH 6.0. The data are expressed as the fraction of the zero-time radioactivity remaining. Each symbol is the mean ± S.E. for three experiments conducted in quadruplicate using three different membrane preparations. Inset: A representation of the experimental conditions.

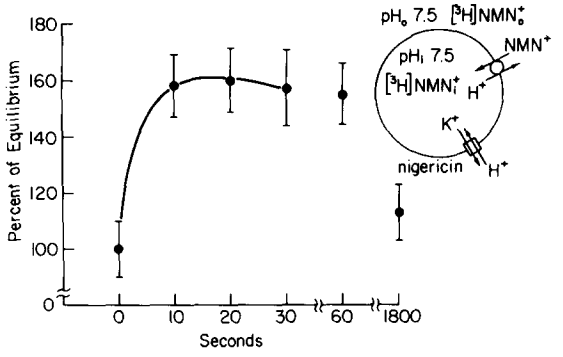

Fig. 5. Nigericin generated H^+ gradient drives NMN transport. Brush-Border membrane vesicles in a medium of 10 mM Hepes, 150 mM potassium gluconate (pH 7.5) and containing 50 μM [^{3}H]NMN were preequilibrated with nigericin (360 nM) for 30 min at 25 °C. The reaction was started by diluting the BBMV (10 μl) 10-fold into a medium of 10 mM Hepes, 300 mM mannitol (pH 7.5) containing 50 μM [^{3}H]NMN of the same specific activity. Transport was examined over the time period indicated. The data are expressed as percent of equilibrium. Each symbol gives the mean ± S.E. of two experiments conducted in quadruplicate with two different membrane preparations. Inset: A representation of a nigericin induced H^+ gradient sufficient to drive NMN transport.

centrations in excess of 10 mM were employed in order to minimize competition by acridine orange.

The validity of the method was ascertained by following the activity of the Na^+/H^+ exchanger which is known to be present in the brush-border membrane [8]. The vesicles were preequilibrated with 165 mM NaCl (see Methods) and diluted 250-times into a medium where the NaCl is replaced by *N*-methyl-D-glucamine chloride creating an outwardly directed Na^+ gradient. As shown in Fig. 6, panel A, the intravesicular compartment was rapidly acidified under these conditions. The presence of NMN/H^+ exchange was shown by preequilibrating with 165 mM NMN and creating an NMN gradient in to out by dilution. Under these conditions, the intravesicular compartment was acidified compared to the absence of an NMN gradient (Fig. 6, panel A). The extent of acidification produced by the NMN gradient was less than that produced by the Na^+ gradient. The initial rate of change in absorbancy was 0.008 units/min per mg of protein in the presence of the NMN gradient, but was 0.052 units/min per mg of protein in the presence of the Na^+ gradient. This difference probably represents the exchange capacity of the two systems.

The issue under study was whether the acidification was directly or indirectly coupled to NMN moving down its chemical gradient. The following series of experiments were designed to resolve this issue. First, the BBMV were preequilibrated with 50 mM NMN and diluted into a medium with or without an identical concentration of NMN (Fig. 6, panel B). In the absence of an NMN gradient, no significant change in the pH of the intravesicular compartment was observed. However, in the presence of the NMN gradient (in to out), a sustained acidification was observed as before (Fig. 6, panel A). The extent of acidification with the 50 mM NMN gradient was the same as that observed with the 165 mM NMN gradient. In two experiments conducted in triplicate and using separate membrane preparations, the rate of change in the absorbance when the vesicles were preequilibrated with 165 mM NMN was 0.008 ± 0.001 (mean ± S.E.) absorbance units/min per mg protein compared to 0.010 ± 0.002 (mean ± S.E.) absorbance units/min per mg protein when the vesicles were preequilibrated with 50 mM NMN. These findings suggest that the transporter is saturated under either condition and remains saturated throughout the course of the experiment. Second, the possibility that the acidification was a consequence of an electrical coupling between NMN and H^+, generated by an NMN diffusion potential (inside negative), was eliminated by repeating the experiment in the presence of a K^+/valinomycin voltage clamp using conditions which have been shown to effectively clamp the membrane [6]. The acidification found in the presence of the K^+/valinomycin clamp (0.008 ± 0.001, mean ± S.E., $n = 2$) was the same as that observed in the absence of the clamp (0.007 ± 0.0005, mean ± S.E., $n = 3$). Third, we attempted to short-circuit the exchange by adding nigericin, e.g., exchanging H^+ for K^+. (The experiment was conducted in the presence of a K^+/valinomycin clamp as well.) When these maneuvers were carried out, the extent of acidification was significantly, but not completely abolished (Fig. 6, panel B). These data were taken as showing that the movement of protons against their concentration gradient (i.e. acidification) is chemically coupled to the countermovement of

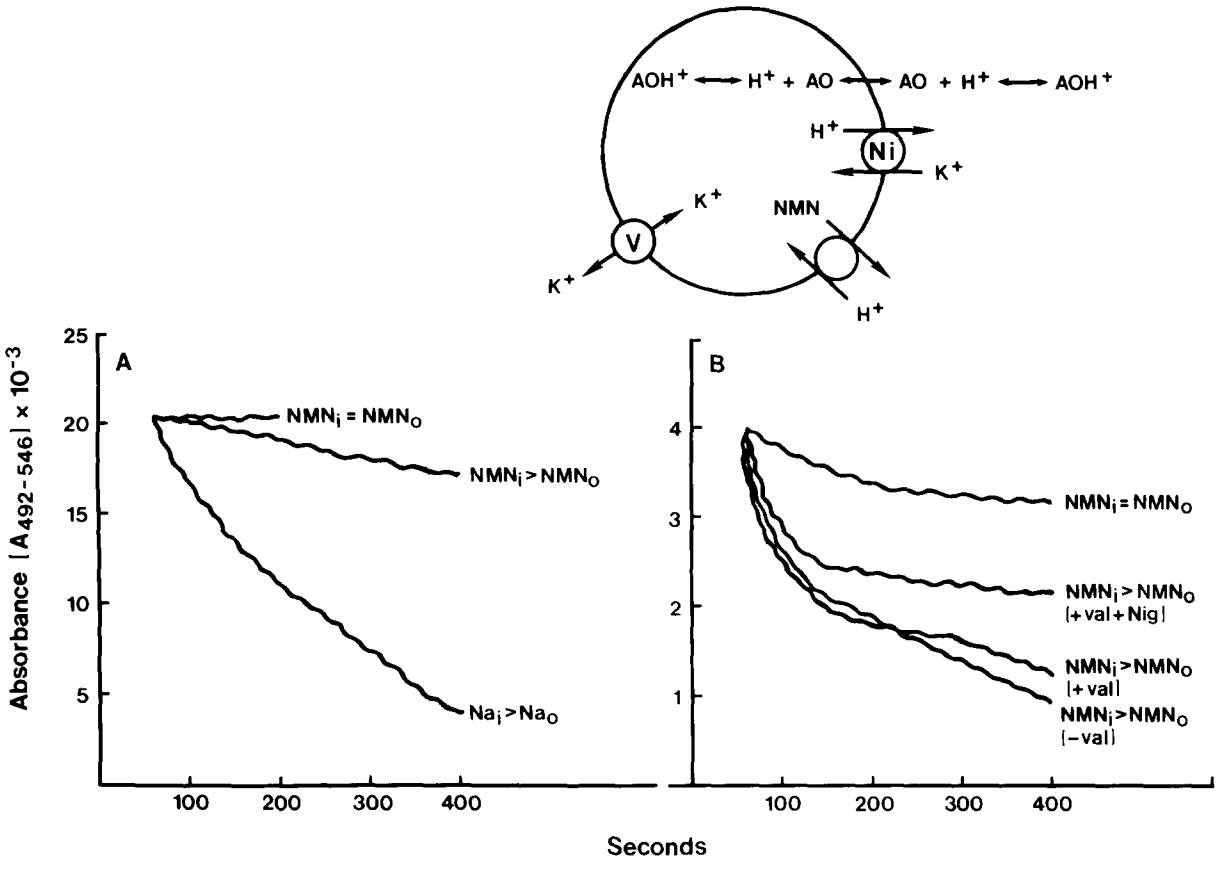

Fig. 6. NMN gradient-stimulated H^+ transport. Gradients were created by diluting brush-border membrane vesicles (panel A, 10 μl BBMV at 11 mg/ml; panel B, 20 μl BBMV at 12.5 mg/ml) preequilibrated with 20 μM acridine orange preloaded with 165 mM NMNCl or 165 mM NaCl (panel A) or 50 mM NMNCl, 115 mM KCl (panel B) in a medium of 2.5 mM Hepes (pH 7) to 2.5 ml of the same medium of the same composition ($NMN_i = NMN_o$) or where the NMNCl or NaCl was replaced with *N*-methyl-D-glucamine chloride ($NMN_i > NMN_o$) or ($Na_i^+ > Na_o^+$). The absorbance of the dye was followed with time. The effect of ionophores was established by adding valinomycin (2 μg/mg protein) or nigericin (1 μg/mg protein) to the preequilibration media. The data are a representative experiment. Inset: A representation of the experimental conditions.

NMN molecules down their concentration gradient.

3. Kinetics

The rate of [^{3}H]NMN influx in BBMV as a function of NMN concentration did not give a simple hyperbolic relationship (data not shown) but could be described by the following equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} + D[S]$$

meaning that NMN influx occurred via two pathways: one which is saturable and can be described by Michaelis-Menten kinetics; and the other which is non-saturable, and therefore represents non-mediated influx. The specifically-mediated component was evaluated by measuring the 5-s uptakes in the absence or in the presence of a 100-fold excess of mepiperphenidol (see Methods). The effect of chemically coupling a H^+ gradient to NMN transport was examined by measuring the kinetics of [^{3}H]NMN influx in the absence and presence of a pH gradient. The results are shown in Fig. 7. Both the K_m and V_{max} were affected by the H^+ gradient. The data presented in Fig. 7 represent a single experiment. In three separate experiments the K_m value determined under pH gradient conditions was 15 ± 4 μM (mean $\pm$ S.E., $n = 3$) com-

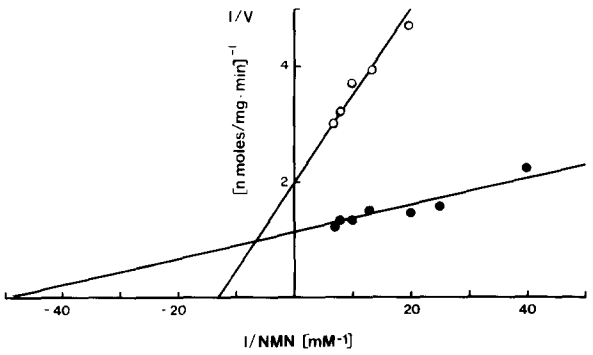

Fig. 7. Lineweaver-Burk plots of H^+ gradient-stimulated (●), ($pH_i = 6.0$, $pH_o = 7.5$) and nongradient-stimulated (○), ($pH_i = pH_o = 7.5$) influx of [^{3}H]NMN. The specifically-mediated NMN transport was determined in quadruplicate at 5 s (see Methods). The data represent a single experiment performed in quadruplicate.

pared to 94 ± 3 μM (mean $\pm$ S.E., $n = 3$) when determined in the absence of a pH gradient. The V_{max} value was 990 ± 215 pmol/min per mg of protein under pH gradient-stimulated conditions and 450 ± 180 pmol/min per mg of protein under non gradient-stimulated conditions.

Discussion

The results demonstrate that the transport of the prototypic organic cation, NMN, is coupled to the countertransport of protons. These findings confirm our original hypothesis of electroneutral exchange of organic cations for H^+ in the dog kidney brush-border membrane [5,6]. Others have supplied confirmatory results for a similar, if not identical, system in the brush-border membrane from other species [19,20,24–27].

We provide evidence that the NMN and proton gradients are chemically coupled; the uphill transport of NMN is eliminated by the dissipation of the pH gradient with uncouplers or ionophores (Figs. 2 and 4), and conversely the uphill transport of NMN is achieved by generation of the pH gradient in situ (Fig. 5). In addition, NMN gradients drive H^+ accumulation (Fig. 6) which is also reduced in the presence of an uncoupler. The functional significance of the coupling is that a proton gradient lowers the k_m and increases the V_{max} for NMN transport. The dimensions and orientation of these effects are consistent with the physiological function of the exchanger, i.e., secretion.

Uphill transport of NMN driven by a pH gradient, is not observed in the presence of either CCCP or gramicidin D. CCCP acts as a mobile proton carrier and rapidly equilibrates hydrogen ions across biological membranes [28]. Commercially available gramicidin D is a mixture of gramicidins A, B and C in the approximate ratio of 72 : 9 : 19. They are a family of closely related polypeptides containing fifteen amino acids [23] and their chemistry and biological applications have been extensively reviewed [23,29]. Gramicidins alter membrane conductance by forming water-filled channels or pores constructed of two molecules of the polypeptide associated in a head-to-head fashion that spans the bilayer [30]. The N-terminus of gramicidin is located in the membrane interior and the C-terminus is at the membrane surface. Although gramicidin has low selectivity in that numerous ions can pass through the channel, it has the highest affinity for protons [23]. Our findings with gramicidin are consistent with disruption of a H^+ gradient. It is possible that a potential difference arising from a H^+ diffusion potential across the BBMV was created by the maneuvers used in the experiments with CCCP and gramicidin D. However, we established that NMN influx and efflux in BBMV are independent of voltage [6], and therefore, even if a small potential difference were present it would not affect the exchange reaction.

Additional evidence for a direct coupling between organic cation and proton transport was the observation that NMN transport was driven in one direction in response to a pH gradient generated in situ (Fig. 5). Nigericin is a naturally occurring carboxylic ionophore having a linear backbone of heterocyclic rings [23]. It noncovalently cyclizes to form a stable liganded complex that acts as a mobile shuttle to move potassium ions down their concentration gradient and returns in a protonated form, thus exchanging K^+ for H^+. Using nigericin, we were able to achieve transient accumulation of NMN away from its equilibrium. The only energy sources present were the H^+ and K^+ gradients. Since we have previously established that K^+ gradients are ineffective as driving forces for NMN transport [6], we conclude that

the uphill transport is driven by the H^+ gradient, generated as described.

We find that high concentrations of the ionophores and uncouplers inhibit NMN transport nonspecifically (Fig. 1). Previously, we reported on a similar finding for valinomycin [6]. Wright [19] also found that valinomycin and CCCP inhibited NMN transport in rabbit BBMV. Similarly, Rafizadeh et al. [20] found an inhibitory effect of valinomycin on the tetraethylammonium (TEA) transport in rabbit BBMV. The causes of these inhibitory effects are unknown. Presumably, they reflect a distortion of the lipid bulk phase of the bilayer. In the present report, we reconfirm that these reagents are useful tools for studying membrane transport, but show also that they must be applied judiciously.

We find also that an NMN gradient drives the concentrative transport of H^+ (Fig. 6). The fact that coupled transport remains in the presence of a K^+/valinomycin voltage clamp, implies that the two gradients are coupled chemically and not indirectly by a voltage. On the other hand, the extent of acidification is lessened, but not completely abolished in the presence of nigericin (Fig. 6, panel B). This finding is the predicted response: protons which accumulate inside the vesicle because of coupled transport to NMN efflux are rapidly expelled via K^+/H^+ exchange mediated by the ionophore, nigericin. In fact, this finding is necessary proof that the transport of organic cations and protons are chemically coupled. The acidification was not completely blocked by the addition of nigericin, however. The simplest explanation of these data is that the amount of ionophore used was insufficient to prevent the generation of a pH gradient across the membrane. Unfortunately, we were unable to use higher concentrations of nigericin because of its deleterious non-specific effects on the transporter (Fig. 1).

An unexpected finding is that acridine orange is a putative substrate for the organic cation transport system. Therefore, to minimize interactions of the dye with the transporter, NMN concentrations were used which would saturate the transporter throughout the course of the experiments. Our results are quite different from those of Hsyu and Giacomini [26] who found that acridine orange affected tetraethylammonium (TEA) transport in rabbit BBMV, but not NMN transport. The significance of this discrepancy is not clear; it may reflect species differences with regard to the renal handling of organic cations.

What is clear is that a pH gradient affects the kinetic parameters (Fig. 7). The effects are compatible with the physiological function of the transporter; a pH gradient increases the affinity and the turnover of the transporter. However, the values of the kinetic constants presented in the present report are dramatically different from those we published earlier [15]. Previously, we followed 30-s uptakes at 37 °C compared to 5-s uptakes at 25 °C used in the present study.

The important questions are what are the mechanistic and physiological importance of the countermovement of H^+ to NMN transport. A shallow pH gradient exists across the apical membrane in the proximal tubule (the tubular fluid is more acidic) so that the physiological importance is that organic cations can be driven out of the cell into the tubular fluid in exchange for a H^+, i.e. concentrative transport. It is equally conceivable that NMN/H^+ exchange is not a concentrative mechanism, but rather is advantageous as a charge compensatory mechanism. The mechanistic details have not been fully elucidated as yet. We have found that DCCD inhibits the exchanger [10] presumably by modifying the H^+ binding site, although this interpretation has not been confirmed. We have published related findings suggesting that the NMN binding site and the H^+ binding site are topographically distinct [5,10]. The proton gradient may have other functions as well. We proposed that disulfide/sulfhydryl exchange is the mechanism underlying the exchange reaction [9]. In this regard, Robillard and Konings [31] have proposed a model whereby disulfide/sulfhydryl exchange can explain proton gradient symport in microorganisms.

In summary, we have confirmed our earlier findings that the transport of organic cations across the brush-border membrane is coupled to the countertransport of protons. We have extended our earlier findings by showing the converse; the transport of protons is coupled to the countertransport of organic cations. We have also presented evidence of the functional consequence of the coupling mechanism.

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