Sodium Uptake across Basolateral Membrane of Rat Distal Colon

Evidence for Na-H Exchange and Na-Anion Cotransport

Vazhaikkurichi M. Rajendran, Matthew Oesterlin, and Henry J. Binder
Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract

This study sought to characterize the mechanism of Na transport across basolateral membrane vesicles of rat distal colon. Both an outward proton gradient and an inward bicarbonate gradient stimulated ^{22}Na uptake. Proton gradient-stimulated ^{22}Na uptake was activated severalfold by the additional presence of an inward bicarbonate gradient, and bicarbonate gradient-stimulated ^{22}Na uptake was significantly enhanced by an imposed intravesicular membrane positive potential. 0.1 mM amiloride inhibited both proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake by 80 and 95%, respectively, while 1 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) inhibited both proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake by 40 and 80%, respectively. Both proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake saturated as a function of increasing Na concentration: the apparent kinetic constants (K_m) for Na for the DIDS-insensitive component of proton gradient-stimulated ^{22}Na uptake was 46.4 mM, while the DIDS-sensitive component of proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake had K_m for Na of 8.1 and 6.4 mM, respectively. Amiloride inhibited both DIDS-insensitive proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake with an inhibitory constant (K_i) of ~35 and 1 μM, respectively. We conclude from these results that proton gradient-stimulated ^{22}Na uptake represents both DIDS-insensitive Na-H exchange and DIDS-sensitive electrogenic Na-OH cotransport, and that the DIDS-sensitive component of proton gradient-stimulated ^{22}Na uptake and bicarbonate gradient-stimulated ^{22}Na uptake may represent the same electrogenic Na-anion cotransport process. (*J. Clin. Invest.* 1991. 88:1379–1385.) Key words: DIDS-sensitive Na uptake • DIDS-insensitive Na uptake • Bicarbonate-stimulated Na cotransport • DIDS-sensitive Na-OH cotransport

Introduction

Recent studies with colonic apical membrane vesicles (AMV)[1] have demonstrated a Na-H exchange with characteristics that differ from the properties of Na-H exchange that has been identified in many other membranes of both polar and nonpolar cells (1). Because this Na-H exchange is not proton activated, we proposed that Na-H exchange of AMV is primarily involved with vectorial Na transport. Thus, we initiated these studies to determine whether a Na-H exchange (i.e., proton gradient-stimulated ^{22}Na uptake) was also located on the basolateral membrane of epithelial cells of rat distal colon. Because preliminary studies revealed that proton gradient-stimulated ^{22}Na uptake by isolated basolateral membrane vesicles (BLMV) was both 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)-sensitive and DIDS-insensitive (2), we planned this investigation to characterize the properties of Na transport in BLMV with particular emphasis on the effect of both proton and bicarbonate gradients on ^{22}Na uptake.

The results presented in this study indicate the presence of both DIDS-insensitive Na-H exchange, with properties that differ somewhat from the Na-H exchange of AMV, and a novel DIDS-sensitive electrogenic Na-anion cotransport process which has affinity for both hydroxyl and bicarbonate but not for other inorganic and organic anions.

Methods

Preparation of basolateral membrane vesicles. BLMV from scraped mucosa of distal colon of Sprague-Dawley rats (200–250 g) were isolated by sucrose density gradient and differential centrifugation method of Biber et al. (3). BLMV was resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and was used for enzyme assays. BLMV used for uptake studies were resuspended in respective media that are specified in figure legends and stored at −70°C. Frozen vesicles were thawed and incubated at room temperature for 90 min before the uptake studies. Uptake rates in fresh and frozen vesicles were quantitatively identical; as a result, all the uptake experiments were performed with frozen vesicles. The purity of BLMV was assessed by the basolateral enzyme marker Na,K-ATPase. The specific activity of Na,K-ATPase in BLMV (1071.8±84.8 μmol inorganic phosphate, (P_i) liberated per mg protein · min) permeabilized with SDS was enriched 12.7-fold more than that of homogenate (84.5±13.8 μmol P_i liberated per mg protein · min). Almost similar enrichment (11.4-fold) was observed for BLMV (539.5±42.6 μmol P_i liberated per mg protein · min) vs. homogenate (47.3±6.8 μmol P_i liberated per mg protein · min) in nonpermeabilized membranes (i.e., in the absence of SDS). The specific activity of Na,K-ATPase in nonpermeabilized membrane is ~ 50% of that observed in membranes permeabilized with SDS. This observation indicates that these BLMV contain a mixed (50:50) population of right side out and inside out vesicles. The relative purity of the basolateral membrane was comparable to that of Biber et al. (3).

Preparation of apical membrane vesicles. AMV of rat distal colon were prepared by the method of Stieger et al. (5) as previously described (4).

Enzyme and protein assays. Na,K-ATPase was assayed by the method of Forbush (6). The specific activity of Na,K-ATPase was expressed as mean±SE from six different preparation. Protein was assayed by the method of Lowry et al. using bovine serum albumin as standard (7).

Uptake studies. ^{22}Na (New England Nuclear Co., Boston, MA) uptake was measured by rapid filtration technique, as described earlier

Address correspondence and reprint requests to Dr. Henry J. Binder, Department of Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

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1. *Abbreviations used in this paper:* AMV, apical membrane vesicles; BLMV, basolateral membrane vesicles; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; MES, 2-[*N*-morpholino]ethanesulfonic acid; NMG, *N*-methyl-D-glucamine.

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(4). Representative experiments that are illustrated represent the mean±SEM of triplicate assays. Standard errors of the mean that are less than 5% are not shown in figures. Kinetic constants were calculated using Enzfitter program in an IBM PC. All experiments except where noted were performed with at least three different membrane preparations from different animals.

Results

The initial experiment was the determination of the effect of an outward proton gradient on ^{22}Na uptake in BLMV. As shown in Fig. 1, an outward proton gradient stimulated ^{22}Na uptake and resulted in transient accumulation ("overshoot"). Because proton gradient-stimulated ^{22}Na uptake was linear for up to at least 8 s at Na concentrations of 0.1 mM and 100 mM (not shown), further studies were performed using an incubation period of 6 s.

The effect of amiloride, an inhibitor of Na-H exchange, was examined on proton gradient-stimulated ^{22}Na uptake. As shown in Fig. 2, proton gradient-stimulated ^{22}Na uptake was inhibited 76% by 0.1 mM amiloride. The effect of several transport inhibitors (all at one mM), DIDS (Fig. 2), bumetanide, furosemide, and ouabain (not shown), on proton gradient-stimulated ^{22}Na uptake was also examined. Of the inhibitors tested only DIDS, an inhibitor of electrogenic Na-HCO_3 cotransport and of anion exchange, significantly inhibited (40%) proton gradient-stimulated ^{22}Na uptake (Fig. 2). To examine whether DIDS inhibits the amiloride-insensitive or the amiloride-sensitive fraction of proton gradient-stimulated ^{22}Na uptake, the effect of simultaneous presence of amiloride and DIDS was studied. As shown in Fig. 2 the simultaneous presence of amiloride and DIDS did not inhibit ^{22}Na uptake more than that which was inhibited by amiloride alone, indicating that DIDS inhibits the amiloride-sensitive portion of proton gradient-stimulated ^{22}Na uptake. These results indicate that BLMV proton gradient-stimulated ^{22}Na uptake is primarily amiloride-sensitive and consists of a DIDS-sensitive and a DIDS-insensitive process.

The previous demonstration of DIDS-sensitive Na-HCO_3 cotransport in the BLMV of rabbit renal proximal tubule (8, 9) suggests that the DIDS-sensitive component of proton gradient-stimulated ^{22}Na uptake in colonic BLMV might represent Na-OH cotransport perhaps via the Na-HCO_3 cotransport process. The DIDS-insensitive component of proton gradient-stimulated ^{22}Na uptake might then represent coupled Na-H exchange. Thus, the effect of an inward bicarbonate gradient on ^{22}Na uptake was examined in absence (pH_o = pH_i = 7.5) (Fig. 3 *A*) and presence (pH_o = 7.5; pH_i = 5.5) (Fig. 3 *B*) of a proton gradient. A bicarbonate gradient, in the absence of proton gradient, stimulated ^{22}Na uptake approximately two- to threefold compared with that in the absence of bicarbonate gradient, but transient accumulation was not observed (Fig. 3 *A*). However, in the presence of proton gradient, a bicarbonate gradient stimulated ^{22}Na uptake by severalfold together with evidence of a transient accumulation. These results are identical to that reported in BLMV of rabbit renal proximal tubules (8, 9). ^{22}Na uptake stimulated by the bicarbonate gradient in the presence of a proton gradient was linear up to 6 s (not shown) and was greater than that in the absence of a proton gradient. As a result, all further experiments were performed for 6 s.

The effect of bicarbonate concentration on ^{22}Na uptake was examined to determine the concentration required for maximal enhancement of bicarbonate gradient-stimulated ^{22}Na uptake. As the results shown in Fig. 4, increasing bicarbonate concentration resulted in enhanced ^{22}Na uptake which saturated with a half-maximal bicarbonate concentration of ~ 40

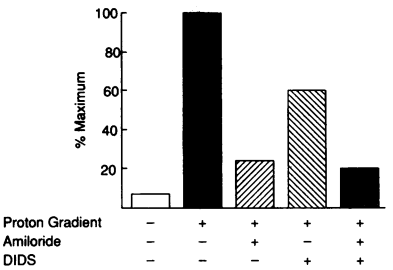

Figure 2. Effect of amiloride and DIDS on proton gradient-stimulated ^{22}Na uptake. BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 150 mM K-gluconate, 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 50 mM MES-Tris (pH 5.5) or 50 mM Hepes-Tris (pH 7.5). The experiment with Hepes-Tris was also performed in presence of amiloride (0.1 mM) and DIDS (1 mM). ^{22}Na uptake in the absence of a proton gradient was performed in MES-Tris, while that in the presence of a proton gradient in Hepes-Tris. Uptake expressed as percentage maximum which was 86.15 pmol/mg protein · 6 s.

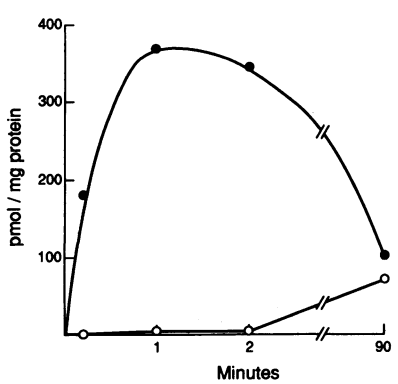

Figure 1. Effect of proton gradient on ^{22}Na uptake. BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSo_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na (0.2–90 min.) was performed by diluting the BLMV into an incubation medium that contained 150 mM K-gluconate, 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 50 mM MES-Tris (pH 5.5) (*open circles*) or 50 mM Hepes-Tris (pH 7.5) (*closed circles*).

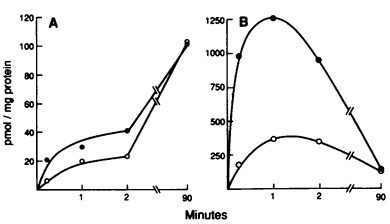

Figure 3. Effect of bicarbonate gradient on ^{22}Na uptake. (*A*) BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM Hepes-Tris (pH 7.5). Uptake of ^{22}Na (0.2–90 min) was performed by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 150 mM K-gluconate (*open circles*) or 150 mM $KHCO_3$ (*closed circles*). (*B*) BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na (0.2–90 min) was performed by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 150 mM K-gluconate (*open circles*) or 150 mM $KHCO_3$ (*closed circles*).

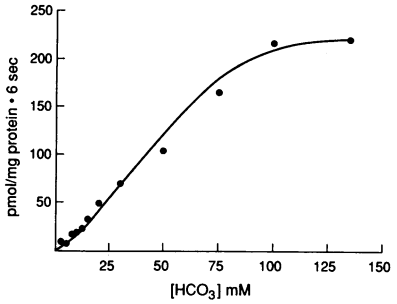

Figure 4. Effect of bicarbonate concentration on ^{22}Na uptake. BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and varying (1–135 mM) concentrations of $KHCO_3$. Isosmolarity was maintained by changing K-gluconate concentrations. Results presented are the absolute values after subtraction of uptake obtained in the absence of bicarbonate.

mM, evidence for the presence of a specific binding site for bicarbonate. The sigmoidal shape of the curve observed in this study suggests that more than one bicarbonate ion is transported with each Na ion. Analysis of this data with a Hill plot yielded a coefficient of ~ 1.7.

Experiments were designed to evaluate the effect of amiloride and DIDS on bicarbonate gradient-stimulated ^{22}Na uptake (Fig. 5). Both amiloride and DIDS significantly inhibited bicarbonate gradient-stimulated ^{22}Na uptake by ~ 98 and 80%, respectively. Although 150 mM bicarbonate in the absence of amiloride produced more than a tenfold increase in proton gradient-stimulated ^{22}Na uptake, bicarbonate failed to stimulate ^{22}Na uptake in the presence of amiloride. In the absence of DIDS, 150 mM bicarbonate increased ^{22}Na uptake by 815.0±2.9 pmol/mg protein • 6 s, but in the presence of DIDS enhanced ^{22}Na uptake by only 184.0±6.7 pmol/mg protein • 6 s. These results are consistent with the possibility that a major fraction of bicarbonate gradient-stimulated ^{22}Na uptake represents DIDS-sensitive Na-HCO_3 cotransport because the DIDS-insensitive fraction is less than 20% of total bicarbonate gradient-stimulated ^{22}Na uptake. Because the amiloride-sensitive component of bicarbonate gradient-stimulated ^{22}Na uptake was substantially greater than either the DIDS-sensitive or the DIDS-insensitive component, these results suggest that amiloride had inhibited both DIDS-insensitive Na-H exchange and DIDS-sensitive bicarbonate gradient-stimulated ^{22}Na uptake.

The demonstration that more than one bicarbonate ion is transported with each Na ion indicates that there is movement of net negative charge across the membrane. Thus, an intravesicular positive membrane potential should stimulate bicarbonate gradient-stimulated ^{22}Na uptake. To examine this possibility the effect of an intravesicular positive membrane potential generated by an inward K gradient and valinomycin was determined. Fig. 6 shows that an inward directed bicarbonate gradient in the absence of pH gradient significantly stimulated ^{22}Na uptake and that the addition of valinomycin further enhanced ^{22}Na uptake. The data presented in Fig. 6 also demonstrates that both DIDS and amiloride inhibited bicarbonate gradient-stimulated ^{22}Na uptake. These results indicate that bicarbonate gradient-stimulated ^{22}Na uptake carries net negative charge and is an electrogenic process.

Experiments were performed to determine the kinetic properties of both DIDS-insensitive and DIDS-sensitive proton gradient, and bicarbonate gradient-stimulated ^{22}Na uptake. The results presented in Fig. 7 demonstrate that increasing Na concentrations saturated both DIDS-insensitive (Fig. 7 *A*) and DIDS-sensitive (Fig. 7 *B*) ^{22}Na uptake that was stimulated by a proton gradient, and bicarbonate gradient-stimulated ^{22}Na uptake (Fig. 7 *C*). Analysis of these data with a Lineweaver-Burk plot yielded different apparent affinity constants (K_m) for Na: thus, the half-maximum saturation concentration for the DIDS-sensitive and DIDS-insensitive components of proton-gradient stimulated ^{22}Na uptake was 8.1 and 46.4 mM, respectively, while for bicarbonate gradient-stimulated ^{22}Na uptake was ~ 6.4 mM. These data suggest that the DIDS-sensitive component of proton gradient-stimulated and bicarbonate gradient-stimulated ^{22}Na uptake may represent the same transport process, which differs from the DIDS-insensitive component of proton gradient-stimulated ^{22}Na uptake.

Because both proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake were sensitive to amiloride, the inhibitory constant (K_i) of amiloride was determined to establish whether these two uptake processes manifest similar or different affinities for amiloride. The results shown in Fig. 8 indicate that increasing concentrations of amiloride progressively inhib-

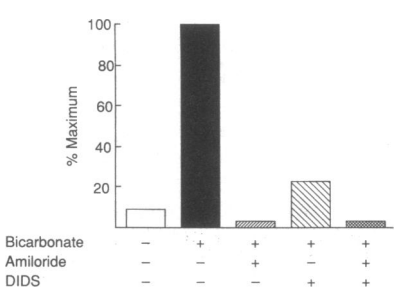

Figure 5. Effect of amiloride and DIDS on bicarbonate gradient-stimulated ^{22}Na uptake. BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 150 mM K-gluconate or 150 mM $KHCO_3$. Experiment with $KHCO_3$ was also performed in presence of amiloride (0.1 M) and DIDS (1 mM). Uptake is expressed as percentage maximum which was 965.9 pmol/mg protein • 6 s.

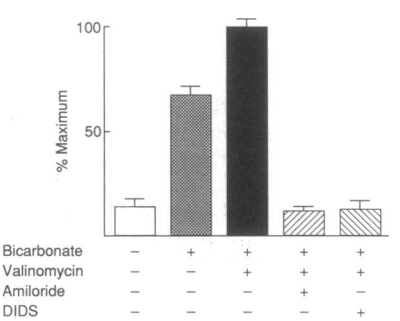

Figure 6. Effect of intravesicular positive membrane potential on bicarbonate gradient-stimulated ^{22}Na uptake. BLMV of rat distal colon were preloaded with 150 mM NMG-gluconate, 10 mM $MgSO_4$, and 50 mM Hepes-Tris (pH 7.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 150 mM K-gluconate or 150 mM $KHCO_3$. Uptake with $KHCO_3$ was also performed in presence of valinomycin (25 μM), DIDS (1 mM), and amiloride (0.1 mM). Uptake is expressed as percentage maximum which was 39.4±1.5 pmol/mg protein • 6 s. The addition of valinomycin significantly stimulated ^{22}Na uptake ($P < 0.01$).

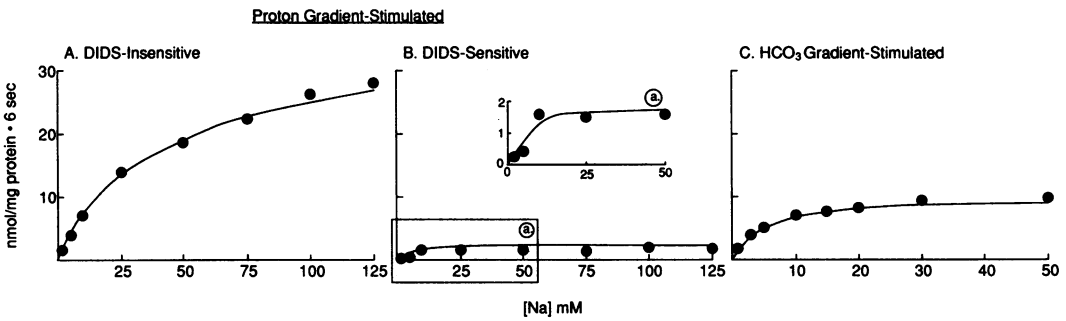

Figure 7. Effect of Na concentration. (*A*) and (*B*) BLMV of rat distal colon were preloaded with 150 mM NMG-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained tracer of ^{22}Na, 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, and varying concentrations of Na-gluconate (2–125 mM). Isosmolarity was maintained by adjusting the concentrations of NMG-gluconate. This experiment was performed in the absence (total proton gradient-stimulated uptake) and presence (DIDS-insensitive proton gradient-stimulated uptake) of 1 mM DIDS. Absolute values of DIDS-insensitive uptake presented in *A* were obtained by subtraction of uptake determined in medium that contained 50 mM MES-Tris (pH 5.5) in place of Hepes-Tris. The DIDS-sensitive uptake values presented in *B* were obtained by subtraction of DIDS-insensitive uptake from total proton gradient-stimulated uptake. Inset *A* in *B* provides an expanded scale of DIDS-sensitive uptake values up to 50 mM Na concentration. (*C*) BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 100 mM NMG-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 150 $KHCO_3$, 10 mM $MgSO_4$, tracer of ^{22}Na, 50 mM Hepes-Tris (pH 7.5), and varying concentration of Na-gluconate (1–50 mM). Medium isosmolarity was maintained by adjusting NMG-gluconate concentration. Absolute values presented were obtained by subtraction of uptake determined in absence of bicarbonate. The best fit curves were drawn using the Michaelis-Menten equation.

ited both proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake. It should be noted that proton gradient-stimulated ^{22}Na uptake in the presence of DIDS was not significantly inhibited by 250 and 500 nM amiloride. In contrast, bicarbonate gradient-stimulated ^{22}Na uptake was inhibited by ~ 20 and 30% by these two amiloride concentrations, respectively. Analysis of these data with a Dixon plot yielded an apparent K_i for amiloride of 35 and 1 μM for DIDS-insensitive component of proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake, respectively. These results provide further confirmation that DIDS-insensitive component of proton gradient- and bicarbonate gradient-stimulated ^{22}Na uptake represent two separate and distinct transport processes.

The significantly lower half-maximal inhibitory concentration of amiloride (K_i) for bicarbonate gradient-stimulated ^{22}Na uptake raised the possibility that bicarbonate might stimulate ^{22}Na uptake via an Na channel as a result of establishing an intravesicular negative membrane potential. However, an intravesicular negative membrane potential, produced by an outward K gradient and valinomycin, did not stimulate ^{22}Na uptake thus excluding the possibility that Na channels are present in BLMV (data not shown).

To characterize the inhibition of DIDS-insensitive proton gradient-stimulated and bicarbonate gradient-stimulated ^{22}Na uptake by amiloride, experiments were also performed with amiloride analogues. Table I presents the results of experiments with amiloride, ethylisopropylamiloride (EIPA), phenamil, and benzamil. These results demonstrate that EIPA is a

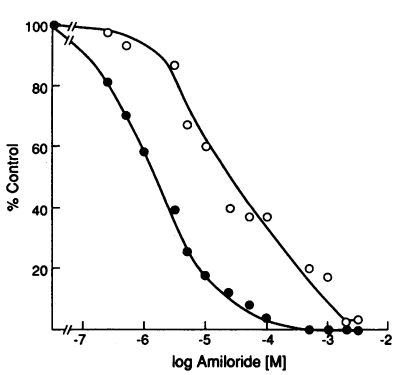

Figure 8. Effect of amiloride concentration. BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 2 mM NMG-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained varying concentrations of amiloride, 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, tracer of ^{22}Na, and either 150 mM K-gluconate with 1 mM Na_2 DIDS (*open circles*) or 150 mM $KHCO_3$ with 2 mM Na-gluconate (*closed circles*). Uptake obtained in absence of amiloride considered 100%.

Table I. Effect of Amiloride and Its Analogues on Proton Gradient- and Bicarbonate Gradient-stimulated ^{22}Na Uptake

Inhibitors	Concentration	DIDS-insensitive proton gradient-stimulated	Bicarbonate gradient-stimulated
	μM	pmol/mg protein · 6 s	
None	—	1,668.3±76.2	5,982.0±86.4
Amiloride	1	1,715.7±11.7‖	3,364.7±291.3§
	10	932.0±51.8‡	1,339.7±89.1§
EIPA	1	1,120.3±21.5‡	1,374.7±48.8§
	10	509.0±54.4§	645.7±40.9§
Benzamil	1	1,880.3±42.4‖	5,586.3±98.1*
	10	1,958.3±48.3*	5,523.7±199.7‖
Phenamil	1	1,735.3±33.3‖	6.032.7±41.5‖
	10	1,613.7±78.3‖	5,324.0±78.4‡

BLMV of rat distal colon were preloaded with 150 mM K-gluconate, 2 mM NMG-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the BLMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, trace of ^{22}Na, and either 150 mM K-gluconate plus 1 mM Na_2 DIDS (proton gradient-stimulated) or 150 mM $KHCO_3$ plus 2 mM Na-gluconate (bicarbonate gradient-stimulated). All experiments were performed in presence of 1 and 10 μM of indicated analogues which were dissolved in DMSO. All medium contained 1% DMSO. * $P < 0.05$, ‡ $P < 0.01$, § $P < 0.001$, compared with control. ‖ Not significantly different from control.

more potent inhibitor of both transport processes than amiloride. Neither benzamil nor phenamil inhibited DIDS-insensitive proton gradient-stimulated or bicarbonate gradient-stimulated ^{22}Na uptake (DIDS-insensitive proton gradient-stimulated ^{22}Na uptake was enhanced by benzamil by less than 12% for reasons that are uncertain). The order of inhibition by these analogues is EIPA ⩾ amiloride ⩾ phenamil = benzamil.

To compare the characteristics of proton gradient-stimulated ^{22}Na uptake in AMV to these results, the effect of a bicarbonate gradient, amiloride, and DIDS on proton gradient-stimulated ^{22}Na uptake was examined in AMV. As the results show in Fig. 9, proton gradient-stimulated ^{22}Na uptake in AMV was inhibited by amiloride but was neither stimulated by a bicarbonate gradient nor inhibited by DIDS. These results indicate the absence of Na-anion cotransport in AMV.

Discussion

This study establishes that both a proton gradient and a bicarbonate gradient stimulates ^{22}Na uptake and provides evidence for the presence of both coupled Na-H exchange and electrogenic Na-anion cotransport in BLMV of rat distal colon. This study also demonstrates that the electrogenic Na-anion cotransport of rat colonic BLMV is sensitive to both DIDS and amiloride. Proton gradient-stimulated ^{22}Na uptake is only partially inhibited by DIDS (Fig. 2) and thus consists of both DIDS-sensitive and DIDS-insensitive fractions. The latter is inhibited by amiloride with a K_i of 35 μM and is probably similar to the several electroneutral Na-H exchanges that have been described in multiple epithelial and nonepithelial cells (10–12). The DIDS-sensitive component of proton gradient-stimulated ^{22}Na uptake has identical properties as bicarbonate gradient-stimulated ^{22}Na uptake in that both are inhibited by DIDS and amiloride and have almost identical K_m values for Na. Therefore, we propose that these results can best be accounted for by the presence of a single DIDS-sensitive Na-anion cotransport process that can be stimulated by either bicarbonate or hydroxyl gradients.

Because an electroneutral Na-H exchange has also been identified in AMV of rat distal colon, it is important to establish that this BLMV preparation is not contaminated by AMV. First, the basolateral membrane marker, Na,K-ATPase, is enriched 12.7-fold in this BLMV preparation (see Methods). Second, K-ATPase and Cl-OH exchange that have been identified in AMV are not present in these BLMV (unpublished observations and reference 9). Third, DIDS-sensitive proton gradient-stimulated ^{22}Na uptake and bicarbonate gradient-stimulated ^{22}Na uptake are not present in AMV (Fig. 9). Finally, the K_m for Na for the DIDS-insensitive component of proton gradient-stimulated ^{22}Na uptake in BLMV (Fig. 7A) is substantially higher than that previously identified in AMV (4). These observations provide compelling evidence that the Na-H exchange identified in the BLMV in these studies is not the Na-H exchange of AMV.

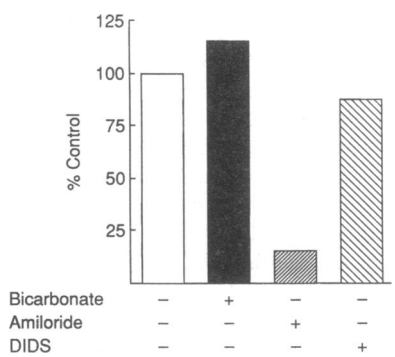

Figure 9. Effect of bicarbonate, DIDS and amiloride on proton gradient-stimulated ^{22}Na uptake in AMV. AMV of rat distal colon were preloaded with 150 mM K-gluconate, 10 mM $MgSO_4$, and 50 mM MES-Tris (pH 5.5). Uptake of ^{22}Na was performed for 6 s by diluting the AMV into an incubation medium that contained 50 mM Hepes-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM ^{22}Na, and either 150 mM K-gluconate or 150 mM $KHCO_3$. Experiment with K-gluconate was also performed in presence of amiloride (0.1 mM) and DIDS (1 mM). Uptake obtained in K-gluconate considered 100%.

Proton gradient-stimulated ^{22}Na uptake (in the absence of HCO_3) could represent either Na-H exchange that is both amiloride-sensitive and partially DIDS-sensitive or the presence of both amiloride-sensitive Na-H exchange and Na-OH cotransport that is sensitive to both DIDS and amiloride. The demonstration that the K_m for Na for the DIDS-sensitive and DIDS-insensitive components significantly differ (8.1±3.7 vs. 46.4±5.0 mM) excludes the possibility that there is a single Na-H exchange with partial sensitivity to DIDS. Further, the close similarity between the K_m for Na for the DIDS-sensitive component of proton gradient-stimulated ^{22}Na uptake and bicarbonate gradient-stimulated ^{22}Na uptake (8.1±3.7 vs. 6.4±0.6 mM) is the basis for the presence of a DIDS-sensitive Na-anion cotransport process with affinity for both hydroxyl and bicarbonate. We are not aware of other demonstrations of bicarbonate-independent, proton gradient-stimulated ^{22}Na uptake that is DIDS-sensitive. Studies of bicarbonate gradient-stimulated Na uptake in BLMV of rabbit renal cortex in the absence of bicarbonate have identified proton gradient-stimulated Na uptake (9). However, Na uptake in these conditions has been low, presumed to be electroneutral Na-H exchange or not examined any further (9).

The results represented in Figs. 5 and 6 establish that a bicarbonate gradient stimulates ^{22}Na uptake that is inhibited by DIDS. This uptake appears to represent an electrogenic cotransport process as an intravesicular positive membrane potential (Fig. 6) enhanced DIDS-sensitive bicarbonate gradient-stimulated ^{22}Na uptake. Similar observations of bicarbonate stimulation of Na uptake have been reported with BLMV of renal proximal tubules (8, 9). In the presence of proton gradient, Grassl and Aronson demonstrated enhancement of Na uptake by a bicarbonate gradient with transient accumulation (9), while in the absence of proton gradient Akiba et al. reported that a bicarbonate gradient accelerated Na uptake but did not result in transient accumulation (8). These previous observations are identical to our own results (see Fig. 3). The demonstration that ^{22}Na uptake saturates as a function of both increasing bicarbonate concentration at a fixed Na (0.1 mM) concentration (Fig. 4) and increasing Na concentration at a fixed bicarbonate (150 mM) concentration (Fig. 7 *C*) is consistent with a carrier-mediated Na-HCO_3 cotransport process.

The demonstration of a sigmoidal curve for bicarbonate gradient-stimulated ^{22}Na uptake indicates that more than one bicarbonate ion is cotransported with each Na ion. As a result, this Na-HCO_3 cotransport system should carry a net negative charge. Similar observations have been reported in several other cells (8, 14–18) and a Hill coefficient of 1.7 was calculated for bicarbonate gradient-stimulated ^{22}Na uptake indicating a stoichiometry of HCO_3 to Na of > 1. This stoichiometry is confirmed by the demonstration that bicarbonate gradient-

stimulated ^{22}Na uptake is stimulated by an intravesicular positive membrane potential (Fig. 6).

The kinetic data presented in Fig. 7 and Table II permits comparison of these transport systems in terms of their kinetic constants. The two electrogenic Na-anion cotransport (Na-OH and Na-HCO_3) systems have almost identical K_m for Na but the V_{max} for the bicarbonate gradient-stimulated system is sixfold greater than the hydroxyl gradient-stimulated system. As a consequence, these results indicate that bicarbonate does not alter the affinity of this transport process for Na but increases the turnover rate of the cotransport process. Comparison of the kinetic constants of DIDS-insensitive Na-H exchange and Na-anion cotransport systems also reveal that the former represents a low affinity, high capacity system, while the latter is a high affinity, low capacity system.

DIDS-sensitive, bicarbonate gradient-stimulated ^{22}Na uptake identified in BLMV of rat distal colon has many but not all of the characteristics of bicarbonate gradient-stimulated ^{22}Na uptake that has previously been described in other epithelial (8, 9, 14–18). As indicated in the data presented in Figs. 5 and 6, bicarbonate gradient-stimulated ^{22}Na uptake is substantially inhibited by DIDS; however, prior studies of this transport process have not reported amiloride sensitivity. In one study in rabbit proximal tubule amiloride was reported not to inhibit HCO_3 stimulated ^{22}Na uptake, while the effect of amiloride was not tested in another study of this transport system (8, 9). Recently, Felipe et al. have presented evidence that amiloride and DIDS inhibit HCO_3 stimulated ^{22}Na uptake in sinusoidal membrane vesicles from rat hepatocytes (28) and in abstract form Zamir, Barry and Ramaswamy have demonstrated that both DIDS and amiloride inhibited bicarbonate stimulated ^{22}Na uptake in BLMV from human small intestine (29). In contrast, another recent study did not observe amiloride inhibition of bicarbonate stimulated ^{22}Na uptake in sinusoidal membranes from rat hepatocytes (30). Because amiloride inhibits both Na-H exchange and Na conductive channels, it is possible that the present inhibition of ^{22}Na uptake by low amiloride concentrations represents amiloride inhibition of Na channels. This possibility is unlikely because one, an imposed intravesicular negative potential did not enhance ^{22}Na uptake excluding the presence of Na channels (results not shown); two, bicarbonate gradient-stimulated ^{22}Na uptake was not inhibited by specific inhibitors of Na channels, benzanil and phenamil; and three, the K_m (6.4 mM) of bicarbonate gradient-stimulated ^{22}Na uptake is relatively low. In contrast, conductive Na channels usually do not saturate (19) or saturate with substantially higher K_m (20, 21). Thus, we conclude that amiloride inhibits bicarbonate gradient-stimulated ^{22}Na uptake. Because amiloride did not inhibit bicarbonate gradient-stimulated [^{14}C]-butyrate uptake in these BLMV (13) and because the K_i for amiloride inhibition of bicarbonate gradient-stimulated ^{22}Na uptake is less than that for DIDS-insensitive component of proton gradient-stimulated ^{22}Na uptake (Fig. 8), it is unlikely that this inhibition of ^{22}Na uptake by amiloride represents a nonspecific effect of BLMV transport processes.

Table II. Comparison of the Properties of DIDS-insensitive (Na-H Exchange) and DIDS-sensitive (Na-OH Cotransport) Proton Gradient-Stimulated, and Bicarbonate Gradient-Stimulated (Na-HCO_3 Cotransport) ^{22}Na Uptake by BLMV and Na-H Exchange of AMV

	BLMV			AMV
	Na-H Exchange	Na-OH Cotransport	Na-HCO_3 Cotransport	Na-H Exchange
Amiloride	Sensitive	Sensitive	Sensitive	Sensitive
DIDS	Insensitive	Sensitive	Sensitive	Insensitive
Km for Na (mM)	46.4±5.0	8.1±3.7	6.4±0.6	10.6±1.5*
Vmax for Na§	37.7±1.6	2.0±0.2	12.4±0.9	1.7±0.08*
Ki Amiloride (μM)	30.0±5.0	—	1.0±0.03	27‡

* Adapted from reference 4. ‡ Adapted from reference 1. § nmol/mg protein · 6 s.

These studies establish that amiloride and its analogue, EIPA, are potent inhibitors of Na-anion cotransport, a previously unobserved phenomenon. Amiloride inhibits several transport systems including Na conductive channels, Na-H exchange, Ca-ATPase, and Na-K,ATPase. Several amiloride analogues have been synthesized with varying specificity. Thus, EIPA is a potent inhibitor of Na-H exchange but does not affect Na channels, while benzamil has high specificity to conductive Na channels but does not inhibit Na-H exchange. The rank order of bicarbonate gradient-stimulated Na uptake by these amiloride analogues corresponds to that for the inhibition of Na-H exchange (Table I). It is possible that amiloride and EIPA might inhibit HCO_3 gradient-stimulated ^{22}Na uptake by inhibiting Na-H exchange. However, this appears unlikely because the presence of an outward directed proton gradient markedly enhances HCO_3 gradient-stimulated ^{22}Na uptake (Fig. 5) and a functioning Na-H exchange will reduce such a proton gradient. Inhibition of Na-H exchange by amiloride or EIPA will prevent dissipation of this proton gradient and thus should enhance rather than inhibit ^{22}Na uptake. It should also be noted that Vanhaesebroeck et al. recently reported that EIPA prevented the cytotoxic effects of tumor necrosis factor in murine L929s fibrosarcoma cells by a mechanism independent of Na-H exchange inhibition (22). Thus, the mechanisms of amiloride (and EIPA) inhibition of bicarbonate gradient-stimulated ^{22}Na uptake may not necessarily be inhibition of Na-H exchange and requires further study.

Bicarbonate gradient-stimulated ^{22}Na uptake has been identified in several different types of epithelial cells (18), but this novel transport process is not uniformly present nor necessarily present in adjacent cell types (23, 24). Bicarbonate gradient-stimulated Na transport is present in both mammalian and nonmammalian tissues and has been characterized in studies with both isolated basolateral membranes (8, 9) and of dispersed individual cells or isolated tissue (i.e., renal tubules) (18). Although electrogenic Na-HCO_3 cotransport is present in hepatocytes (25) and parietal cells (26), previous studies have not established its presence in other intestinal epithelial cells.

Although these experiments were designed to examine the effect of proton and bicarbonate gradients on Na transport, they also provide information regarding bicarbonate movement across the basolateral membrane. These studies indicate that a major fraction of bicarbonate movement is electrogenic and Na dependent. Because bicarbonate-dependent Cl uptake has not been identified in these membranes (unpublished observations) and because Cl-HCO_3 exchanges are frequently the primary transport mechanism for transmembrane bicarbonate movement, it is likely that basolateral bicarbonate movement is mediated by the DIDS-sensitive electrogenic Na-HCO_3 co-

transport process described in these studies. These studies do not provide information whether this basolateral transport process regulates transepithelial bicarbonate movement or is critical in the regulation of intracellular pH. If this transport process is primarily involved with the former function, it may participate in the regulation of bicarbonate secretion, a well established colonic transport process (27). Alternatively, as a regulator of cell pH, this bicarbonate transport process may modulate either base loading or base extrusion or both.

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