Characterization of apical membrane Cl-dependent Na/H exchange in crypt cells of rat distal colon

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Rajendran, Vazhaikkurichi M., John Geibel, and Henry J. Binder. Characterization of apical membrane Cl-dependent Na/H exchange in crypt cells of rat distal colon. Am J Physiol Gastrointest Liver Physiol 280: G400–G405, 2001.—A novel Cl-dependent Na/H exchange (Cl-NHE) has been identified in apical membranes of crypt cells of rat distal colon. The presence of Cl is required for both outward proton gradient-driven Na uptake in apical membrane vesicles (AMV) and Na-dependent intracellular pH recovery from an acid load in the crypt gland. The present study establishes that Cl-dependent outward proton gradient-driven 22Na uptake is saturated with increasing extravesicular Na concentration with a Michaelis constant (K_m) for Na of -24.2 mM; 2) is saturated with increasing outward H concentration gradient with a hyperbolic curve and a K_H for H of ~1.5 mM; 3) is inhibited by the Na/H exchange (NHE) inhibitors amiloride, ethylisopropylamiloride, and HOE-694 with an inhibitory constant (K_i) of ~480.2, 1.1, and 9.5 mM, respectively; 4) is inhibited by 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid, an anion exchange inhibitor at low concentration and a Cl channel blocker at high dose, and by 5-nitro-2(3-phenylpropylamino)benzoic acid, a Cl channel blocker, with a K_i of ~280.6 and 18.3 mM, respectively; and 5) substantially stimulated Cl-NHE activity by dietary Na depletion, which increases plasma aldosterone and inhibits NHE in surface cell AMV. These properties of Cl-NHE are distinct from those of NHE1, NHE2, and NHE3 isoforms that are present in colonic epithelial cells; thus these results suggest that the colonic crypt cell Cl-NHE is a novel NHE isoform.

ELECTRONEUTRAL Na/H EXCHANGE (NHE) that extrudes intracellular H and absorbs extracellular Na is responsible for several physiological cell functions, including intracellular pH regulation, volume regulation, and transepithelial Na absorption (1, 6, 8, 16, 26). NHEs with distinct kinetic and pharmacological properties have been localized in apical and basolateral membranes of epithelial cells (5, 9). Recent molecular studies have identified the expression of at least three NHE isoforms (NHE1, NHE2, and NHE3 isoforms) in colonic epithelial cells (2, 3, 11, 26).

Colonic NHE isoforms have different cell and tissue distribution as well as transport characteristics. NHE2 and NHE3 isoforms are present in apical membranes, whereas the NHE1 isoform is expressed in basolateral membranes (2, 11, 26). The NHE3 isoform is expressed only in surface cells, but NHE1 and NHE2 isoforms are localized to both surface and crypt cells (Refs. 2, 3; see Table 1). Studies of 22Na uptake that demonstrated NHE activity in apical membrane vesicles (AMV) isolated from surface cells established that this NHE function is Cl independent (17). In contrast, recent studies identified a novel Cl-dependent Na/H exchange (Cl-NHE) in apical membranes of crypt epithelial cells of rat distal colon by demonstrating that both H concentration (IH) gradient-driven 22Na uptake and Na-dependent pH recovery from an acid load required the presence of Cl (18). The Cl dependence of Cl-NHE appears to represent a Cl channel, as Cl-NHE function is inhibited by both NHE inhibitors and Cl channel blockers (19). It is not known whether Cl-NHE is an existing NHE isoform that in the presence of one or more Cl channels manifests Cl dependence or is a novel NHE isoform. The present study, therefore, was initiated to identify the kinetic properties of Cl-NHE. The observed results establish that the properties of Cl-NHE are distinct and differ from those of the NHE isoforms that have previously been identified in colonic epithelial cells and suggest that Cl-NHE is a novel isoform of the existing gene family of NHE isoforms.

METHODS

Vesicle preparation. Crypt cells were isolated from distal colon of both normal and Na-depleted diet-fed rats (Sprague-Dawley, 200–250 g) by the method of Lomax et al. (14), as described previously (18). Na-depleted animals were fed a Na-free diet (20 g/day), prepared in our laboratory, as described previously (10), for 6–7 days. AMV were prepared from crypt colonocytes by the modified method of Stieger et al. (23), as described earlier (18, 20). Protein was assayed by the method of Lowry et al. (15). Purity of the crypt cell apical membrane was assessed by ouabain-sensitive H-K-ATPase enrichment (10- to 12-fold), as described earlier (18, 21).

Uptake studies. The initial rate of Cl-dependent proton gradient-driven 22Na (NEC, Boston, MA) uptake in AMV was performed for 6 s using a “rapid uptake” machine, as described previously (19). In brief, AMV preloaded with 150 mM KCl and 50 mM MES-Tris (pH 5.5) were incubated in medium containing 50 mM HEPES-Tris (pH 7.5), 150 mM KCl, 0.1 mM 22Na, 25 μM valinomycin, and either 0 or 10 μM sodium depletion; aldosterone; regulation; kinetics

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CL-DEPENDENT Na/H EXCHANGE

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Fig. 1. Effect of Cl on proton gradient-driven 22Na uptake. Colonic crypt apical membrane vesicles (AMV) from normal animals were preloaded with either 50 mM MES-Tris (pH 5.5) and 150 mM potassium gluconate or 50 mM MES-Tris (pH 5.5) and 150 mM KCl. Uptake in AMV loaded with 50 mM MES-Tris and 150 mM potassium gluconate was measured for 6 s by incubation in medium containing 150 mM potassium gluconate, 0.1 mM sodium gluconate, 22Na trace, 25 μM valinomycin, and 50 mM of either MES-Tris (pH 7.5; open bars) or HEPES-Tris (pH 7.5; filled bars). Uptake in AMV loaded with 50 mM MES-Tris and 150 mM KCl was measured for 6 s by incubation in medium containing 150 mM KCl, 0.1 mM sodium gluconate, 22Na trace, 25 μM valinomycin, and 50 mM of either MES-Tris (pH 5.5) or HEPES-Tris (pH 7.5). The presented results represent triplicate determinations of 3 different membrane preparations.

5-ethylisopropylamiloride (EIPA). EIPA-sensitive proton gradient-driven 22Na uptake presented was calculated by subtracting the uptake in the presence of 10 μM EIPA from that in its absence. Some of the experiments were also performed in the presence of 25 mM Cl, as previous studies established that maximal Cl-NHE activity was observed in the presence of 25 mM Cl (19). Specific details of each experiment are given in the legends for Figs. 1–7. All of the experiments were repeated at least three times with different membrane preparations. Results presented represent means ± SE of triplicate assays of a typical experiment.

RESULTS

Figure 1 presents the initial rate of 22Na uptake in AMV isolated from normal rat distal colonic crypt cells that was measured in the presence of either Cl or gluconate. As shown in Fig. 1, 22Na uptake was almost identical both in the presence [extracellular pH (pH\textsubscript{o})/pH\textsubscript{i}: 7.5/5.5] and absence (pH\textsubscript{o}/pH\textsubscript{i}: 5.5/5.5) of an outward proton gradient in medium with gluconate. In contrast, 22Na uptake was substantially stimulated by an outward proton gradient in medium that contained Cl (Fig. 1). These results are consistent with previous observations (18).

Kinetic studies were performed to establish the characteristics of Cl-NHE. As shown in Fig. 2, increasing extravesicular Na concentration ([Na]) from 3 to 100 mM stimulated and saturated an outward-directed [H] gradient-driven 22Na uptake. A Lineweaver-Burke plot of these data yielded a Michaelis constant (K\textsubscript{m}) for Na of −24.2 ± 2.9 mM and a maximal velocity (V\textsubscript{max}) of 309.6 ± 21.3 pmol·mg protein\textsuperscript{-1}·s\textsuperscript{-1}.

Experiments were also designed to determine whether Cl-NHE is responsible for vectorial Na absorption and/or pH\textsubscript{i} regulation by assessing the presence of a proton modifier site. Thus the effect of increasing outward-directed [H] gradient on Cl-NHE activity was measured. With a fixed pH\textsubscript{o} of 7.5, decreasing pH\textsubscript{i} from 7.5 to 5.5 resulted in an increase in the initial rate of 22Na uptake with a hyperbolic curve (Fig. 3). An apparent K\textsubscript{m} for a proton of outward-directed [H] gradient-driven 22Na uptake was 1.5 ± 0.3 μM (pH 5.8). In additional studies Cl-NHE activity was measured at different [H] but with identical pH gradients (pH\textsubscript{o}/pH\textsubscript{i}: 7.5/6.5 vs. 6.5/5.5). As shown in Fig. 4A, the initial rate of Cl-NHE activity was almost similar at both pH gradient conditions. Previous studies have demonstrated that Cl-NHE also functions as an Na/Na exchanger (18). Therefore, Na/Na exchange was measured as outward Na gradient-stimulated 22Na uptake at different proton concentrations. As shown in Fig. 4B, Na/Na exchange was also identical at both low and high proton concentrations. These observations are not
consistent with the presence of a proton modifier site and suggest that the function of Cl-NHE is not associated with pH regulation but is linked to vectorial Na absorption.

Previous studies have demonstrated that inhibitors both of NHE function and of Cl channel activity inhibit Cl-NHE activity (18, 19). Therefore, the kinetics of these inhibitors on Cl-NHE function was also determined. Increasing concentrations of amiloride resulted in progressive inhibition of the initial rate of outward-directed [H] gradient-driven 22Na uptake with an apparent inhibitory constant ($K_i$) of 480.2 ± 8.6 μM (Fig. 5). This observation is consistent with our initial observation that suggested that Cl-NHE in AMV from crypt cells is relatively resistant to amiloride (19).

Experiments were also performed with two specific inhibitors of NHE, EIPA and 3-methyl sulfonyl-4-piperidinoebenzoyl guanidine (HOE-694). The apparent $K_i$ of EIPA of Cl-NHE was 1.1 ± 0.3 μM (Fig. 5). Similarly, Cl-NHE activity was moderately sensitive to HOE-694 with an apparent $K_i$ of 9.5 ± 2.2 μM (Fig. 5).

Prior studies of Cl-NHE demonstrated that Cl-dependent outward-directed [H] gradient-driven 22Na uptake by crypt AMV was also inhibited by the Cl channel blockers 5-nitro-2(3-phenylpropylamino)benzoic acid (NPPB) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS; see Ref. 19). Experiments were therefore performed to establish the kinetics of both DIDS and NPPB inhibition of Cl-NHE. The apparent $K_i$ of DIDS was 280.6 ± 12.6 μM, whereas the $K_i$ of NPPB for Cl-NHE was 18.3 ± 3.9 μM (Fig. 6). Other transport inhibitors, bumetanide (an Na-K-2Cl co-transport inhibitor), ouabain (an Na-K-ATPase inhibitor), benzamil (an Na channel blocker), and cimetidine [an inhibitor that inhibits basolateral NHE (12)], did not inhibit Cl-dependent proton gradient-driven 22Na uptake (data not shown).

Dietary Na depletion with resulting increased levels of serum aldosterone inhibits electroneutral Na-Cl absorption and NHE in AMV prepared from surface cells.
Thus the effect of dietary Na depletion on Cl-NHE activity was also determined. As shown in Fig. 7, Cl-NHE activity in AMV from a Na-depleted animal was 46% higher than that in AMV from normal animals. Kinetic studies were also performed to establish whether the increased Cl-dependent NHE activity in Na-depleted rats was a result of a change in affinity for Na or an increased number/turnover rate. As shown in Fig. 2, similar to AMV prepared from control animals, increasing [Na] also saturated Cl-dependent NHE in AMV from Na-depleted animals. Lineweaver-Burke plot analyses yielded a $K_m$ for Na of $21.4 \pm 2.2$ mM and a $V_{max}$ of $732.3 \pm 29.3$ pmol/mg protein s$^{-1}$. These observations differ qualitatively from those of AMV from surface cells of Na-depleted animals (11, 20). These results establish that the characteristics of Cl-NHE are distinct from those of other NHE isoforms that are present in colonic epithelial cells.

**DISCUSSION**

Na absorption in the rat distal colon is electroneutral and is primarily a result of the NHE3 isoform, which is located in the apical membrane of surface epithelial cells (2). Because studies of fluid movement in the isolated microperfused colonic crypt revealed the presence of Na-dependent net fluid absorption (22), the mechanism of Na absorption in the colonic crypt was uncertain, since the NHE3 isoform is not present in the apical membrane of crypt cells (2). The demonstration that NHE in crypt AMV required the presence of Cl (Fig. 1) and that NHE in surface cell AMV was Cl independent (18) suggested that Na-dependent net fluid absorption in the isolated colonic crypt was secondary to Cl-NHE.

The initial studies of Cl-NHE established that Na-dependent recovery of pH$_i$ to an acid load also required lumen Cl, which was inhibited by amiloride (18). Additional studies revealed that [H] gradient-driven Na uptake by crypt AMV in the presence of Cl was relatively amiloride resistant in that 250 $\mu$M amiloride did not inhibit 50% of Cl-NHE activity (18). Furthermore, the requirement for Cl was not specific, as other halides could also stimulate [Na] gradient-driven $^{22}$Na uptake (Cl$>$Br$>$F$>$I). Subsequent studies examined...
the role of Cl and found that both intravesicular and extravesicular Cl were equally effective in stimulation of [H] gradient-driven 22Na uptake and that 5 mM Cl resulted in 58% of maximal Cl-NHE activity (19).

These present studies were designed to characterize the properties of the novel Cl-dependent NHE that we have identified in apical membranes in crypt but not in surface epithelial cells of the rat distal colon (18, 19). To date, at least six NHE isoforms have been identified, and at least three are present in epithelial cells of rat distal colon (2, 3, 6, 11, 26). The NHE1 isoform has been identified on the basolateral membrane, is likely responsible for the regulation of one or more cell functions, e.g., volume and pHi, and has often been referred to as a “housekeeper.” NHE function in the basolateral membranes is not affected by dietary Na depletion, and NHE1 message or protein is also not altered in dietary Na-depleted rats (11). NHE2 and NHE3 isoforms are also present in the distal colon but are localized to the apical membrane. Because NHE2 and NHE3 isoforms when expressed in PS 120 cells do not require Cl (M. Donowitz, personal communication), the absence of any NHE activity in the absence of Cl (Fig. 1) established that Cl-NHE is the only NHE isoform present in crypt apical membranes.

The results presented in Figs. 2–4 indicate that the kinetic properties of Cl-NHE are distinct from those of other NHE isoforms that are present in colonic epithelial cells. The apparent $K_m$ for Na of NHE1, NHE2, and NHE3 isoforms are approximately between 15 and 18 mM (4, 13), which is not physiologically different from the apparent $K_m$ for Na of Cl-NHE (24.2 mM). The kinetics for intravesicular [H] for Cl-NHE yielded Michaelis-Menten kinetics with a Hill coefficient of $\approx 1$ (Fig. 3). In contrast, studies of NHE1, NHE2, and NHE-3 isoforms in transfected PS 120 cells did not show Michaelis-Menten kinetics and revealed Hill coefficients of $\approx 2$ (13). However, studies with AMV prepared from surface cells of rat distal colon in which both NHE2 and NHE3 isoforms are probably present also did not reveal evidence of a proton modifier site (17). The absence of a proton modifier site in Cl-NHE may also be explained by the following reasons: 1) a modifier site may require other intracellular signaling factors, which are absent in isolated vesicles; and 2) sensing capacity of the proton modifier site may be altered during membrane preparation.

Comparison of the kinetics of amiloride and the amiloride analogs that are more specific NHE inhibitors also reveals that the kinetic properties of Cl-NHE are dissimilar from those of the other NHE isoforms present in colonic epithelial cells (see Table 1). The $K_i$ of Cl-NHE for amiloride was substantially greater than those of NHE1, NHE2, and NHE3 isoforms, whereas $K_i$.

Table 1. Properties of Cl-NHE and comparison to NHE isoforms present in colon epithelial cells

<table>
<thead>
<tr>
<th>Cell distribution</th>
<th>Membrane distribution</th>
<th>CI-NHE</th>
<th>NHE1</th>
<th>NHE2</th>
<th>NHE3</th>
</tr>
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<tbody>
<tr>
<td>Crypt</td>
<td>Apical</td>
<td>Surface/crypt</td>
<td>Surface/crypt</td>
<td>Surface</td>
<td></td>
</tr>
<tr>
<td>$K_m$ for Na, µM</td>
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<td>24.2</td>
<td>15</td>
<td>18</td>
<td>17</td>
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<tr>
<td>$K_i$ for amiloride, µM</td>
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<td>1–3</td>
<td>1–3</td>
<td>39</td>
</tr>
<tr>
<td>$K_i$ for EIPA, µM</td>
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<td>0.02</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>$K_i$ for HOE-694, µM</td>
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<td>9.5</td>
<td>0.3</td>
<td>5</td>
<td>650</td>
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<td>Increased</td>
<td>Unchanged</td>
<td>Reduced</td>
<td>Inhibited</td>
</tr>
</tbody>
</table>

Cl-NHE, Cl-dependent Na/H exchange; NHE, Na/H exchange; $K_m$, Michaelis constant; $K_i$, inhibitory constant; EIPA, ethylisopropylamiloride. Data obtained from Refs. 4, 5, 11, 13, 18, 26, and 27 and Figs. 1, 3, and 5.
values of Cl-NHE for both EIPA and HOE-694 were similar only to those of the NHE2 isoform (Fig. 4).

The effect of dietary Na depletion and aldosterone on apical membrane NHEs provides additional evidence that the properties of Cl-NHE are distinct from those of both NHE2 and NHE3 isoforms. Recent studies have demonstrated that NHE3 isoform-specific NHE activity is completely inhibited in AMV from dietary Na-depleted animals (11). Furthermore, dietary Na depletion results in a substantial decrease in NHE3 mRNA and protein in the rat distal colon (11). Parallel studies revealed that dietary Na depletion produced a moderate decrease in NHE2 transport function, mRNA abundance, and protein expression (11). In contrast, Fig. 7 provides evidence that dietary Na depletion increases Cl-NHE transport function, which is a result of an increase in $V_{max}$ without any change in $K_m$ (Fig. 2).

The molecular identification of Cl-NHE has not been established, but these present studies provide compelling evidence that the properties of Cl-NHE are distinct and dissimilar from other colonic NHE isoforms. It is possible that Cl-NHE might represent an unrelated transport protein with both Na+ transport function and Cl channel activity. However, the inhibition of Na+ transport function by amiloride, EIPA, and HOE-694 (Fig. 5) suggests that Cl-NHE likely represents a novel isoform that is related to the existing gene family. Further characterization of Cl-NHE, including its subcellular localization and tissue distribution, is needed to better understand its role in Na+ transport and Cl channel function.

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