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Mechanisms of Na^+ transport in human distal colonic apical membrane vesicles

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Abstract

Apical membrane vesicles purified from mucosal scrapings obtained from distal segments of organ donor colons and a ^{22}Na-uptake technique were used to characterize the mechanism(s) of Na^+ transport into these vesicles. An outwardly directed H^+ gradient (pH $5.5_{in}/7.5_{out}$) markedly increased uptake of ^{22}Na into these vesicles. Osmolarity studies demonstrated that ^{22}Na was taken up into the intravesicular space with minimal binding observed to the surface of the vesicles. Voltage clamping in the presence of K^+/valinomycin reduced the H^+ gradient-dependent ^{22}Na uptake into these vesicles by ~45% and generation of an inside negative membrane potential significantly increased ^{22}Na uptake. Under non voltage clamped conditions, H^+ gradient-dependent ^{22}Na uptake into these vesicles was significantly inhibited by specific inhibitors of Na^+–H^+ exchange (DMA, HMA and EIPA) as well as by inhibitor of epithelial Na^+ channels (phenamil). Under voltage clamped conditions, H^+ gradient-dependent ^{22}Na uptake, however, was unaffected by phenamil (20 μM), but was almost completely inhibited by DMA, HMA and EIPA (20 μM each). The mechanism of amiloride inhibition of electroneutral Na^+–H^+ exchange was noncompetitive with a K_i for amiloride of 340 μM. Electroneutral ^{22}Na uptake exhibited saturation kinetics with an apparent K_m for Na^+ of 8.7 ± 1.7 mM and a V_{max} of 2.02 ± 0.45 nmol/mg per 5 s. The Na^+–H^+ exchange demonstrated cation specificity similar to the Na^+–H^+ exchangers described in other epithelia. These studies demonstrate for the first time that Na^+ transport across the apical membranes of human distal colon involves both conductive Na^+ uptake and an electroneutral Na^+–H^+ exchange process.

Key words: Sodium ion–proton exchange; Sodium ion conductance; Human distal colon; Human proximal colon

1. Introduction

The absorption of sodium is one of the most important physiological functions of the mammalian large intestine [1,2]. In vivo perfusion experiments as well as studies with intact human colonic tissues in vitro indicate that the human colon is important for the conservation of Na^+, Cl^- and H_2O while secreting K^+ and HCO_3^- [2–6]. Na^+ transport mechanisms in the mammalian large intestine have been shown to involve two different processes: (1) electroneutral NaCl absorption which is chloride-dependent and insensitive to low concentrations of amiloride (10^{-4}–10^{-5} M); and (2) electrogenic Na^+ absorption, which is independent of chloride and inhibited by low concentrations (10^{-4}–10^{-5} M) of amiloride, thought to inhibit luminal membrane sodium channels [1–11]. The relative contribution of these pathways has been shown to differ in various species as well as in different segments of the colon in the same species [1–12]. Until recently, however, investigations of Na^+ absorption in the human colon have been limited due to the low availability of human colonic tissue for investigational purposes and lack of techniques to isolate and purify human colonic

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DMA, 5-(*N*,*N*-dimethyl)amiloride; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HMA, 5-(*N*,*N*-hexamethylene)amiloride; Mes, 2-(*N*-morpholino)ethanesulfonate; *N*-MG, *N*-methyl-D-glucamine; *N*-MGG, *N*-methyl-D-glucamine gluconate; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene; Tris, tris(hydroxymethyl) aminomethane.

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plasma membrane vesicles. Recently, however, our laboratory has been successful in the acquisition of human colonic tissue from organ donors and has developed a method for the isolation and purification of apical plasma membrane vesicles from colonic mucosa [13,14].

Studies, to date, which have attempted to elucidate the mechanism(s) responsible for Na^+ transport in the human colon using either in vitro short circuit current or in vivo perfusion techniques have yielded conflicting results. For example, Hawker et al. [6], based on studies which employed short circuit current techniques and isotopic flux measurements, suggested that all of the Na^+ absorption in the human distal colon involved an electrogenic process. Other studies utilizing voltage-clamped conditions also suggested that electrogenic Na^+ transport accounted for almost all of the amiloride-sensitive short circuit current in the human distal colon [15,16], whereas, amiloride-sensitive Na^+ conductance was shown to be absent from proximal human colon [11]. Sellin and DeSoignie [17], however, employing the same technique suggested that while there was an aboral gradient of increasing electrogenic Na^+ transport, Na^+ absorption in both the proximal and distal human colon involved both electroneutral NaCl transport and electrogenic Na^+ transport. Schiller et al. [18], utilizing in vivo perfusion studies with amiloride inhibition, also suggested that most of the Na^+ absorption in normal human proximal and distal colon might represent an electroneutral or an amiloride-insensitive electrogenic process, since in their studies low concentrations of amiloride (10^{-4}–10^{-5} M) reduced the Na^+ absorption in the proximal and distal human colon by only ~33% and 25%, respectively. Thus, the above studies highlight the inconsistencies reported in the Na^+ transport mechanism(s) in proximal and distal human colon, and also suggest that, similar to human proximal colon, an electroneutral Na^+ absorptive process may also be present in the human distal colon.

Recent studies from our laboratory have demonstrated the presence of an electroneutral, amiloride-sensitive Na^+–H^+ antiporter as well as a conductive Na^+ uptake pathway in human proximal colonic apical membrane vesicles, and have partially characterized the Na^+–H^+ exchanger in this region of the large intestine [13]. The present studies were undertaken to elucidate the mechanism(s) of Na^+ transport in the human distal colon employing purified apical membrane vesicles isolated from distal organ donor colons [14]. In contrast to the results of a number of previous studies using other techniques, which indicated that the electrogenic Na^+ transport was predominant in the human distal colon, the present studies utilizing purified vesicles demonstrate, for the first time, that Na^+ transport across human distal colonic apical membranes involves both electroneutral and conductive pathways. Moreover, the electroneutral Na^+ transport pathway involves a Na^+–H^+ antiporter, whereas the conductive pathway appears to represent apical membrane epithelial type Na^+ channels.

2. Materials and methods

2.1. Materials

Valinomycin, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene (SITS), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), amiloride, ouabain, and acetazolamide were obtained from Sigma (St. Louis, MO). Bumetanide was a generous gift from Hoffman La Roche (Nutley, NJ). 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA), 5-(*N*,*N*-dimethyl)amiloride (DMA), phenamil, and 5-(*N*,*N*-hexamethylene)amiloride (HMA) were synthesized as described in Ref. [19]. Stock solutions (1 M) of *N*-methyl-D-glucamine gluconate (*N*-MGG) (pH 5.5 or 7.5) were made by titrating 1 M *N*-methyl-D-glucamine (*N*-MG) with crystalline D-gluconic acid lactone. All other materials were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ) unless otherwise stated.

2.2. Isolation of human distal colonic apical membrane vesicles

Colonic tissue from eight healthy adult organ donors was obtained immediately after harvest of transplantation of organs. The cecum was discarded and the remaining large intestine divided into two equal parts: proximal and distal. The luminal contents were thoroughly washed and the mucosa was scraped from the seromuscular layer of the colon, and stored at −70°C. Purified distal apical membranes were prepared from thawed mucosal scrapings utilizing a method which involves divalent cation precipitation and differential centrifugation, as recently described by our laboratory [13,14]. The purity of the membrane vesicles and the degree of contamination with intracellular organelles were assessed by appropriate marker enzymes. The specific activity ratios (purified apical membrane/crude homogenate) for the apical membrane enzyme marker, cysteine-sensitive alkaline phosphatase, were approximately 9–10 in all membrane preparations. The corresponding values for succinate dehydrogenase, NADPH–cytochrome-*c* reductase, and sodium/potassium-dependent adenosine triphosphatase, marker enzymes for mitochondrial, microsomal, and basolateral membranes, respectively, ranged from 0.5 to 2.2 in all membrane preparations [13,14]. Membrane vesicles were loaded with appropriate buffers by utilizing the buffers for the last two centrifugation and final resuspension steps and used within 2–3 h of preparation.

Membrane protein was assessed by the method of Bradford [20] using bovine plasma γ-globulin as standard. The orientation of these vesicles was found to be ~ 75–80% right-side out, as assessed by the estimation of marker enzyme cysteine-sensitive alkaline phosphatase in the absence or presence of 0.05% Triton X-100 to open the vesicles.

2.3. ^{22}Na uptake studies

Uptake of ^{22}Na was measured at 25°C by a rapid Millipore filtration technique as described previously [21,22]. Briefly, the membrane vesicles were preloaded with either 100 mM mannitol, 50 mM *N*-MGG, 50 mM K^+ gluconate, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5) or 100 mM mannitol, 50 mM *N*-MGG, 50 mM K^+ gluconate, 50 mM Tris-Hepes, 10 mM $MgSO_4$, pH 7.5. Changes in the composition of the resuspension media are given in the legends or tables. Experiments were started by the addition of 80 μl of the incubation medium consisting of ~ 0.5 μCi of ^{22}Na (1.25 mM), 100 mM mannitol, 50 mM *N*-MGG, 50 mM K^+ gluconate, 50 mM Tris-Hepes, 10 mM $MgSO_4$ (pH 7.5) with or without 20 μM valinomycin to 20 μl of the membrane vesicles (80–100 μg membrane protein). After designated periods of time the reaction was terminated by the addition of 2 ml ice-cold buffer containing 150 mM LiCl, 50 mM Tris-Hepes, 10 mM $MgSO_4$, pH 7.5. The diluted sample was immediately filtered through a 0.65-μm Millipore filter (HAWP) using a Millipore manifold filtration assembly. Filters were further washed two times with 5 ml of cold stopping solution, dissolved in scintillation fluid (Filtercount, Packard Instruments, Downers Grove, IL), and the radioactivity measured in a Packard TR-1600 (Packard Instruments, Downers Grove, IL) liquid scintillation counter.

2.4. Statistical analysis

All experiments were performed using three to four membrane preparations isolated from distal colons of different organ donors. Results are expressed as means ± S.E. Paired or unpaired Student's *t*-tests were used in statistical analysis as appropriate. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Contribution of electroneutral vs. electrogenic Na^+ transport in H^+ gradient-dependent ^{22}Na uptake

Initial studies were performed to examine and compare the effects of an outwardly directed transmembrane H^+ gradient (pH $5.5_{in}/7.5_{out}$) on ^{22}Na uptake into these vesicles under voltage clamped (in the presence of K^+/valinomycin) and non-voltage clamped conditions. H^+ gradient-dependent ^{22}Na uptake at 5 s (initial linear uptake) was examined. For voltage clamped conditions, the vesicles were loaded with a pH 5.5 buffer consisting of 100 mM mannitol, 50 mM *N*-MGG, 50 mM K^+-gluconate, 50 mM Tris-Mes and 10 mM $MgSO_4$, and diluted into an incubation medium containing 100 mM mannitol, 50 mM *N*-MGG, 50 mM K^+ gluconate, 10 mM $MgSO_4$, 1.25 mM ^{22}Na, 20 μM valinomycin and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). The uptake in the absence of a transvesicular pH gradient (pH $5.5_{in}/5.5_{out}$) was subtracted from the uptake in the presence of a pH gradient i.e. (pH $5.5_{in}/7.5_{out}$) to calculate H^+ gradient-dependent ^{22}Na uptake. In non-voltage clamped studies, 50 mM K^+ gluconate in the incubation medium and intravesicular medium (described above) was replaced with equimolar *N*-MGG in the absence of valinomycin. The imposition of voltage clamping markedly reduced the H^+ gradient-stimulated ^{22}Na uptake to 55 ± 10% of control levels, i.e. the absence of voltage clamping. These data, therefore, indicate the presence of both potential sensitive (~ 45%) and electroneutral (~ 55%) H^+ gradient-dependent ^{22}Na uptake into these vesicles.

To further characterize the potential sensitive and electroneutral Na^+ transport, the effects of specific inhibitors of Na^+–H^+ exchange (amiloride analogs DMA, HMA and EIPA) and Na^+ channels (phenamil) on H^+ gradient-dependent ^{22}Na uptake under non-voltage clamped conditions were examined. As shown in Fig. 1, in the absence of voltage clamping, H^+ gradient-dependent ^{22}Na uptake was significantly inhibited ($P < 0.05$) by 20 μM of DMA, HMA, EIPA and phenamil to approximately 50% of control values. These findings again indicate that in the absence of voltage clamping, H^+ gradient-dependent Na^+ uptake may involve almost equal contributions of Na^+–H^+ exchanger and epithelial Na^+ channel(s).

To further confirm the presence of a potential sensitive Na^+ pathway into these vesicles, the effects of applying an intravesicular negative ($K_i > K_o$ + valinomycin) or positive ($K_i < K_o$ + valinomycin) membrane potential on ^{22}Na uptake in the absence of a transvesicular pH gradient (pH $7.5_{in}/7.5_{out}$) were assessed. These results were compared with vesicles in the absence of a transvesicular potential, i.e. voltage clamping ($K_i = K_o$ + valinomycin). As shown in Fig. 2, applying an inside negative membrane potential ($K_i > K_o$ + valinomycin) significantly ($P < 0.05$) increased ^{22}Na uptake (initial linear 5 s uptake) to 210 ± 57% compared to control (voltage clamped) vesicles. Imposition of an inside positive potential ($K_i < K_o$ + valinomycin), however, failed to significantly ($P > 0.05$) influence ^{22}Na uptake (80 ± 18% of control). These

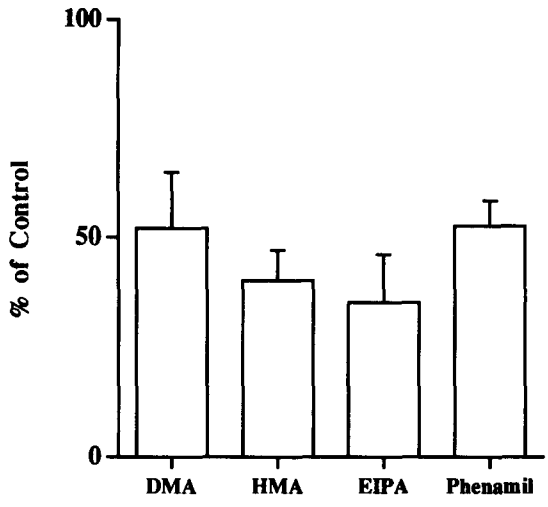

Fig. 1. Effect of amiloride analogs on H^+ gradient-dependent ^{22}Na uptake under non-voltage clamped conditions. Membrane vesicles were preloaded with 100 mM *N*-MGG, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). ^{22}Na (1 mM) uptake was determined at 25°C by diluting these vesicles into a reaction medium containing 100 mM *N*-MGG, 100 mM mannitol, 10 mM $MgSO_4$, 1.25 mM ^{22}Na and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). Uptake was determined at 5 s in the presence and absence of 20 μM (final concentrations) of each inhibitor. Uptake represents H^+ gradient-dependent ^{22}Na uptake calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$ and presented as % of control (mean control value: 357 pmol/mg per 5 s). Values represent means ± S.E. of 5–7 separate preparations.

results clearly demonstrate the presence of a potential-sensitive Na^+ uptake pathway in human distal colonic apical membrane vesicles. To characterize the electroneutral Na^+ transport pathway in these vesicles, voltage clamped conditions were, therefore, used for all subsequent experiments.

3.2. Characterization of electroneutral Na^+ transport

3.2.1. Effects of an outwardly directed H^+ gradient on the time-course of ^{22}Na uptake

The initial studies were performed to determine the effects of an outwardly directed transmembrane H^+ gradient (pH $5.5_{in}/7.5_{out}$) on the time-course of ^{22}Na uptake into these vesicles under voltage clamped conditions (in the presence of K^+/valinomycin). As shown in Fig. 3, ^{22}Na uptake in the presence of an outwardly directed pH gradient ($5.5_{in}/7.5_{out}$) was significantly higher ($P < 0.05$) at each time point up to 3 min compared to the absence of a pH gradient ($5.5_{in}/5.5_{out}$). The peak ^{22}Na uptake (3 min) in the presence of an outwardly directed H^+ gradient (pH $5.5_{in}/7.5_{out}$) was also significantly higher ($P < 0.05$) than the uptake of ^{22}Na at equilibrium (180 min). Thus, ^{22}Na uptake demonstrated an 'overshoot phenomenon' in these vesicles, indicative of the presence of a carrier-mediated Na^+ transport process which is stimulated by a transmembrane H^+ gradient in human distal colonic apical membrane vesicles.

3.2.2. Effect of medium osmolarity on equilibrium ^{22}Na uptake

To further confirm that ^{22}Na was transported into the intravesicular space rather than non-specifically bound to the membrane, the effect of increasing osmolarity of the medium on the equilibrium uptake of ^{22}Na (1 mM Na^+, 120 min, pH $5.5_{in}/7.5_{out}$) was studied (Fig. 4). Osmolarity was altered by varying sucrose concentrations in the extravesicular medium. The ^{22}Na uptake at equilibrium conditions was decreased with increasing medium osmolarity. This suggests that the membrane vesicles were intact, responsive to changes in medium osmolarity and accumulated ^{22}Na into closed intravesicular space. The relationship between the uptake and the reciprocal of osmolarity was linear. Extrapolation of the line to infinite osmolarity (where the intravesicular volume should theoretically be zero) showed that a small binding component of ^{22}Na existed in these vesicles (Fig. 4).

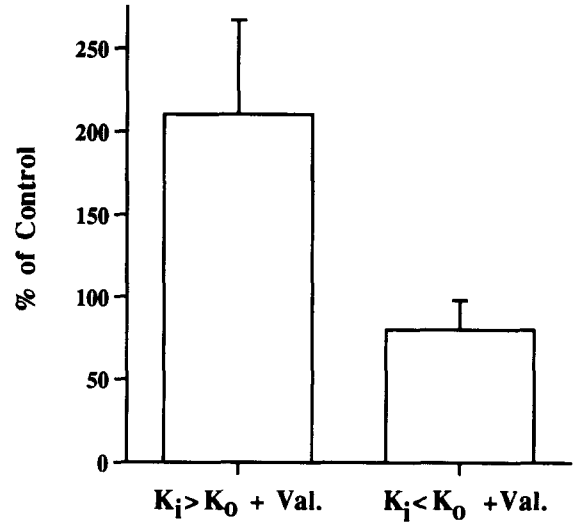

Fig. 2. Effect of transmembrane potential difference on ^{22}Na uptake in the absence of a pH gradient. Membrane vesicles were preloaded with 100 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Hepes, 10 mM $MgSO_4$ (pH 7.5) and diluted into the incubation media containing 100 mM mannitol, 10 mM $MgSO_4$, 50 mM Tris-Hepes (pH 7.5), 1.25 mM ^{22}Na, 20 μM valinomycin and either 100 mM K^+-gluconate (for voltage clamping, i.e. $K_i = K_o$ + valinomycin) or 100 mM *N*-MGG (for inside negative membrane potential, i.e. $K_i > K_o$ + valinomycin). For inside positive membrane potential ($K_i < K_o$ + valinomycin), vesicles were preloaded with 100 mM *N*-MGG, 100 mM mannitol, 10 mM $MgSO_4$, 50 mM Tris-Hepes (pH 7.5) and diluted into the incubation media containing 100 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 50 mM Tris-Hepes (pH 7.5), 1.25 mM ^{22}Na and 20 μM valinomycin. Initial 5 s uptakes were measured and expressed as % of control (voltage clamped vesicles) (mean control value: 63 pmol/mg per 5 s). Values represent means ± S.E. of three separate membrane preparations.

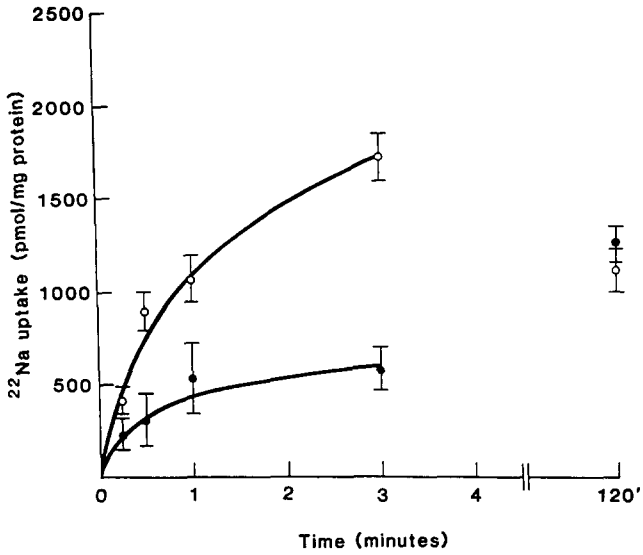

Fig. 3. Time-course of H^+ gradient-dependent ^{22}Na uptake. Membrane vesicles (~ 80–100 μg protein) were preloaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). ^{22}Na (1 mM) uptake was determined at 25°C by diluting these vesicles (20 μl) into a reaction medium (80 μl) containing 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM MgSO4, 20 μM valinomycin, 1.25 mM ^{22}Na and either 50 mM Tris-Hepes (pH 7.5) (○) or 50 mM Tris-Mes (pH 5.5) (●). Values represent means ± S.E. of 5–7 separate preparations.

3.2.3. *Effect of transport inhibitors on ^{22}Na uptake*

Inhibition by the diuretic amiloride has been shown to be one of the important characteristics of an electroneutral Na^+–H^+ exchange process in a variety of cell types [7,14,21–24]. The effects of amiloride and other transport inhibitors (1 mM) on initial uptake rates of ^{22}Na into these vesicles were evaluated in the presence of voltage clamped conditions. As shown in Fig. 5, acetazolamide (a carbonic anhydrase inhibitor), bumetanide (coupled sodium and chloride transport inhibitor), DIDS (anion exchange inhibitor), furosemide (coupled sodium and chloride transport inhibitor) and SITS (anion exchange inhibitor) had no effect on ^{22}Na uptake. Amiloride inhibited ^{22}Na uptake by approximately 75%. Taken together, these data suggest that H^+ gradient-stimulated Na^+ uptake in human distal colonic apical membrane vesicles involves a Na^+–H^+ antiport process.

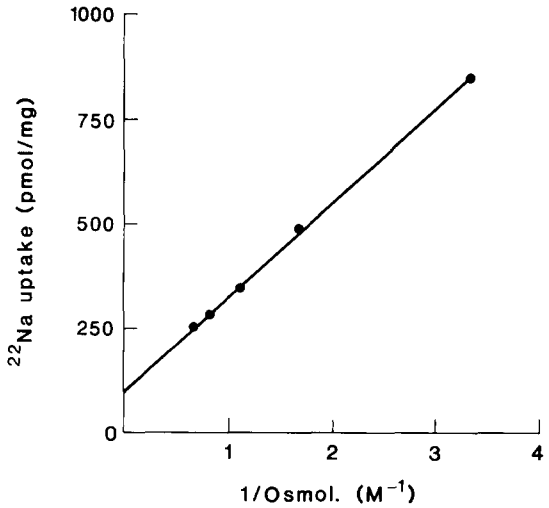

Fig. 4. Effect of medium osmolarity on equilibrium Na^+ uptake. Vesicles were preloaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). ^{22}Na uptake (1 mM) at 180 min was determined at 25°C by diluting the vesicles into an incubation medium at pH 7.5. Osmolarity was altered by varying the sucrose concentration in extravesicular medium. The results are representative of three separate membrane preparations.

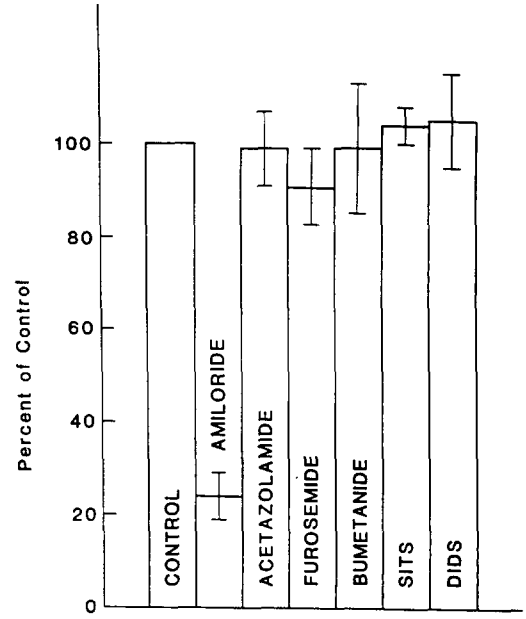

Fig. 5. Effects of transport inhibitors on H^+ gradient-dependent ^{22}Na uptake. Membrane vesicles were loaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). The incubation medium consisted of 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin, 1.25 mM ^{22}Na and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). Uptake was determined at 5 s in the presence and absence of 1 mM concentrations (final) of each inhibitor. Uptake represents H^+ gradient-dependent ^{22}Na uptake calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$ and presented as % of control (mean control value: 155 pmol/mg per 5 s). Values represent means ± S.E. of four or five separate preparations.

3.2.4. *Effect of amiloride analogs on ^{22}Na uptake*

Amiloride (1 mM), in the above studies inhibited ^{22}Na uptake by about 75%. This partial inhibition by amiloride could have been due to either (i) Na^+–H^+ exchanger of human colonic apical membranes is relatively less sensitive to amiloride; or (ii) the remaining 25% of the ^{22}Na uptake may represent contribution by pathways other than Na^+–H^+ exchange. In order to clarify this issue, the effect of more specific inhibitors of Na^+–H^+ exchange (amiloride analogs DMA, HMA

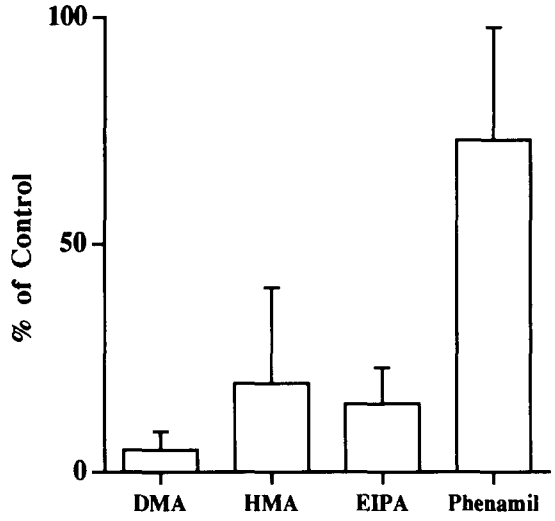

Fig. 6. Effect of amiloride analogs on H^+ gradient-dependent ^{22}Na uptake under voltage clamped conditions. Membrane vesicles were loaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). The incubation medium consisted of 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin, 1.25 mM ^{22}Na and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). Uptake was determined at 5 s in the presence and absence of 20 μM concentrations (final) of each inhibitor. Uptake represents H^+ gradient-dependent ^{22}Na uptake calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$ and are presented as % of control (mean control value: 193 pmol/mg per 5 s). Values represent means ± S.E. of three separate preparations.

and EIPA) [19], and Na^+ channels (phenamil) on H^+ gradient-dependent ^{22}Na uptake under voltage clamped conditions was examined. As shown in Fig. 6, under these conditions, 20 μM (final concentrations) of DMA, HMA and EIPA significantly ($P < 0.05$) inhibited ^{22}Na uptake (ranging from 80–95% inhibition) compared to control. Phenamil (20 μM), however, failed to significantly ($P > 0.05$) inhibit ^{22}Na uptake under these conditions (73 ± 25% of control). These data clearly demonstrate that in the presence of voltage clamping, H^+ gradient-dependent ^{22}Na uptake mainly represents an electroneutral Na^+–H^+ exchange with minimal contribution, if any, from epithelial Na^+ channels.

3.2.5. Amiloride inhibition of ^{22}Na uptake

Dose-dependent relationship. The effect of various amiloride concentrations (0–1 mM) on H^+ gradient-dependent ^{22}Na uptake was examined by the ^{22}Na uptake technique in voltage clamped vesicles. A dose-dependent effect of amiloride on H^+ gradient-dependent ^{22}Na uptake was observed (not shown). Dixon plot analysis (Fig. 7) of the data on the effect of various concentrations of amiloride demonstrated a K_i for amiloride of 340 μM. Calculations from the Lineweaver–Burk plot of Fig. 9, also gave same K_i values (~ 300 μM) for amiloride. This calculated K_i is similar to the K_i of Na^+–H^+ antiporters of other transporting epithelia [10,23–27].

Dose-dependent inhibition of ^{22}Na uptake by EIPA. Similar to studies of amiloride inhibition, the effect of increasing concentrations of EIPA (0–50 μM) on H^+ gradient-dependent ^{22}Na uptake under voltage clamped conditions was also examined. As shown in Fig. 8, EIPA inhibited H^+ gradient-dependent ^{22}Na uptake in a dose-dependent manner and K_i for EIPA (calculated from a Dixon plot, not shown) of about 7.5 μM was obtained.

3.2.6. Kinetics of Na^+–H^+ exchange and mechanism of amiloride inhibition

Saturation kinetics is also an important characteristic of a carrier-mediated Na^+–H^+ exchange process. To examine the kinetics of Na^+–H^+ exchange and mechanism of amiloride inhibition in human distal colonic apical membrane vesicles, ^{22}Na uptake studies were performed under voltage clamped conditions in the presence of varying Na^+ concentrations (2–25 mM), in the presence and absence of 250 μM amiloride. The uptake of ^{22}Na into these vesicles was linear for at least 5 s up to a concentration of Na^+ of 50 mM (data not shown). The results in Fig. 9, show a Lineweaver–

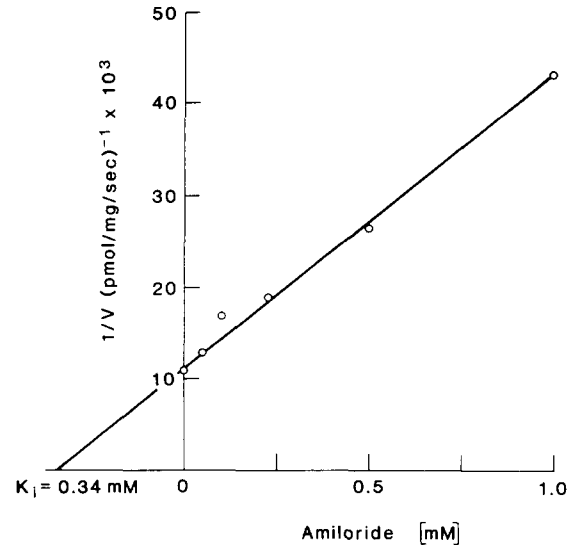

Fig. 7. Dixon plot of the effect of amiloride on H^+ gradient-dependent ^{22}Na uptake. Vesicles preloaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5) were diluted into a reaction medium containing 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). Uptake was determined at 5 s in the presence and absence of indicated concentrations (final) of amiloride (0–1 mM). Uptake represents H^+ gradient-dependent ^{22}Na uptake calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$. The calculated K_i for amiloride was 0.34 mM. Values are representative of five separate membrane preparations.

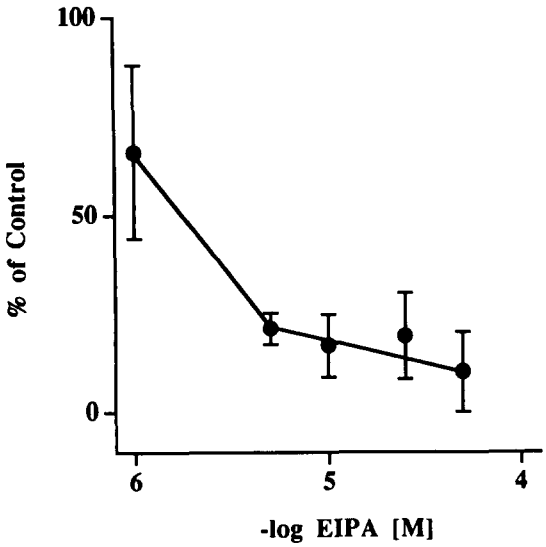

Fig. 8. Dose–response of EIPA inhibition on H^+ gradient-dependent ^{22}Na uptake. Vesicles preloaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5) were diluted into a reaction medium containing 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5). Uptake was determined at 5 s in the presence and absence of indicated concentrations (final) of EIPA (0–50 μM). Uptake represents H^+ gradient-dependent ^{22}Na uptake (5 s) calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$ and represented as % of control (mean control value: 162 pmol/mg per 5 s). Values represent means ± S.E. of three separate preparations.

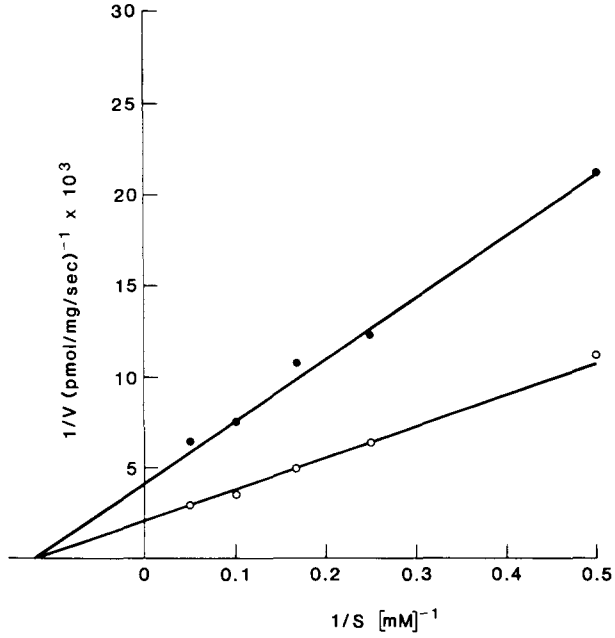

Fig. 9. Kinetics of Na^+–H^+ exchange and mechanism of amiloride inhibition. H^+ gradient (pH $5.5_{in}/7.5_{out}$)-dependent ^{22}Na uptake was determined at increasing extravesicular concentrations of Na^+ (2–25 mM) in the presence (•) or absence (○) of 250 μM amiloride. Vesicles preloaded with 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5) were diluted into the incubation medium containing 50 mM *N*-MGG, 50 mM K^+-gluconate, 100 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5) and 2–25 mM (final conc.) of $^{22}Na^+$-gluconate. Total salt concentration was kept constant on both sides of vesicles by altering the *N*-MGG concentration. S in the *X*-axis represents Na^+ concentration. ^{22}Na uptake (5 s) was determined and results shown are representative of 4–6 separate membrane preparations. The actual uptake values were corrected by subtracting the uptake in the absence of a pH gradient (pH $5.5_{in}/5.5_{out}$). Lineweaver–Burk plot demonstrated a straight line with an apparent K_m for Na^+ of 8.7 ± 1.7 mM and a V_{max} of 2.02 ± 0.45 nmol/mg per 5 s for controls (absence of amiloride).

Burk plot of H^+ gradient-stimulated ^{22}Na uptake vs. Na^+ concentrations in control and in the presence of 250 μM amiloride. These plots demonstrated straight lines and conformed to simple Michaelis–Menten kinetics, indicating saturation kinetics of the antiporter with respect to Na^+. In control vesicles, an apparent K_m for Na^+ of 8.7 ± 1.7 mM and a V_{max} of 2.02 ± 0.45 nmol/mg per 5 s was calculated from this plot. These results are consistent with the presence of a carrier-mediated Na^+–H^+ antiport process in human distal colonic apical membrane vesicles. Amiloride, however, failed to influence the apparent K_m for Na^+, but significantly decreased ($P < 0.05$) V_{max} of the antiporter to 1.19 nmol/mg per 5 s. This data demonstrates that amiloride inhibited Na^+–H^+ antiporter of human distal colonic apical membrane vesicles in a non-competitive manner.

3.2.7. Effect of cations on H^+ gradient-dependent ^{22}Na uptake

Demonstration of cation specificity for the exchanger is another important characteristic of a Na^+–H^+ antiporter. Various cations, i.e. K^+, NMG^+, $choline^+$, Cs^+, Li^+ and NH_4^+ were used to determine the cation specificity of the human distal colonic apical membrane antiporter. In these studies the effect of 20

Table 1
Effect of cations on H^+ gradient-dependent ^{22}Na uptake [a]

Cations	% of control
Li^+	20 ± 5
Na^+	30 ± 3
$Choline^+$	112 ± 8
Cs^+	104 ± 12
NH_4^+	61 ± 7
NMG^+	91 ± 9

[a] Values are depicted as percent of control and represent means ± S.E. of 3 separate membrane preparations. Vesicles were preloaded with 100 mM K^+-gluconate, 100 mM mannitol, 50 mM Tris-Mes, 10 mM $MgSO_4$ (pH 5.5). ^{22}Na (1 mM) uptake was measured at the 5 s time point by incubating in buffer that contained 100 mM K^+-gluconate, 60 mM mannitol, 10 mM $MgSO_4$, 20 μM valinomycin, 1.25 mM ^{22}Na and either 50 mM Tris-Hepes (pH 7.5) or 50 mM Tris-Mes (pH 5.5) in the presence of 20 mM concentrations of chloride salts of each of the various cations. ^{22}Na uptake (5 s) in the presence of K^+ was considered 100% and calculated by subtracting values in the absence of a pH gradient, i.e. $5.5_{in}/5.5_{out}$.

mM concentrations of these cations on the uptake of 1 mM ^{22}Na was evaluated. As shown in Table 1, NH_4^+ and Li^+ significantly inhibited ^{22}Na uptake, indicating that these cations could effectively substitute for Na^+ in the Na^+–H^+ exchange mechanism. Theoretically, inhibition by NH_4^+ ions could also be secondary to dissipation of pH gradient by diffusion of NH_3 into the vesicles. This, however, is considered unlikely in our studies since very high buffering capacity (50 mM Tris-Mes) and very early (5 s) time points of uptake were used. NMG^+, $choline^+$, K^+, and Cs^+, however, failed to inhibit ^{22}Na uptake and were, therefore, unable to substitute for Na^+ in the Na^+–H^+ exchange mechanism.

4. Discussion

Prior studies in a number of epithelial tissues have demonstrated that electroneutral Na^+ absorption may involve a Na^+–H^+ exchange process [10,21–27]. The latter transport process(es), moreover, have been shown to exist in virtually all living cells and appear to be involved in a number of diverse physiological functions, including maintenance of intracellular pH, volume regulation, proliferative response(s) to growth factors and transepithelial transport of Na^+, Cl^- and HCO_3^- (for review see Ref. [28]). While the majority of the previous studies in human colon, utilizing in vivo perfusion or in vitro short circuit current techniques, have indicated that Na^+ absorption in the human distal colon appeared to be electrogenic, several other studies [17,18] suggested that both electroneutral and electrogenic Na^+ transport processes may exist in this segment of the human colon. The present studies, performed in isolated human distal colonic apical membrane vesicles, confirm and extend the latter observations, i.e. the presence of both electroneutral and conductive Na^+ transport mechanism(s) at the luminal membrane domain of human distal colonocytes. Furthermore, the electroneutral Na^+ transport appears to involve a Na^+–H^+ exchange mechanism in these vesicles, whereas the electrogenic process may be represented by epithelial Na^+ channels.

Several lines of evidence are consistent with the presence of an electroneutral Na^+–H^+ exchange process in human distal colonic apical membranes, including: (1) the presence of H^+ gradient-stimulated ^{22}Na uptake, which was only partially potential sensitive (45%); (2) potential-insensitive H^+ gradient-stimulated ^{22}Na uptake exhibited inhibition by amiloride, DMA, HMA and EIPA, but not by the inhibitors of other transport processes; and (3) this process demonstrated saturation kinetics and cation specificity for Li^+ and NH_4^+ similar to other Na^+–H^+ antiporters [10,21–27].

The Na^+–H^+ exchanger of the human distal colonic apical membrane vesicles appears to share a number of characteristics of Na^+–H^+ antiporters of other cell types [10,21–27]. For example, its K_i of amiloride inhibition of Na^+ uptake of 340 μM is similar to that obtained for several other apical membrane Na^+–H^+ antiporters, including those of human jejunal (99 μM) and ileal (140 μM) brush-border membranes, rat colonic apical membranes (425 μM) and rabbit ileal brush-border membranes (230 μM) [10,21–27]. Furthermore, its K_m for Na^+ (8.7 ± 1.7 mM) is similar to values previously reported for its counterparts present in the plasma membranes of the human [10,25,27] and rat small intestine [22] and the rat colon [23,26]. Its V_{max} for Na^+ (2.02 ± 0.45 nmol/mg protein per 5 s) is, however, higher compared to previously reported values for rat colonic plasma membranes [21,23] and lower than values for Na^+–H^+ antiporters in the brush-border membranes of the human small intestine [10,25,27] and rabbit ileum [24]. Kinetic studies demonstrated that, like the apical membranes of rat colonocytes [26] and the plasma membranes of LLC-PK cells [29], amiloride inhibition of Na^+ transport in these vesicles was non-competitive in nature. This is in contrast to several other Na^+–H^+ exchangers in other cell types which are inhibited by this diuretic in a competitive manner (for review see ref. [30]). Substrate specificity studies of Na^+–H^+ antiporters of a number of cell types have also demonstrated that Li^+ and NH_4^+ could also substitute for Na^+ in Na^+–H^+ exchange [10,21–27,30]. In agreement with these studies, the present studies also demonstrate that Li^+ and NH_4^+, but not other cations, e.g. *N*-methylglucamine$^+$, $choline^+$, K^+ and Cs^+, could serve as alternative substrates for this antiporter in human distal colonic apical membrane vesicles. The order of substrate specificity of this antiporter was $Li^+ > Na^+ > NH_4^+$, which is similar to the substrate specificity of Na^+–H^+ antiporters of human jejunal [25] and ileal [27] apical membranes as well as antiporters of the renal cortex of several animal species [31–33].

Prior studies from our laboratory [13] have characterized the Na^+ transport mechanisms of human proximal colonic apical membranes. In view of the large number of previous studies demonstrating the presence of marked segmental variations in Na^+ transport mechanism(s) in mammalian colon (for review see Ref. [2]), it was also of interest to compare and contrast the Na^+ transport characteristics of human distal colonic apical membrane vesicles obtained in the present experiments with those of its proximal counterpart [13]. As shown in Table 2, the transport characteristics of both proximal and distal Na^+ transport appear to be similar except for small quantitative differences, e.g. K_m, V_{max} for Na^+, cation specificity and K_i for amiloride and EIPA. Although our observations are in contrast to some of the previous studies [6,11,15,16], it

Table 2
Comparison of human colonic proximal and distal apical membrane Na^+ transport [a]

Parameter	Proximal [b]	Distal [c]
K_m (mM)	11.8±2.4	8.7±1.7
V_{max} (nmol/mg per 5 s)	2.5±0.6	2.0±0.5
K_i (μM) for EIPA	10.0	7.5
K_i (μM) for amiloride	325	340
Amiloride inhibition	Non-competitive	Non-competitive
Cation specificity	$Li^+ = Na^+ > NH_4^+$	$Li^+ > Na^+ > NH_4^+$
Electroneutral Na^+ uptake	~60%	~55%
Electrogenic Na^+ uptake	~40%	~45%

[a] Values represent mean or means±S.E. of 3–7 independent membrane preparations.
[b] Values taken from Ref. [13].
[c] Values obtained in the present studies.

should, however, be noted that our results confirm and extend the previous in vitro studies of Sellin and DeSoigne [17] and in vivo perfusion studies of Schiller et al. [18] suggesting the presence of both electroneutral and electrogenic Na^+ transport pathways in proximal as well as distal regions of the human large intestine.

Recent studies have shown the existence of a family of Na^+–H^+ exchangers (NHEs) consisting of several isoforms including NHE-1, 2, 3, 4 and β-NHE [34–37]. Among these, NHE-1 and NHE-3 have been the most extensively studied isoforms of this exchanger [38,39]. For example, NHE-1 has been shown to be ubiquitously expressed and its protein product localized in the basolateral membranes of polarized epithelial cells [38]. This exchanger (NHE-1) has been shown to be highly sensitive to amiloride and involved in a number of 'housekeeping' functions, e.g. maintenance of intracellular pH, volume regulation and cell proliferation [35,38]. The NHE-3 isoform has been shown to be relatively less sensitive to amiloride, suggested to be localized on the apical plasma membranes of intestinal and kidney epithelial cells and involved in vectorial Na^+ absorption [39,40]. The molecular nature of the Na^+–H^+ antiporter described in the present studies is not known at present, however, based on its apical membrane localization and its relative insensitivity to amiloride it may represent the NHE-3 or a variant of this isoform. Preliminary studies from our laboratory (unpublished observations) have, in fact, demonstrated, utilizing an RNase protection assay, the presence of NHE-3 message in both proximal and distal human colonic mucosa, lending additional support to our present findings of the existence of an electroneutral Na^+–H^+ exchange process on the apical membranes of epithelial cells of the distal human colon. Further studies will be required to address this issue in detail.

In summary, Na^+ transport in human distal colonic apical plasma membrane vesicles involves both electroneutral and conductive pathways. Electroneutral Na^+ transport represents a Na^+–H^+ antiport process similar to a number of other epithelial cell types, whereas the conductive pathway appears to involve Na^+ channels. In future studies, it would be of interest to characterize the human distal colonic Na^+ channels and to identify the NHE isoforms, i.e. NHE-1, 2, 3 or 4 of the human colon, their regional distribution as well as to define their roles in NaCl absorption.

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