Characterization of Cl-HCO₃ exchange in basolateral membrane of rat distal colon

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Submitted 29 August 2002; accepted in final form 16 June 2003

Ikuma, Mutsuhiro, John Geibel, Henry J. Binder, and Vazhaikkurichi M. Rajendran. Characterization of Cl-HCO₃ exchange in basolateral membrane of rat distal colon. Am J Physiol Cell Physiol 285: C912–C921, 2003.—So- dium-independent Cl movement (i.e., Cl-anion exchange) has not previously been identified in the basolateral membranes of rat colonic epithelial cells. The present study demonstrates Cl-HCO₃ exchange as the mechanism for ³⁶Cl uptake in basolateral membrane vesicles (BLMV) prepared in the presence of a protease inhibitor cocktail from rat distal colon. Studies of ³⁶Cl uptake performed with BLMV prepared with different types of protease inhibitors indicate that preventing the cleavage of the COOH-terminal end of AE2 protein by serine-type proteases was responsible for the demonstration of Cl-HCO₃ exchange. In the absence of voltage clamping, both outward OH gradient (pH₈O/pH₉I: 7.5/5.5) and outward HCO₃ gradient stimulated transient ³⁶Cl uptake accumulation. However, voltage clamping with K-ionophore, valinomycin, almost completely (87%) inhibited the OH gradient-driven ³⁶Cl uptake, whereas HCO₃ gradient-driven ³⁶Cl uptake was only partially inhibited (38%). Both electroneutral HCO₃ and OH gradient-driven ³⁶Cl uptake were 1) completely inhibited by DIDS, an anion exchange inhibitor, with a half-maximal inhibitory constant (Kᵢ) of ~26.9 and 30.6 µM, respectively, 2) not inhibited by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), a Cl channel blocker, 3) saturated by increasing extravascular Cl concentration with a Kₘ for Cl of ~12.6 and 14.2 mM, respectively, and 4) present in both surface and crypt cells. Intracellular pH (pHᵢ) was also determined with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetomethyl ester (BCECF-AM) in an isolated superfused crypt preparation. Removal of Cl resulted in a DIDS-inhibitable increase in pHᵢ both in HCO₃-buffered and in the nominally HCO₃-free buffered solutions (0.28 ± 0.02 and 0.11 ± 0.02 pH units, respectively). We conclude that a carrier-mediated electroneutral Cl-HCO₃ exchange is present in basolateral membranes and that, in the absence of HCO₃, Cl-HCO₃ exchange can function as a Cl-OH exchange and regulate pHᵢ across basolateral membranes of rat distal colon.

crypt glands; superfusion; intracellular pH; membrane vesicles; ³⁶Cl uptake; Cl-anion exchange

TRANSCELLULAR TRANSPORT of electrolytes across epithelial cells requires specific transport processes in both apical and basolateral membranes. Na-H and Cl-HCO₃ exchanges coupled via intracellular pH (pHᵢ) have been suggested as being responsible for electroneutral Na-Cl absorption, in which Na enters via an apical membrane Na-H exchange while exiting via the basolateral Na-pump in rat distal colon (6). Studies with apical membrane vesicles (AMV) have established Cl-anion exchange (i.e., Cl-HCO₃ and Cl-OH exchanges) as the mechanism for Cl uptake across the apical membrane in rat distal colon (12, 20). These studies have also established that Cl-HCO₃ and Cl-OH exchanges of colonic AMV are distinct and separate anion exchanges and proposed that the former is responsible for transcellular Cl absorption, whereas the latter may be responsible for pHᵢ regulation (20). Although Cl-HCO₃ exchange has been established as a mechanism for Cl entry, an exit mechanism for Cl across basolateral membrane has not been identified.

Immunohistochemical studies have established the localization of anion exchange-2 (AE2) isoform-specific polypeptide in basolateral membranes of human and mouse intestine, rabbit kidney, and rat colon (2, 4, 7, 23). However, our previous studies did not identify Cl-anion exchange in basolateral membrane vesicles (BLMV) isolated from rat distal colon (20). Recent immunoblot study with rabbit kidney basolateral membranes indicated that AE2-specific protein was readily identified in membranes isolated in the presence of a protease inhibitor cocktail (7). As a result, this study concluded that the COOH-terminal end of AE2 protein was cleaved during the sucrose gradient centrifugation (7). The present study was, therefore, initiated to determine whether Cl-anion exchange is present in BLMV when isolated in the presence of one or more protease inhibitor(s). The results of this study indicate that an electroneutral, carrier-mediated, DIDS (4,4'-diisothiocyanato-2,2'-disulfonic acid)-sensitive Cl-HCO₃ exchange is present and that this Cl-HCO₃ exchange is most likely encoded by AE2 isoform in BLMV of rat distal colon.

MATERIALS AND METHODS

Vesicle preparation. BLMV were isolated from nonfasting normal male rat (Sprague-Dawley, 200–250 g; Charles River,
Raleigh, NC) distal colon by the modified method of Biber et al. (5) that has been described previously (26). To isolate surface and crypt cell-specific BLMV, scraped mucosa and isolated crypt cells were used, respectively. Crypt cells were isolated by the divalent chelation techniques of Weiser (34), as described previously (24). In this modified method, BLMV were prepared in the presence of serine-type (PMSF 88.0, aprotinin 5.0, leupeptin 1.0), cysteine-type (chymostatin 1.0, benzamidine 208.0), aspartate-type (pepsatin A 5.0), and metalo-enzyme-type (phosphoramidon 1.1) protease inhibitors, as well as a cocktail of these four types of inhibitors to which preservatives (amino caproic acid 131.0, sodium metasulphite 190.0) were added. Homogenates of mucosal scraping and isolated crypt cells were layered on a continuous (13–37%) sucrose gradient and centrifuged isokinetically at 28,000 rpm (SW40 rotor; L-7-55 Beckman Ultracentrifuge, Palo Alto, CA). Three to four milliliters of top layer collected from continuous gradients were homogenized by using a Potter-Elvehjem homogenizer and were layered on continuous/discontinuous sucrose (13–15/37.5/55%) gradients. After centrifugation at 39,000 rpm for 2 h (SW40 rotor; L-7-55 Beckman Ultracentrifuge), membrane bands were seen at junctions between 15 and 37.5% (fraction I) and between 37.5 and 55% (fraction II) sucrose gradients for scraped mucosa, whereas only fraction II was seen for isolated crypt cells. Membrane fractions I and II collected were diluted 10-fold with 10 mM Tris-HCl buffer (pH 7.4) and pelleted by centrifugation at 50,000 rpm for 1 h (Ti50.1 rotor, L-7-55 Beckman Ultracentrifuge). The final membrane pellets were resuspended in appropriate media and used for both Na,K-ATPase assay and uptake studies. Similar to previous observations, Na,K-ATPase activity was enriched 13.9-fold in membrane fraction I (mucosal homogenate vs. fraction I: 70.0 ± 3.6 vs. 975.3 ± 28.9 pmol P, liberated·mg protein$^{-1}·h^{-1}$) and 11.9-fold enrichment in fraction II (isolated crypt cell homogenate vs. fraction II: 43.3 ± 2.4 vs. 513.7 ± 32.3 pmol P, liberated·mg protein$^{-1}·h^{-1}$) compared with activity in homogenates of scraped mucosa and isolated crypt cells, respectively. The specific activities of Na,K-ATPase in fraction II obtained from scraped mucosa and isolated crypt cells were identical. However, the Na,K-ATPase activities in fraction II from mucosal scraping were enriched only 7.3-fold (70.0 ± 3.6 vs. 513.7 ± 32.3 pmol P, liberated·mg protein$^{-1}·h^{-1}$). The substantially lower enrichment in fraction II from mucosal scraping was due to the higher Na,K-ATPase activity seen in homogenate of scraped mucosa than in homogenate of isolated crypt cells (43.3 ± 2.4 vs. 70.0 ± 3.6 pmol P, liberated·mg protein$^{-1}·h^{-1}$) but not due to contamination by other proteins. The observations that both fraction I and fraction II were seen in scraped mucosa, whereas only fraction II was seen in isolated crypt cells, indicate that scraped mucosa consists of both surface and crypt cells, whereas isolated crypt cells consist only of crypt cells. Similar observations have been reported for rabbit colon (35). Thus the membrane fraction I and fraction II were used as surface- and crypt cell-specific BLMV, respectively.

Preliminary studies established that outward HCO$_3$/OH gradient-driven $^{36}$Cl uptake (Cl-HCO$_3$/OH exchange) was not identified in BLMV prepared in the absence of protease inhibitors (Fig. 1, control). In contrast, in BLMV prepared in the presence of the protease inhibitor cocktail, Cl-HCO$_3$/OH exchange was identified (Fig. 1). To establish whether this preservation of vesicle function was a result of one or more specific types of protease inhibitors, additional studies of Cl-HCO$_3$/OH exchange were performed with BLMV prepared in presence of either serine-type, cysteine-type, aspartate-type, or metalo-enzyme-type protease inhibitors. These studies established the presence of Cl-HCO$_3$/OH exchange in BLMV prepared in the presence of serine-type protease inhibitors (Fig. 1) and that the uptake was identical to that in BLMV prepared in the presence of either protease inhibitor cocktail (Fig. 1) or a commercial preparation of multiple protease inhibitors COMPLET (Roche Applied Science, Indianapolis, IN) (not shown). BLMV prepared in the presence of cysteine-type protease inhibitor exhibited 30% of maximal Cl-HCO$_3$/OH exchange activity, whereas BLMV prepared in the presence of either aspartate-type or metalo-enzyme-type protease inhibitors exhibited Cl-HCO$_3$/OH exchange activity that is identical to that in BLMV prepared in the absence of protease inhibitors. All further experiments to characterize Cl-HCO$_3$/OH exchange used BLMV prepared in the presence of the cocktail of protease inhibitors.

Apical membrane vesicles. (AMV) from surface cells of rat distal colon were prepared by the method of Stieger et al. (32), as described previously (25). Protein was assayed by the method of Lowry et al. (17) using bovine serum albumin as standard.

Western blot. SDS-PAGE was performed by the standard protocol as described earlier (25). To avoid aggregation, the...
protein samples (30 mg) of rat colonic basolateral membranes were warmed to 37°C for 20 min before being loaded. Proteins were electrophoretically transferred from SDS-PAGE to nitrocellulose (Biorad; Gelman Sciences, Ann Arbor, MI) in 192 mM glycine, 25 mM Tris, pH 8.3, 20% methanol overnight at 30 V. After nonspecific sites were blocked with TBST consisting of 20 mM Tris, 137 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk, pH 7.5, immunostaining was performed with affinity-purified AE2 polyclonal antibodies (4) and horseradish peroxidase-conjugated anti-rabbit IgG (Amer- sham). Antibody-specific bands were visualized by Supersignal enhanced chemiluminescence (Pierce chemicals, Rock- ford, IL).

Although our earlier study did not identify [36Cl]-HCO3/OH exchange activity in BLMV (20), our recent immuno-chemical study demonstrated the expression of AE2 isoform-specific protein in basolateral membranes of rat distal colon (23). In addition, previous studies have also shown that the COOH-terminal end of AE2 protein was cleaved during basolateral membrane preparation from rabbit proximal tubules (7). Thus Western analyses of BLMV prepared in the presence and absence of protease inhibitors were performed to establish whether AE2 proteins are intact during BLMV preparation. A polyclonal antibody (kindly provided by Dr. Seth L. Alper, Harvard University School of Medicine, Bos- ton, MA) that had been raised against a 14-amino acid (1,224–1,237) polypeptide of the COOH-terminal end of mouse AE2 protein was used (7). As shown in Fig. 2A (lanes 3 and 4), AE2 antibody identified an approximate 170-kDa protein in BLMV prepared in presence of serine-type pro- tease inhibitors. In contrast, a faint band was seen in BLMV prepared in the absence of protease inhibitors (Fig. 2A, lanes 1 and 2). To establish whether serine-type protease inhibitors help preserve other transport proteins other than AE2 pro- tein, Western analyses were also performed in BLMV, pre- pared in the presence and absence of serine-type protease inhibitors, using a Na/H exchange isoform 1 (NHE1) polyclonal antibody (kindly provided by Dr. Mark Donowitz, Johns Hopkins University, Baltimore, MD) that had been raised against the 158 amino acids of the COOH-terminal cytoplasmic domain of human NHE1 (18). As shown in Fig. 2B, NHE1 antibody identified an approximate 92-kDa pro-tein in BLMV that was prepared in the presence (lane 2) and in the absence (lane 1) of serine-type protease inhibitors. These observations indicate that serine-type protease inhibitors prevent COOH-terminal cleavage of AE2 protein, but serine-type proteases do not alter either NHE1 protein (Fig. 2B) or NHE function (28) in BLMV of rat distal colon.

ATPase assay. Na,K-ATPase activity was measured in both homogenate and basolateral membrane vesicles by the method of Porbus (11), as previously described (10). Activi- ties are presented (in nmol Pi liberated·mg protein·h−1). Results presented represent means ± SE of triplicate assays of three different membrane preparations.

Uptake studies. An outward OH/HCO3 gradient-, OH gra- dient-, and HCO3 gradient-driven [36Cl] uptake was performed by the rapid filtration techniques, as previously described (20). [36Cl] (9.39 mCi/mg; NEN, Boston, MA) obtained in the form of HCl was titrated to pH 7.2–7.4 with N-methyl-d-glucamine (NMG-[36Cl]). In brief, OH/HCO3 gradient-driven [36Cl] uptake [BLMV preloaded with 50 mM MOPS-Tris (pH 7.5), 150 mM KHCO3, and 10 mM NMG-glucanate] were incubated in medium containing either 50 mM MES-Tris (pH 7.5) or 150 mM K-gluconate or 10 mM NMG-glucanate at pH 7.5. OH/HCO3 gradient-driven [36Cl] uptake was calculated by subtracting uptake in incubation medium with MOPS-Tris (pH 7.5) from that in medium with MES-Tris (pH 7.5). OH gradient-driven [36Cl] uptake [BLMV preloaded with 50 mM MOPS-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate] were incubated in medium containing either 50 mM MOPS-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate or 50 mM MES-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate. OH-gradient-driven [36Cl] uptake was calculated by subtracting uptake in incubation medium with MOPS-Tris (pH 7.5) from that in medium with MES-Tris (pH 7.5). OH gradient-driven [36Cl] uptake [BLMV preloaded with 50 mM MOPS-Tris (pH 7.5), 150 mM KHCO3, and 10 mM NMG-glucanate] were incubated in medium containing either 50 mM MOPS-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate or 50 mM MOPS-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate. OH-gradient-driven [36Cl] uptake was calculated by subtracting uptake in incubation medium with K-glucanate from that in medium with KHCO3. HCO3 gradient-driven [36Cl] uptake [BLMV preloaded with 50 mM MOPS-Tris (pH 7.5), 150 mM KHCO3, and 10 mM NMG-glucanate] were incubated in medium containing either 50 mM MOPS-Tris (pH 7.5), 150 mM K-glucanate, and 10 mM NMG-glucanate or 50 mM MOPS-Tris (pH 7.5), 150 mM KHCO3, and 10 mM NMG-glucanate. HCO3 gradient-driven [36Cl] uptake was calculated by subtracting uptake in incubation medium with K-glucanate from that in medium with KHCO3. HCO3 containing media were gassed with 25% CO2 balanced with O2, whereas media without HCO3 were gasssed with 100% O2. Electroneutral [36Cl] uptakes were measured in the presence of 25 μM vali- nomycin. All uptake studies were repeated at least three times with different membrane preparations. The presented results are means ± SE of triplicate assays of representative experiments. Standard errors that are <5% of mean are not shown.

pH measurement in superfused colonic crypt. Crypts were incubated by isolating small (5- to 10-mm) pieces of colon in a Ca2+-free, 10 mM EGTA Ringer solution (34). Colonic cryp- ters were then transferred to a temperature-controlled perfusion chamber with a bottom made of a glass coverslip that had been precoated with Cell-Tak, a biological cell ad- hesive. The crypt cells in the perfusion chamber were superfused at a rate of 3 ml/min; the temperature controller en- sured a temperature of 37.0 ± 0.05°C adjacent to the cryp- t, as monitored by a thermistor. To exclude the possibility of significant differences in pH regulation in different portion of the crypt, all pH measurements presented in this manuscript were performed in cells of the middle portion of each crypt. To measure pH, 5 μM BCECF-AM was added to the perfusate solution for ~10 min and kept in dye-free solution for 5 min before any data were acquired.

Fig. 2. Western blot analyses of BLMV prepared in the presence and absence of serine-type protease inhibitors by anion exchange-2 (AE2) (A) and Na/H exchange isoform 1 (NHE1) (B) antibodies. A: an ~175- kDa protein identified by AE2 antibodies is maximally present in BLMV prepared in presence of serine-type protease inhibitors (lanes 3 and 4), whereas it is only minimally present in BLMV prepared in the absence of protease inhibitors (lanes 1 and 2). B: an approximate 92-kDa protein identified by NHE1 antibodies is almost equally present in BLMV prepared in the absence (lane 1) and in the presence (lane 2) of serine-type protease inhibitors. Molecular mass markers are indicated at right.
recorded. The pHi recording was performed on the perfusion chamber that was mounted on the stage of an inverted microscope (Nikon Diaphot; Nikon, Melville, NY). BCECF was excited at 490 and 440 nm by using a computer-controlled monochromator placed in front of a 75-watt xenon lamp. A dichroic mirror centered at 510 nm reflected the excitation light to the perfusion chamber and transmitted the emitted fluorescence through a 535-nm filter. Fluorescent images were obtained with an intensified charge-coupled device video camera. A set of images was taken every 2 or 3 s. The 490/440 fluorescence intensity ratio data were converted to pH values by using the nigericin calibration technique (28). Over the pH range of 6.5–7.5, fluorescence varied in a linear fashion with extracellular pH. Data obtained from several cells in a single crypt represent the single observation for that animal; n represents the number of animals.

*Perfusion solution.* Both HCO3/CO2-free HEPES-buffered medium (referred to as nominally HCO3-free buffered solution) and HCO3/CO2-buffered medium (referred to as HCO3-buffered solution) were used. The nominally HCO3-free buffered solution contained (in mM) 125.0 NaCl, 5.0 KCl, 1.2 CaCl2, 1.2 MgCl2, and 32.0 HEPES/Tris (pH 7.4) and was gassed with 100% O2. The HCO3-buffered solution contained (in mM) 117.0 NaCl, 5.0 KCl, 1.0 CaCl2, 1.2 MgCl2, and 24.0 NaHCO3 and was gassed with 95% O2–5% CO2 to maintain pH at 7.4. The osmolalities of solutions were adjusted to 312 mosmol/kgH2O.

*Statistics.* Results are reported as means ± SE. Student’s t-test was used to determine statistical significance between two groups. For more than two group comparisons, a one-way analysis of variance (ANOVA) was performed. A P value of <0.05 was considered significant.

**RESULTS**

**36Cl uptake studies.** Experiments were designed to establish whether HCO3/OH gradient-driven 36Cl uptake seen in BLMV prepared in the presence of protease inhibitors are Cl-HCO3 exchange or Cl-OH exchange. In these studies, the effect of an outward OH gradient [extracellular pH (pHe)/pHi: 5.5/7.5] and an outward HCO3 gradient on 36Cl uptake was examined. As shown in Fig. 3, both an outward OH gradient (Fig. 3A) and outward HCO3 gradient (Fig. 3B) induced transient 36Cl accumulation in BLMV surface cells. Simultaneous presence of both HCO3 and OH gradients resulted in a rate of 36Cl uptake that was greater than that observed in the presence of either a HCO3 gradient or a OH gradient alone. It should be noted in AMV from surface cells that both OH and HCO3 gradient-driven 36Cl uptakes are present, but OH gradient-driven 36Cl uptake is greater than that of HCO3 gradient-driven 36Cl uptake (20). In contrast, in BLM, HCO3 gradient-driven 36Cl uptake is greater than that of OH gradient-driven 36Cl uptake (Fig. 3, A and B).

To determine whether OH gradient-driven and HCO3 gradient-driven 36Cl uptake are the result of a tightly coupled electroneutral anion exchange and/or a coupled electrogenic diffusion process, 36Cl uptake was also performed under voltage-clamp conditions. As shown in Fig. 3A, voltage clamping with K (Kout = Kin = 150 mM) and valinomycin (K ionophore, 25 mM) almost completely inhibited the OH gradient-driven 36Cl uptake. In contrast, voltage clamping only partially inhibited HCO3 gradient-driven 36Cl uptake (Fig. 3B). These results differ substantially from those that were observed with AMV, in which voltage clamping did not inhibit either OH gradient- or HCO3 gradient-driven 36Cl uptake (20). These results suggest that the major component of OH gradient-driven and a small component of HCO3 gradient-driven 36Cl uptake in BLMV are an electrogenic process that is a result of electrodiffusional coupling.

A linear rate of 36Cl uptake was established for both OH gradient-driven and HCO3 gradient-driven 36Cl uptake. As shown in Fig. 4, both OH gradient-driven (Fig. 4A) and HCO3 gradient-driven (Fig. 4B) 36Cl uptake were linear for at least 15 s under both voltage-clamp and non-voltage-clamp conditions. Initial rate of OH gradient- and HCO3 gradient-driven 36Cl uptake was inhibited by voltage clamping by 87% (3.7 ± 0.2 vs.

![Fig. 3. Effect of outward OH gradient and HCO3 gradient on 36Cl uptake in colonic BLMV. A: transient accumulation of 36Cl uptake is present in the presence of an outward-directed OH gradient (pHeout/pHin: 5.5/7.5; [●]), compared with that in the absence of OH gradient (pHeout/pHin: 7.5/7.5; [○]). The OH gradient-driven transient 36Cl uptake was almost completely inhibited by voltage clamping ([■]). B: transient accumulation of 36Cl uptake is present in the presence of an outward-directed HCO3 gradient (HCO3out < HCO3in; pHout/pHin: 7.5/7.5; [●]), compared with that in the absence of HCO3 gradient (HCO3out = HCO3in: 7.5/7.5; [○]). Simultaneous presence of outward gradients of both HCO3 gradient and OH gradient (HCO3out < HCO3in; pHout/pHin: 5.5/7.5; [□]) further stimulated the 36Cl uptake. Both HCO3/OH gradient-driven ([●] and HCO3 gradient-driven (not shown) 36Cl uptake were partially inhibited to the same level by voltage clamping.](http://ajpcell.physiology.org/)

*AJP-Cell Physiol* • VOL 285 • OCTOBER 2003 • www.ajpcell.org
0.5 ± 0.1 nmol/mg protein at 15 s) and by 38% (4.5 ± 0.3 vs. 2.8 ± 0.2 nmol/mg protein at 15 s). The fraction that was inhibited by voltage clamping represents electrodiffusional coupling, whereas the remaining voltage-insensitive OH gradient-driven (13%) and HCO3 gradient-driven (62%) 36Cl uptake represents an electroneutral anion exchange process. In view of the small component of OH gradient-driven 36Cl uptake that is electroneutral, studies were specifically performed to establish the presence of Cl-OH, as well as Cl-HCO3 exchange. 36Cl uptake was performed under voltage-clamp conditions in the presence of outward OH gradient (pHo/pHi: 5.5/7.5) and in the absence of pH gradient conditions at both low (pHo/pHi: 5.5/5.5) and high (pHo/pHi: 7.5/7.5) OH concentrations. As shown in Fig. 5, initial rate of 36Cl uptake was significantly increased in the presence of outward OH gradient compared with that in its absence. The OH gradient-driven 36Cl uptake was completely inhibited by 1 mM DIDS. The 36Cl uptake in the absence of OH gradient at either low or high OH concentrations was not significantly different from DIDS-insensitive uptake seen in the presence of OH gradient. These results indicate that the OH gradient-driven 36Cl uptake occurs via a DIDS-sensitive anion exchange process. Subsequent experiments were designed to characterize OH gradient-driven and HCO3 gradient-driven electroneutral 36Cl uptake at 12 s under voltage-clamp conditions.

The effect of various transport inhibitors was performed on OH gradient- and HCO3 gradient-driven 36Cl uptake. As shown in Fig. 6, both OH gradient-driven (Fig. 6A) and HCO3 gradient-driven (Fig. 6B) 36Cl uptake were completely inhibited by 1 mM DIDS, an anion exchange inhibitor. Bumetanide, a Na-K-2Cl cotransport inhibitor, inhibited only 15 and 17% of OH gradient and HCO3 gradient-driven 36Cl uptake, respectively. Acetazoamide, a carbonic anhydrase inhibitor, inhibited both OH and HCO3 gradient-driven 36Cl uptake by 22%. Neither OH nor HCO3 gradient-driven 36Cl uptake was affected by amiloride, a Na/H exchange inhibitor; NPPB, a Cl channel blocker; or ouabain, a Na,K-ATPase inhibitor. These results establish that both HCO3 and OH gradient-driven 36Cl uptake in basolateral membranes occur via an anion exchange (Cl-HCO3/Cl-OH exchange) process.

Kinetic studies were performed to establish whether both OH gradient-driven and HCO3 gradient-driven 36Cl uptakes were mediated by one or more than one anion exchange processes. As shown in Fig. 7, both OH gradient- and HCO3 gradient-driven 36Cl uptakes were stimulated and saturated with hyperbolic curve with increasing extravesicular Cl concentrations. Kinetic
parameters of these data were calculated: $K_m$ for Cl of $-14.2 \pm 2.6$ and $12.6 \pm 1.2$ mM; $V_{max}$ $1.2 \pm 0.4$ and $5.2 \pm 0.8$ nmol/mg protein 12 s for OH gradient- and HCO$_3^-$ gradient-driven $^{36}$Cl uptake, respectively. As shown in Fig. 8, increasing DIDS concentration in incubation medium progressively inhibited both OH gradient-driven and HCO$_3^-$ gradient-driven $^{36}$Cl uptake. Hill plot analyses of these data yielded a $K_i$ for DIDS of $-26.9 \pm 3.6$ and $30.6 \pm 5.6$ mM for OH gradient- and HCO$_3^-$ gradient-driven $^{36}$Cl uptake, respectively. These results suggest that both OH gradient- and HCO$_3^-$ gradient-driven $^{36}$Cl uptakes may be mediated either by the same transport process or by different transport processes with similar affinities for Cl and DIDS.

Previous studies indicate that Cl-HCO$_3^-$ exchange was present in AMV only from surface and not from crypt cells, whereas Cl-OH exchange was present in AMV from both surface and crypt cells (22). Therefore, studies were performed to determine the activity of Cl-anion exchange in BLMV isolated from crypt cells. Similar to surface cell BLMV, under voltage-clamp conditions both OH gradient-driven and HCO$_3^-$ gradient-driven $^{36}$Cl uptakes were present in crypt cell BLMV (Fig. 9, A and B). Both OH and HCO$_3^-$ gradient-driven $^{36}$Cl uptake were completely inhibited by 1 mM DIDS. These observations indicate that Cl-anion exchanges are uniformly present in BLMV of both surface and crypt cells.

Additional studies were designed to compare the effect of several cations on Cl-anion exchange in BLMV and in AMV compared with their effect on Cl-anion exchange in oocytes injected with AE2 cRNA. In earlier studies, Humphreys et al. (14) have shown that NH$_4^+$ activates AE2 expressed in oocytes, and our previous immunocytochemical studies localized AE2 protein to basolateral membranes of both surface and crypt cells of rat distal colon (23). In the present study, we examined the effect of outward Cl gradient-driven $^{36}$Cl uptake (Cl-CI exchange, an alternate mode of Cl-HCO$_3^-$ exchange) in the presence of several cations (NH$_4^+$, NMG, Na, and K) in BLMV and compared with AMV isolated from colonic surface cells (Fig. 10). As shown in Fig. 10A, in BLMV the outward Cl gradient-driven $^{36}$Cl uptake was significantly greater ($4.1 \pm 0.3$ vs. $5.9 \pm 0.8$ nmol·mg protein$^{-1}·12$ s$^{-1}$; $P < 0.05$) in the presence of NH$_4^+$ than in the presence of the other cations. In contrast, in AMV the effect of these cations on outward gradient-driven $^{36}$Cl uptake was almost identical (Fig. 10B). Thus NH$_4^+$ increases Cl-anion exchange activity in BLMV and in AE2 cRNA injected oocytes (14) but not in AMV.

$pH_i$ studies. These $^{36}$Cl uptake studies establish the presence of an electroneutral Cl-anion exchange in basolateral membranes of rat distal colon. To determine whether these Cl-anion exchanges participate in the regulation of $pH_i$, studies of $pH_i$ were performed in isolated superfused colonic crypts. Basal $pH_i$ of crypt

![Fig. 7. Effect of increasing Cl concentrations on OH gradient-driven and HCO$_3^-$ gradient-driven $^{36}$Cl uptake under voltage-clamp conditions in colonic BLMV. Increasing extravesicular Cl concentration saturated both OH gradient-driven ($\diamond$) and HCO$_3^-$ gradient-driven ($\bullet$) $^{36}$Cl uptake with an apparent $K_m$ for Cl of $14.2 \pm 2.6$ and $12.6 \pm 1.2$ mM, $V_{max}$ $1.2 \pm 0.4$ and $5.2 \pm 0.8$ nmol/mg protein 9 s, respectively. The best fit curves were drawn using a Michaelis-Menten equation, whereas kinetic constants were calculated using a nonlinear regression with Enzfitter program.](http://ajpcell.physiology.org/)

![Fig. 8. Effect of increasing DIDS concentrations on OH gradient-driven and HCO$_3^-$ gradient-driven $^{36}$Cl uptake under voltage-clamp conditions in colonic BLMV. Increasing DIDS concentration inhibits both OH-gradient-driven ($\circ$) and HCO$_3^-$ gradient-driven ($\bullet$) $^{36}$Cl uptake with an apparent $K_i$ for DIDS of $26.6 \pm 3.6$ and $30.6 \pm 5.6$ mM, respectively.](http://ajpcell.physiology.org/)
cells was determined both in a HCO₃-buffered solution and in a nominally HCO₃-free buffered solution. Basal pHᵢ values were significantly higher in cells bathed in the nominal absence of HCO₃ (7.48 ± 0.03; n = 6) than those exposed to the HCO₃-buffered solution (7.24 ± 0.03; n = 6; P < 0.01). Removal of HCO₃/CO₂ was followed by a rapid increase in pHᵢ (data not shown). Similarly, changing from the nominally HCO₃-free buffered solution to the HCO₃-buffered solution resulted in a prompt intracellular acidification.

The effect of the removal of Cl from the superfusion solution on pHᵢ is illustrated in Fig. 11. Basal pHᵢ was monitored for 10 min before bath Cl was removed both in the HCO₃-buffered and in the nominally HCO₃-free buffered solutions. Removal of Cl resulted in an increase in pHᵢ both in HCO₃-buffered (0.28 ± 0.02 pH units; n = 6) and in the nominally HCO₃-free buffered solutions (0.11 ± 0.02 pH units; n = 6) (Fig. 11 and Table 1). In the HCO₃-buffered solution, the changes in dpHᵢ/dt after Cl removal were greater than those observed in the nominally HCO₃-free buffered solution (Table 1). The readdition of Cl to the superfusion solution resulted in a prompt acidification and a return of pHᵢ to basal level. There was no significant difference in steady-state pHᵢ before the removal of Cl compared with that observed after its readdition. These observations after the removal and readdition of Cl (i.e., Cl-dependent alkalinization) are consistent with a Cl-anion exchange but may represent either intracellular base accumulation as a result of prevention of basolateral membrane Cl-anion exchange or intracellular Cl leaving the cell in exchange for HCO₃/OH entry; either process will result in an intracellular alkalinization.

To distinguish between these two possibilities, experiments were also performed in which the effect of an anion exchange inhibitor, DIDS, was determined. DIDS (2 mM) prevented the increase in pHᵢ that was observed after the readdition of Cl to the bath solution (from 7.24 ± 0.01 to 7.32 ± 0.03; n = 4; not significant) (Fig. 12A). DIDS (2 mM) also significantly inhibited
Cl-dependent alkalinization in nominally HCO₃⁻-free buffered solution (from 7.48 ± 0.08 to 7.48 ± 0.06; n = 4, N.S.) (Fig. 12B). These experimental findings indicate that this DIDS-sensitive Cl-HCO₃ exchange may also function as Cl-OH exchange and regulate pHi.

**DISCUSSION**

These studies provide compelling evidence for the presence of a Cl-HCO₃ exchange in the basolateral membrane of colonic epithelial cells employing two different experimental approaches: 1) ³⁶Cl uptake by BLMV and 2) pHi determinations during superfusion of colonic crypt cells. Although active Cl absorption is a well-accepted transport process in mammalian colon, almost all prior studies of Cl transport in colonic epithelial cells have focused on apical membrane transport mechanisms, primarily Cl-HCO₃ exchange (1, 20). In contrast, the mechanism of Cl movement across the basolateral membrane other than that associated with Na-K-2Cl cotransport and Cl secretion (9, 36) has not been examined in detail in earlier studies. By combining two complementary methods, we provide convincing demonstration of Cl-HCO₃ exchange in colonic basolateral membranes.

The experiments performed with BLMV demonstrated the presence of electroneutral, carrier-mediated Cl-HCO₃ exchanges (Fig. 3). Outward-directed HCO₃⁻ gradients stimulated transient accumulation of ³⁶Cl by BLMV. Because Cl-HCO₃ exchanges are inhibited by DIDS, these observations are consistent with the presence of anion exchange (AE) proteins in the basolateral membrane. Similar AE transport processes have been identified in several epithelial and nonepithelial tissues, including the small and large intestine (3, 16, 20, 29, 33). The role(s) of this anion exchange is not known, but this Cl-anion exchange could be responsible for Cl movement across the basolateral membrane, either as a component of overall Cl absorption or in the regulation of pHi.

The studies of pHi that are presented in Figs. 11 and 12 and Table 1 are important in that they also demonstrate the presence of a Cl-HCO₃ exchange in basolateral membranes in distal colon and suggest that Cl-HCO₃ exchange may be critical in the regulation of pHi. An increase in pHi was noted whenever bath Cl was removed (Fig. 11 and Table 1), an observation that is consistent with the presence of a Cl-anion exchange in the basolateral membrane. The demonstration that the intracellular alkalinization that occurred after the removal of Cl was inhibited by DIDS is also compatible with the presence of a Cl-anion exchange.

The BLMV used in these studies were prepared by a standard method but with one very important change, the inclusion of protease inhibitors. These studies also established that BLMV prepared in the presence of serine-type protease inhibitors exhibited both Cl-HCO₃ exchange activity and intact AE2 protein, whereas only minimal Cl-HCO₃ exchange and AE2 protein expression were present in BLMV prepared without any protease inhibitors (Figs. 1 and 2A). The addition of protease inhibitors to the method for BLMV preparation was based on the recent report in which a Cl-anion exchange isoform (AE2) protein was identified in several epithelial and nonepithelial tissues (3, 16, 20, 29, 33). The role(s) of this anion exchange is not known, but this Cl-anion exchange could be responsible for Cl movement across the basolateral membrane, either as a component of overall Cl absorption or in the regulation of pHi.

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Table 1. Effect of Cl removal on intracellular pH

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Steady-State pH&lt;sub&gt;i&lt;/sub&gt;</th>
<th>dpH&lt;sub&gt;i&lt;/sub&gt;</th>
<th>dpH&lt;sub&gt;i&lt;/sub&gt;/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominally HCO₃⁻-free buffered (n = 6)</td>
<td>7.48 ± 0.03</td>
<td>7.59 ± 0.02*</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>HCO₃⁻ buffered (n = 6)</td>
<td>7.24 ± 0.03</td>
<td>7.52 ± 0.01*</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>**P&lt;0.01 NS</td>
<td>&lt;0.01 NS</td>
<td>&lt;0.01 NS</td>
<td>0 &lt; 0.05</td>
</tr>
</tbody>
</table>

Intracellular pH (pHi) was measured using fluorescent dye BCECF both under basal conditions and after Cl removal (i.e., Cl-free conditions), as described in MATERIALS AND METHODS. The pH<sub>i</sub> difference between basal and Cl-free conditions represents dpH<sub>i</sub>, whereas the dpH<sub>i</sub>/dt represents the rate in pH<sub>i</sub> change/min. Results presented are means ± SE of pH<sub>i</sub> measured in 6 crypts from 3 different distal colons. Student t-test was used to determine the statistical significance. *Basal steady-state pH<sub>i</sub> comparison between before and after Cl removal (P < 0.01). **Comparison between nominally HCO₃⁻-free buffered and HCO₃⁻-buffered solutions. NS, not significant.
to different protocols used for BLMV preparation or to the presence of both AE2 and AE3 isoform-specific proteins in human colonic basolateral membranes (33). Human colonic BLMV in this prior study were prepared by the Percoll gradient centrifugation (33), whereas rat BLMV in the present experiments were prepared by sucrose gradient centrifugation. It is possible that AE2 protein is not inactivated by proteases during Percoll gradient centrifugation (33). Alternatively, AE3 isoform may also encode Cl-anion exchange and may not be inactivated by proteases. Thus the inactivation of Cl-anion exchange by proteases is selective in that NHE1 protein (Fig. 2B, lane 1), Na/H exchange, Na-HCO3 cotransport, and Na,K-ATPase have been identified in colonic BLMV prepared in the absence of protease inhibitors in rat distal colon (13, 26, 27).

Previous studies have characterized Cl-anion exchange in apical membranes of rat distal colon (12, 20, 22). Comparison of the prior experiments with these present observations indicates that apical membrane and basolateral membrane Cl-anion exchanges are distinct transport processes. In AMV, the Km for Cl of Cl-OH exchange is greater than that for Cl-HCO3 exchange; in contrast, the Km for Cl-HCO3 exchange in BLMV is almost similar to that of Cl-OH exchange. These experimental findings indicate that neither BLMV nor AMV are contaminated by AMV or BLMV, respectively. This conclusion is also supported by the observation that in BLMV but not AMV Cl-Cl exchange is activated in the presence of NH4 (Fig. 10).

The present experiments provide evidence that a DIDS-sensitive mechanism is responsible for base loading. Although these observations are most consistent with a Cl-anion exchange, a DIDS-sensitive NaHCO3 cotransport mechanism has previously been identified in the basolateral membrane of rat distal colon (26). Several observations, however, make it unlikely that this Cl-dependent alkalinization is related to NaHCO3 cotransport. First, Cl-dependent alkalinization was observed either in the presence or absence of HCO3 (Fig. 11). Second, the removal of bath Na did not prevent Cl-dependent alkalinization (unpublished observation). Third, NaHCO3 cotransport does not require Cl (26). Even though NaHCO3 cotransport may also regulate pHi (8, 21, 31), NaHCO3 cotransport is not responsible for the Cl-dependent changes in pHi observed in these present experiments.

The experiments of 36Cl uptake described in these experiments provide substantial evidence that Cl-HCO3 and Cl-OH exchanges are present in colonic BLMV (Figs. 3–5). This conclusion is supported by the pHi studies in which a Cl-anion exchange mechanism was identified that functioned in both the presence and absence of HCO3. Although these observations, however, do not distinguish between the presence of specific Cl-OH and Cl-HCO3 exchanges and a single Cl-anion exchange with affinity for both OH and HCO3, we favor the latter possibility because the Km for Cl and Kf for DIDS for both Cl-HCO3 exchange and Cl-OH exchange are almost identical (Figs. 7 and 8). In addition, Cl-OH and Cl-HCO3 have similar distribution in BLM of surface and crypt cells.

This study also suggests that basolateral Cl-HCO3 exchange might be encoded by AE2 isoforms. Recent molecular studies have identified several anion exchange isoforms that include AE1, AE2, AE3, PAT1, and pendrin from various tissues (3, 19, 23, 30). Whereas PAT1 and pendrin proteins have been shown to express in apical membranes of gastric tubular membranes and apical membranes of proximal tubules, respectively (15, 19), only AE1, AE2, and DRA have been identified to be present in colonic epithelial cells (23). AE1 has been proposed to encode apical Cl-HCO3 exchange in surface cells of rat distal colon, because apical Cl-HCO3 exchange activities and AE1-specific mRNA expression that are downregulated during dietary Na depletion (hyperaldosterone) are present only in surface but not in crypt cells (23). In contrast, DRA has been identified in apical membranes of both surface and crypt cells of rat distal colon (23), whereas DRA and both AE2-specific mRNAs and proteins (that are localized to basolateral membrane) that are not altered by dietary Na depletion are expressed in both surface and crypt cells (2). In addition, NH4 that enhances AE2 function in oocytes (14) stimulates Cl-Cl exchange in BLMV (Fig. 10). Thus, it is likely that Cl-anion exchange that these present experiments have characterized is encoded by AE2.

REFERENCES


