Role of Cl channels in Cl-dependent Na/H exchange

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Rajendran, Vazhaiikkurichi M., John Geibel, and Henry J. Binder. Role of Cl channels in Cl-dependent Na/H exchange. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G73–G78, 1999.—A novel Na/H exchange activity that requires Cl was recently identified in the apical membrane of crypt cells of the rat distal colon. This study explores the nature of the coupling of Cl and Na/H exchange. A concentration of 100 µM 5-nitro-2-(3-phenylpropylamino)benzoic acid, a Cl channel blocker, inhibited the Cl dependence of both proton gradient-driven $^{22}$Na uptake from crypt cell apical membrane vesicles and Na-dependent intracellular pH recovery from an acid load during microperfusion of the crypt lumen. Cl-dependent proton gradient-driven $^{22}$Na uptake was inhibited by 94% by 500 µM DIDS but only by 1% by 10 µM DIDS, an anion exchange inhibitor at low concentrations but a Cl channel blocker at high concentrations. In addition, a polyclonal antibody to the cystic fibrosis transmembrane conductance regulator (CFTR) inhibited Cl-dependent proton gradient-driven $^{22}$Na uptake by 38%. These results indicate that the Cl dependence of Na/H exchange in the apical Cl channel membrane involves a Cl channel and not a Cl/anion exchange and permit the speculation that this Cl channel activity represents both CFTR and the outward rectifying Cl conductance.

SODIUM AND CHLORIDE absorptive and secretory processes have been extensively studied in several epithelia, including the small and large intestine. More than one mechanism can account for the close interrelationship between Na and Cl transport, including coupled cotransport (e.g., Na-K-2Cl cotransport), electrical or pH-coupled Na and Cl transporters (e.g., Na/H and Cl/HCO$_3^-$ exchanges), and Na-dependent Cl/HCO$_3^-$ exchange (4, 9, 16, 23).

An additional mechanism of Cl and Na interdependence was recently described in the rat distal colon (19). Cl-dependent Na/H exchange was identified in the apical membrane of colonic crypt cells using studies of $^{22}$Na uptake by isolated apical membrane vesicles (AMV) and intracellular pH (pH$_i$) determinations in response to an acid load (19). Whereas minimal Cl-dependent Na/H exchange activity was noted in apical membranes of surface cells, Na/H exchange in apical membranes of crypt cells was almost exclusively Cl dependent (19). Distinct differences exist in the characteristics of Cl-dependent and Na-dependent Na/H exchanges in the rat distal colon in that Cl-dependent Na/H exchange in crypt cells is relatively amiloride resistant compared with the amiloride sensitivity of Cl-independent Na/H exchange in surface cells (19).

The nature of the Cl dependence of Na/H exchange in the apical membrane of crypt epithelial cells is not known. It has not been established whether the Cl dependence of Na/H exchange represents 1) the coupling of a Cl transport protein with a known Na/H exchange isoform or with a previously unidentified Na/H exchange isoform or 2) the presence of a novel transport protein with both Cl transport and Na/H exchange activities. Because several mechanisms of Cl transport are present in the mammalian colon, including crypt epithelial cells, the Cl transport mechanism linked to Na/H exchange in the colonic crypt could represent either a Cl/anion exchange or a Cl channel (e.g., cystic fibrosis transmembrane conductance regulator (CFTR) or outward rectifying Cl conductance (ORCC)). Presently, available information does not distinguish between these possibilities.

This study was designed to determine whether Cl-dependent Na/H exchange was associated with a Cl/anion exchange or a Cl channel by determining the effect of various inhibitors of both anion exchange and Cl channels on the Cl dependence of both proton gradient-driven $^{22}$Na uptake by AMV and Na-dependent pH$_i$ recovery to an acid load in microperfusion experiments. The results of this study indicate that the Cl dependence of Na/H exchange in the apical membrane of colonic crypt cells represents a Cl channel, not a Cl/anion exchange, that is linked to Na/H exchange function.

METHODS

AMV Preparation

AMV were isolated from distal colonic crypt cells that were isolated by the diencephalation and sequential separation of Lomax et al. (12) as previously described (19). In brief, segments of distal colon were removed from male Sprague-Dawley rats (200–250 g) and washed in ice-cold buffer medium that contained (in mM) 96 NaCl, 27 sodium citrate, 0.8 KH$_2$PO$_4$, 5.6 Na$_2$HPO$_4$, 1.5 glucose, and 0.5 dithiothreitol (buffer A, pH 7.4). After a 15-min incubation in buffer A at 37°C, everted colonic segments were transferred to a prewarmed colonic solution buffer that contained (in mM) 112 NaCl, 5 KCl, 30 EDTA, 29 HEPES, and 0.5 dithiothreitol (buffer B, pH 7.1). Surface-to-crypt cell fractions were sequentially isolated by shaking and incubation in buffer B at 37°C (6 complete exchanges of 10 min each). Crypt cells were sedimented by centrifugation (Beckman GS-6KR; GH-3.8 rotor) at 500 g for 2 min. Fractions 5 and 6 were used to isolate crypt cells and then to isolate AMV with the use of the method of Stieger et al. (26) as previously described (20). Purity of the AMV preparation was periodically verified by the 10- to 12-fold enrichment of ouabain-sensitive H-K ATPase (21). Uptake studies were performed by rapid filtration techniques.

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that have been previously described (20). Protein was mea-
sured by the method of Lowry et al. (13).

All experiments were performed with at least three differ-
ent membrane preparations. Data presented are means of
triplicate assays of a typical experiment. Standard errors are
not provided when they are <5% of the mean.

pH Measurements

All pH experiments were performed on isolated perfused
colonic crypts. The methods for isolation and perfusion of
colonic crypts have previously been described in detail (21,
24). Briefly, individual colonic crypts were harvested from
distal colon that was isolated and hand dissected at 4°C in
HEPES-based Ringer solution. After dissection, individual
crypts were transferred to the stage of an inverted microscope
and attached to a series of concentric glass pipettes, cannu-
lated, and perfused in vitro. After perfusion was established,
the crypt was warmed to 37°C in a perfusion chamber that
completely exchanged the volume four to eight times per
second. The crypt was loaded with the pH-sensitive 2-
carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) (10
µM). After dye uptake by crypt cells, intracellular esterases
cleaved the dye molecules, leaving the fluorescent, cell-
impermeant pH marker BCECF trapped within the cytosol.
All pH measurements were carried out using a dual-
excitation monochrometer system. Data were collected using
an intensified charge-coupled device system. To record pHi in
vitro, fluorescent data were calibrated in vitro for the pH experi-
ments and were converted to pHi values using the nigericin
calibration technique (25). Nigericin (1 µg/ml) was placed into
the crypt axis. The intensity information as well as the
images from each excitation wavelength were recorded to the
hard disk of a personal computer and stored to either an
optical hard drive or to the computer disk. This method of
data storage permitted subsequent analysis of the data
following the experiment and examination of additional areas
of interest following completion of the experiments. The
fluorescent data were calibrated in vitro for the pH experi-
ments and were converted to pH values using the nigericin
measurement.

Figure 1 shows that the presence of Cl, but not a Cl concentra-
tion gradient, stimulated proton gradient-driven 22Na uptake. The presence of 5 mM Cl stimulated 58% of maximal 22Na uptake, whereas 22Na uptake was not stimulated significantly in the presence of 3 mM Cl (data not shown). Maximal 22Na uptake was observed in the presence of 25 mM Cl, which was not signifi-
cantly enhanced any further by increasing Cl concentrations up to 100 mM. These results did not establish a preferential vesicular side for Cl action. This failure to identify a preferential vesicular side for
Cl stimulation of Na/H exchange is more consistent with the movement of Cl through a Cl channel than via a
Cl/anion exchange.

Effect of Cl channel blockers and anion exchange
inhibitors on Cl-dependent Na/H exchange. To deter-
mine the possible role of a Cl channel and/or Cl/anion
exchange on Cl-dependent Na/H exchange, the effects
of 5-nitro-2-(3-phenylpropylamino)benzoxoic acid (NPPB),
a Cl channel blocker (8), and DIDS, an anion exchange
inhibitor at low concentration but a Cl channel blocker
at high concentration (11, 18), were examined on Cl-
dependent Na/H exchange activity. As shown in Fig.
2A, Cl-dependent Na/H exchange was inhibited 20, 96,
and 100% by 10, 100, and 500 µM NPPB, respectively.
Cl-dependent Na/H exchange was inhibited 8, 8, and
94% by 10, 100, and 500 µM DIDS, respectively (Fig.

Solutions

The composition of the standard Na-containing, Cl-
containing perfusion solution was (in mM) 125 NaCl, 5 KCl,
1.2 CaCl2, 1.2 MgSO4, 2 Na2HPO4, 10.2 glucose, and 32.2
HEPES. In the Na-free solutions, Na was replaced with
equivalent amounts of N-methyl-D-glucamine; in the Cl-free
solutions, Cl was replaced by equivalent amounts of cyclo-
estra. Solutions and Ca was increased to 4.0 mM. The solutions
were adjusted to a final osmolality of 300–310 mosmol/kgH2O. pH
was adjusted to 7.4 at 37°C.

RESULTS

36Cl Uptake Studies

Effect of Cl concentration on Cl-dependent Na/H
exchange. Initial experiments were performed both to
establish the optimal Cl concentration required to
stimulate Cl-dependent Na/H exchange and to identify
the sidedness of Cl interaction (i.e., an extravesicular
vs. an intravesicular site) with Cl-dependent Na/H
exchange. In this series of studies, Cl-dependent proton
gradient-driven 22Na uptake was examined in the
presence of different Cl concentration gradients: in-
ward (Clout > Clin; Fig. 1, right), outward (Clout < Clin,
Fig. 1, middle), and no (Clout = Clin; Fig. 1, left) Cl
gradients. Figure 1 shows that the presence of Cl, but
not a Cl concentration gradient, stimulated proton
gradient-driven 22Na uptake. The presence of 5 mM Cl
stimulated 58% of maximal 22Na uptake, whereas 22Na
uptake was not stimulated significantly in the presence
of 3 mM Cl (data not shown). Maximal 22Na uptake was
observed in the presence of 25 mM Cl, which was not
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Cl-dependent Na/H exchange was inhibited 8, 8, and
94% by 10, 100, and 500 µM DIDS, respectively (Fig.

Fig. 1. Effect of Cl concentrations on Cl-dependent Na/H exchange. Left and middle: crypt apical membrane vesicles (AMV) were pre-
loaded with 50 mM MES- Tris (pH 5.5) containing 0.1 mM MgSO4;
and various concentrations of KC1 (0, 5, 10, 25, 50, and 100 mM) and
potassium gluconate (150, 145, 140, 125, 100, and 50 mM). Uptake
was performed by diluting the AMV in medium containing 50 mM
HEPES-Tris (pH 7.5), 0.1 mM MgSO4, 0.1 mM sodium gluconate,
trace amounts of 22Na, and varying concentrations of KCl and
potassium gluconate identical to that of AMV (Clout = Clin; where
Clout and Clin are the outward and inward Cl concentration gradients,
respectively) or 150 mM potassium gluconate (Clout < Clin). Right:
AMV were preloaded with 50 mM MES-Tris (pH 5.5) containing 0.1
mM MgSO4 and 150 mM potassium gluconate. Uptake was per-
duced by diluting the AMV in Tris medium containing 50 mM
HEPES-Tris (pH 7.5), 0.1 mM MgSO4, 0.1 mM sodium gluconate,
trace amounts of 22Na, and varying concentrations of KCl (0, 5, 10, 25, 50,
and 100 mM) and potassium gluconate (150, 145, 140, 125, 100, and
50 mM). Absolute proton gradient-driven uptake was calculated by
subtracting uptake in absence of pH gradient from that in presence of
pH gradient.
Inhibition of Cl-dependent Na/H exchange function by NPPB and DIDS at high but not low concentrations indicates that an apical Cl channel, not a Cl/anion exchange, is responsible for the Cl dependence of Na/H exchange.

These results with DIDS and NPPB are consistent with the presence of apical membrane Cl channels, e.g., CFTR and/or ORCC. To distinguish between these two possibilities, the effect of a polyclonal antibody to CFTR (3) on Cl-dependent Na/H exchange activity was next examined. Both 1 and 2 µg CFTR antibody reduced Cl-dependent proton gradient-driven 22Na uptake by 38% (Fig. 3). Parallel studies performed with irrelevant antibody did not alter Cl-dependent Na/H exchange activity (data not shown). In addition, CFTR antibody did not affect proton gradient-driven 22Na uptake by AMV isolated from surface cells (data not shown). Because CFTR antibody only partially altered Cl-dependent proton gradient-driven 22Na uptake, these results permit the speculation that the Cl channel coupled to Na/H exchange in colonic crypt cell AMV represents both CFTR and ORCC.

Effect of Cl channel blocker and anion exchange inhibitor on Cl channel. Studies were designed to examine the presence of Cl channel activity in AMV prepared from colonic crypt cells. The effects of an intravesicular-positive potential, generated by an inward K gradient and valinomycin, on 36Cl uptake were examined to establish whether functional Cl channels are present in these apical membranes. As shown in Fig. 4, 36Cl uptake was substantially stimulated by the imposition of an intravesicular-positive potential. In addition, stimulation of 36Cl uptake by an intravesicular-positive potential was inhibited 44, 99, and 97% by 10, 100, and 500 µM NPPB, respectively (Fig. 4A). 36Cl uptake was also inhibited 5, 18, and 68% by 10, 100, and 500 µM DIDS, respectively (Fig. 4B). Because the dose responses of both DIDS and NPPB on Cl-dependent proton gradient-driven 22Na uptake and potential-driven 36Cl uptake were similar,
these results suggest that a Cl channel is linked to Cl-dependent Na/H exchange.

pHi Regulation Studies

Effect of a Cl channel blocker on pHi regulation. The effect of the Cl channel blocker, NPPB, was examined to establish whether the Cl dependence of Na-dependent pHi regulation in isolated perfused crypt cells represents a Cl channel. When both Na and Cl were removed from the bath and lumen, pHi was reduced and did not return to baseline pHi (Fig. 5A). Similar to our previous studies (19), addition of luminal Na, in the presence of Cl, increased pHi, pHi recovered to resting pHi values only when both Cl and Na were returned to the luminal perfusate. If Na was returned to the luminal perfusate before the addition of Cl to the lumen, pHi recovery did not occur (Fig. 5B). To confirm that the Cl dependence was a result of an apical Cl channel, the Cl channel inhibitor, NPPB (10 µM), was added to the luminal perfusate following the bilateral removal of Na and Cl. When Na and Cl were returned to the apical perfusate, a 96% inhibition of the Cl- and Na-dependent pHi recovery was observed (Fig. 5C). Addition of NPPB to the lumen in an Na-containing perfusate showed a small but reproducible decrease (0.03 ± 0.01 pH units; n = 25) in pHi. In similar studies the apical removal of Cl in the presence of luminal Na resulted in a modest intracellular acidification (0.04 ± 0.01 pH units; n = 25). These results suggest that a Cl channel is coupled to an apical membrane transport protein with Na/H exchange function and that inhibition of this Cl channel prevents activation of Cl-dependent Na/H exchange activity.

DISCUSSION

Colonic epithelial cells arise from progenitor cells in the base of the crypt and migrate along the crypt to become mature surface epithelial cells that, over a 3- to 6-day period, are sloughed into the colonic lumen. Despite this close relationship of crypt and surface cells, a separation of surface and crypt cell epithelial function has been a long-established concept of intestinal epithelial cell fluid and electrolyte transport (1). Until recently, absorptive processes have been generally considered to be localized solely to surface epithelial cells and secretory processes to crypt epithelial cells. Recent studies, however, provide abundant evidence that absorptive and secretory processes are present both in surface and in crypt epithelial cells (10, 24).

Electroneutral Na-Cl absorption is the primary transport mechanism responsible for Na absorption in the rat distal colon and is the result of the pH coupling of Na/H exchange and Cl/HCO3 exchanges (1, 9). To date, five distinct Na/H exchange isoforms have been cloned and sequenced (15), and recent studies established that the Na/H exchange isoform NHE3 message and protein are located exclusively in surface and not in crypt epithelial cells of the rat distal colon (2). Because studies of fluid absorption in isolated colonic crypts demonstrated Na-dependent fluid absorption (24), it is unlikely that the NHE3 isoform is the mechanism of Na-dependent fluid absorption in isolated colonic crypt cells. As a result, the identity of the Na transport process responsible for the Na-dependent fluid absorptive process in the crypt had to differ from that in surface cells. Subsequent studies designed to establish the nature of Na uptake across the apical membrane of crypt epithelial cells in the rat distal colon established the presence of a novel Na/H exchange, Cl-dependent Na/H exchange, in the luminal membrane of crypt cells but not in surface epithelial cells of the distal colon (19). The present studies were designed to characterize the nature of the Cl dependence of Na/H exchange in

![Fig. 5. Effect of NPPB on the Cl dependence of Na-dependent intracellular pH (pHi) recovery to an acid load during colonic crypt microperfusion. pHi was measured in individual crypt cells with the use of 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, as previously described in studies of pHi regulation in colonic crypts (19, 21). Initially, the crypt was exposed to a Ringer solution with 125 mM Na and 110 mM Cl in both the lumen and bath. The cell was then exposed to an Na-free and Cl-free perfusion solution, which resulted in a rapid decrease in pHi. A: readdition of Na and Cl to the luminal perfusate restored pHi to the initial baseline levels. B: when Na was added to the perfusate in the absence of Cl, pHi recovery did not occur. C: addition of 10 µM NPPB to the luminal perfusate before the readdition of Na and Cl significantly inhibited pHi recovery to baseline values. These tracings are representative of studies performed in 80 cells from 5 animals.
the colonic crypt. Because both Cl/anion exchange and
Cl channels are present in the rat distal colon (1), the
experimental strategy was to determine the effect of
inhibitors of Cl/anion exchange and Cl channels on
Cl-dependent Na/H exchange function. Our results
suggest that the Cl dependence of Na/H exchange in the
apical membrane in the colonic crypt represents a Cl
channel, not a Cl/anion exchange. This conclusion is
based primarily on the effects of NPPB, a Cl channel
blocker, and DIDS, an inhibitor of Cl/anion exchanges
at low concentrations but of Cl channels at high concen-
trations (8, 11). First, Cl-dependent proton gradient-
driven $^{22}$Na uptake was not significantly inhibited by
10 µM DIDS but was inhibited by 500 µM DIDS (Fig. 2B).
Second, 100 µM NPPB inhibited Cl-dependent proton gradient-driven $^{22}$Na uptake (Fig. 2A). Third, $^{36}$Cl uptake by crypt cell AMV induced by a positive
electrical potential was also inhibited by 100 µM NPPB
(Fig. 4). Finally, fourth, 10 µM NPPB also inhibited the
Cl-dependent component of Na-dependent recovery of
pH, to an acid load (Fig. 5C). These observations provide compelling evidence that a Cl channel is respon-
sible for Cl-dependent Na/H exchange in the apical
membrane of the colonic crypt cell.

At least two different Cl channels are present in
apical membranes of the epithelial cells: CFTR and
ORCC (6, 7). Although these two Cl channels are best
characterized by patch-clamp studies, differences in
the specificity of some Cl channel blockers can provide
information suggesting the nature of apical Cl chan-
nels. NPPB inhibits both CFTR and ORCC (6, 7), whereas
DIDS, at high concentrations, primarily inhib-
its ORCC but may also inhibit CFTR (6, 11). A partial
role for CFTR in CI-dependent Na/H exchange is con-
firmed by the 38% inhibition of Cl-dependent Na/H
exchange by a CFTR polyclonal antibody (Fig. 3) (3).
Taken together, these observations indicate that two
distinct apical Cl channels, CFTR and ORCC, are
linked to Na/H exchange in the colonic crypt, as Cl-
dependent proton gradient-driven $^{22}$Na uptake was almost completely inhibited by 500 µM DIDS and 100
µM NPPB and partially inhibited by a CFTR antibody.

Cl-dependent Na/H exchange is most likely the only
Na/H exchange in the apical membrane of crypt epithe-
llial cells, as neither proton gradient-driven $^{22}$Na uptake
nor Na-dependent pH, recovery from an acid load was
detected in crypt cells in the absence of Cl. Although
Cl-independent Na/H exchange is readily identified in
AMV prepared from surface cells (17, 19, 20), it is
uncertain whether Cl-dependent Na/H exchange is also
present in surface cell apical membranes, despite the
demonstration that a relatively small component of
proton gradient-driven $^{22}$Na uptake in AMV prepared
from surface cells was Cl dependent (19). This latter
phenomenon could represent either contamination of
the surface cell AMV preparation with crypt cell apical
membranes or the presence of Cl-dependent Na/H
exchange in surface cell apical membranes (19).

Sodium and chloride movement in the intestine is the
primary driving force for fluid absorption and secretion
in both small and large intestine, and several different
mechanisms have been identified to explain the inter-
relationship of these two ions. Parallel absorption of
sodium and chloride can be explained by both cotrans-
port processes and the coupling of parallel and indepen-
dent ion exchanges. Thus Na-K-2Cl cotransport is
responsible for Na and Cl movement across the basolat-
eral membrane and is critical for active Cl secretion
(16). Although Na-Cl cotransport is present in nonepi-
thelial and other epithelial cells (4), its presence has
not been unequivocally established in the intestinal
traci. In contrast, parallel Na/H and Cl/HCO$_3^-$
exchanges that are coupled by pH can also explain
electroneutral Na-Cl absorption in the small and large
intestine (9). Na and Cl absorption can also be coupled
by an electrical gradient. Thus both electrogenic Na
absorption in the distal colon via apical amiloridesen-
sitive Na channels and glucose-stimulated Na ab-
sorption mediated by SGLT1 in the small intestine will
result in an increase in lumen-negative electrical poten-
tial difference, which acts as the driving force for transepithelial Cl movement.

In the absence of parallel transepithelial movement
of Na and Cl, other mechanisms of Na and Cl depen-
dence of Cl and Na transport, respectively, have also
been described. Na-dependent Cl/HCO$_3^-$
exchange, which is distinct from Na-independent Cl/HCO$_3^-$
regulates pH, at the basolateral membrane (23). Previous ex-
periments on transepithelial $^{36}$Cl fluxes in the rat proximal
colon established that electroneutral Na/H exchange in
normal animals is Cl dependent in the absence of net Cl
absorption (5). Because studies of proton gradient-
driven $^{22}$Na uptake by AMV prepared from proximal
colon did not indicate the presence of Cl-dependent
Na/H exchange (17), the mechanism of Cl-dependent
Na/H exchange in the apical membrane of the rat
proximal colo is not yet available.

In conclusion, the present studies establish a Cl
dependence of both proton gradient-driven $^{22}$Na uptake
and Na-dependent pH, recovery to an acid load that is
1) largely inhibited by NPPB, a Cl channel blocker, 2)
largely inhibited by DIDS only at high concentrations
at which it is also a Cl channel blocker, and 3) partially
inhibited by a CFTR polyclonal antibody. This pattern
of inhibition of Cl-dependent Na/H exchange is consis-
tent with the possibility that apical membrane Cl
channels are coupled to either a known or to a previ-
ously unidentified Na/H exchange. It is not known
whether these Cl channels represent an integral compo-
nent of a transport protein with both Cl channel and
Na/H exchange activities or the close coupling of a Cl
channel with either a previously identified Na/H
exchange isofor or a novel and as yet unidentified Na/H
exchange isofor.

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1 Both Cl/OH and Cl/HCO$_3^-$ exchanges are present on apical membranes of surface cells of rat distal colon (18). Preliminary studies have established that Cl/HCO$_3^-$ exchange is not present on apical membranes of crypt cells of rat distal colon (18a). Cl/OH exchange is present in both surface and crypt cells.
Affinity-purified CFTR\textsubscript{502–511} polyclonal antipeptide antibody and irrelevant antibody were kindly provided by Drs. John Dedman and Marcia Kneisel, University of Cincinnati.

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