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Regulation of Intestinal Epithelial Brush Border Na^+/H^+ Exchanger Isoforms, NHE2 and NHE3, in C2bbe Cells

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Abstract. Until recently, studies to characterize the intestinal epithelial Na^+/H^+ exchangers had to be done in nonepithelial, mutated fibroblasts. In these cells, detection of any Na^+/H^+ exchange activity requires prior acid loading. Furthermore, most of these experiments used intracellular pH changes to measure NHE activity. Because changes in pH_i only approximate Na^+/H^+ exchange activity, and may be confounded by alterations in buffering capacity and/or non-NHE contributions to pH regulation, we have used ^{22}[Na] unidirectional apical to cell uptake to measure activities specific to NHE2 or NHE3. Furthermore, we performed these measurements under basal, *nonacid-stimulated* conditions to avoid bias from this nonphysiological experimental precondition. Both brush border NHEs, when expressed in the well-differentiated, intestinal villuslike Caco-2 subclone, C2bbe (C2), localize to the C2 apical domain and are regulated by second messengers in the same way they are regulated *in vivo.* Increases in intracellular calcium and cAMP inhibit both isoforms, while phorbol ester affects only NHE3. NHE2 inhibition by cAMP and Ca^{++} involves changes to both K_{Na} and V_{max}. In contrast, the same two second messengers inhibit NHE3 by a decrease in V_{max} exclusively. Phorbol ester activation of protein kinase C alters both V_{max} and K_{Na} of NHE3, suggesting a multilevel regulatory mechanism. We conclude that NHE2 and NHE3, in epithelial cells, are basally active and are differentially regulated by signal transduction pathways.

Key words: Intestinal absorption — C2bbe — Protein kinase A — Protein kinase C — Calcium signaling — Thapsigargin

Introduction

A major mechanism for the absorption of sodium across the brush border (apical) membrane of the intestine occurs by Na^+/H^+ exchange (NHE) mediated by specific NHE transport proteins. Of the six isoforms cloned to date, NHE2 and NHE3 have been localized to the brush border membrane of intestinal epithelial cells (Hoogerwerf et al., 1996; Bookstein et al., 1997). However, functional characterization of these isoforms in intestinal epithelial cells has been hampered by our inability to reliably differentiate their activities in the same cell and, therefore, has been performed most frequently using nonepithelial NHE-negative mutant fibroblasts (Levine et al., 1993; Orlowski, 1993; Kapus et al., 1994; Kurashima et al., 1998). Thus, disparities observed when compared with the regulation by the same second messengers of *in vivo* intestinal Na absorption could be a result of the lack of tissue and/or cell-specific factors.

An appropriate choice for a cell line to study these brush border exchangers would be intestinal in character, homogeneous and well differentiated in culture. Both NHE2 and NHE3 are found in villus brush border in the

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Abbreviations: C2bbe cells (C2), amiloride analogue 5-(N,N-dimethyl)amiloride (DMA), (4-isopropyl-3-methanesulfonyl-benzoyl) guanidine methanesulfonate (HOE-642, HOE), phorbol 12-myristate 13-acetate (PMA), 8-chlorophenylthio-cAMP (CPT-cAMP), electroneutral sodium/hydrogen exchanger protein (NHE), ubiquitous "housekeeping" isoform one (NHE1), epithelial specific isoforms two (NHE2) and three (NHE3).

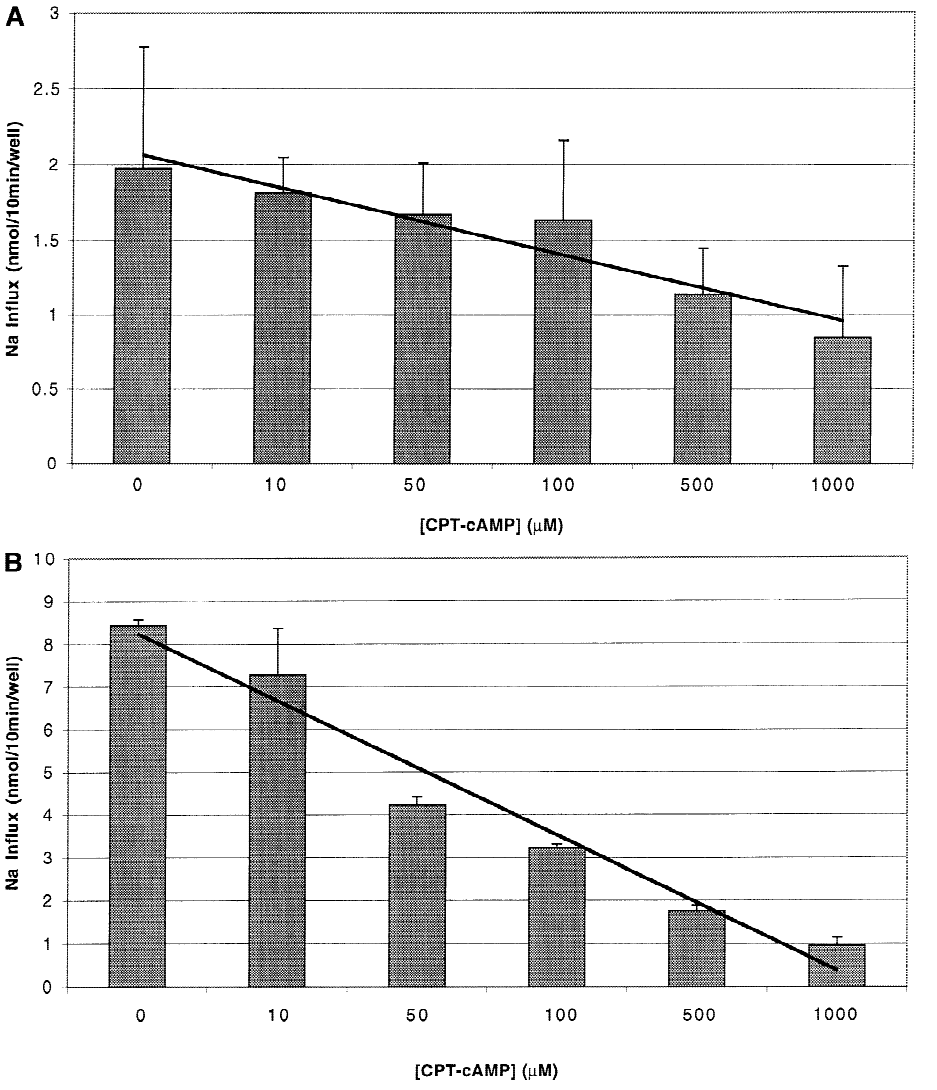

Fig. 1. NHE2 and NHE3 Dose Responses to CPT-cAMP and Thapsigargin. These and subsequent experiments were done without prior acid loading. ^{22}[Na] unidirectional influx was measured from the apical domain only. HOE inhibited influx defines activity of NHE2. DMA inhibits both NHE3 and NHE2. Therefore, NHE3 activity is HOE-inhibited influx minus DMA-inhibited influx. Panels *A* and *B* demonstrate the dose response to CPT-cAMP by NHE2 and NHE3, respectively. A trend line has been drawn across each set of bars. Panels *C* (NHE2) and *D* (NHE3) show responses to thapsigargin. All experiments were done in the presence of an extracellular Na concentration of 20 mM.

small intestine and surface absorptive cells in the colon (Bookstein et al., 1993; Hoogerwerf et al., 1996; Bookstein et al., 1997). Therefore, it is reasonable to expect that intestinal-specific and differentiation-dependent proteins may be required to regulate these exchangers correctly. The C2bbe (C2) cell line is clonally derived from the Caco-2 human colonic adenocarcinoma strain (Peterson & Mooseker, 1992) and has the advantage of a homogenous cell population which differentiates at confluence, developing an extensive brush border and expressing enzymes characteristic of the mature enterocyte. Prior studies have demonstrated that these cells express an endogenous brush border NHE2 and, when transfected with NHE3 cDNA, correctly localize NHE3 protein to the brush border (McSwine et al., 1998). With the development of a novel benzoylguanidine derivative, HOE-642 (Weichert et al., 1997), we are also now able to reliably distinguish NHE2 activity from NHE3.

Activity, as well as regulation, of these brush border exchangers in polarized cells appears to differ in two important ways from nonpolarized fibroblasts (McSwine et al., 1998). First, there is a basal, nonacid loaded brush border sodium/hydrogen exchanger activity and, second, their activities respond to common second messengers just as has been observed in native intestinal brush borders.

In this study we have analyzed the kinetics of second messenger effects on the brush border NHEs to better

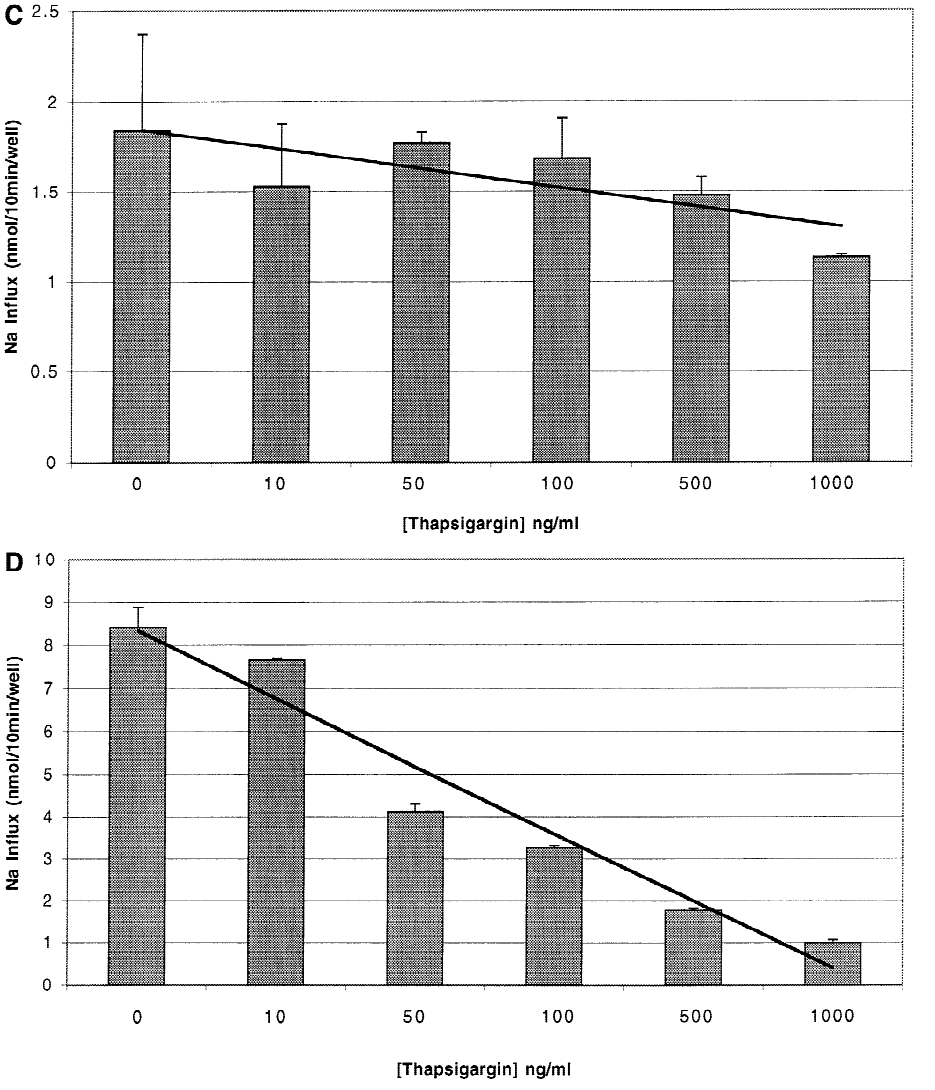

Fig. 1. *(Continued)*

understand their underlying regulatory mechanisms. We have used radioactive ^{22}[Na] unidirectional, apical-to-cell uptake to measure activities specific to NHE2 or NHE3. The present studies demonstrate that second messengers cAMP and intracellular calcium inhibit NHE3 by a decrease in V_{max} exclusively. Phorbol ester activation of protein kinase C, however, alters both V_{max} and K_{Na} of NHE3, suggesting a different regulatory mechanism. In contrast, cAMP and Ca^{++} inhibit NHE2 basal activity (not affected by phorbol ester) by mechanisms involving changes in affinity to Na (K_{Na}) and V_{max}.

Materials and Methods

Materials

High glucose DMEM (Dulbecco's modified Eagle's medium), penicillin, streptomycin and transferrin were purchased from (Life Technologies, Gibco/BRL). Fetal calf serum was supplied by Summit Biotech (Ft. Collins, CO). Tissue culture dishes and Transwells came from Corning/Costar (Corning, NY). ^{22}[Na]Cl (1,000 Ci/g) and ^{3}[H]-mannitol (22.5 Ci/mmol) was purchased from NEN. Amiloride analogue 5-(N,N-dimethyl)amiloride (DMA) was a gift of Dr. Thomas Kleyman at the University of Pennsylvania. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO). Alexis Biochemical (San Diego) supplied 8-chlorophenylthio-cAMP (CPT-cAMP) and thapsigargin. HOE-642 [(4-isopropyl-3-methanesulfonyl-benzoyl) guanidine methanesulfonate] was a gift from Drs. Wolfgang Scholz and Hans-Jochen Lang (HOECHST AG, Frankfurt/Main Germany).

NHE2 and NHE3 Transfection of C2 Cells

C2bbe (C2) cells, a generous gift from M. Mooseker (Yale University, CT) or acquired from ATCC (Bethesda, MD), were grown in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% fetal calf serum (Summit Biotech, Ft. Collins, CO), 10 μg/ml human

transferrin, 2 mM glutamine, 50 μg/ml streptomycin and 50U/ml penicillin. The C2bbe cell line expresses a brush border NHE2, but no NHE3 under culturing conditions used here. Because the endogenous NHE2 basal activity was very low in the absence of acid loading, we transfected rat NHE2 cDNA into the parent cell line. Rat NHE3 cDNA was also transfected into the C2 cells. Cells were transfected with the rat cDNA for NHE2 or NHE3 subcloned in the vector pCB6+ (J. Stinski, University of Iowa, IA), in which the exogenous DNA is under the control of the cytomegalovirus promoter. G418 resistant clones (C2N2 or C2N3) were screened for the specific rat mRNA and protein. Apical domain specific expression of the transfected NHE2 and NHE3 was verified by examining HOE-642- or dimethylamiloride (DMA)-sensitive apical and basolateral fluxes (McSwine et al., 1998). For each experiment, C2N2 or C2N3 cells were seeded at confluent density (10^7 cells/Transwell)* on rat-tail-collagen coated Transwells (Corning, 5 cm^2 with 0.4 μ pores), because, once plated on collagen matrix, the C2 cells will not spread to cover the surface. *Confluence was defined as the number of cells producing a dense monolayer over the same surface area on plastic. The cells were then cultivated for 14 days, a time when maturation was essentially complete (McSwine et al., 1998).

Measurement of basal activities of each NHE isoform by ^{22}[Na], unidirectional, apical to cell flux

Analyses of brush border fluxes in C2N2 or C2N3 transfectants were done using paired controls on the same day from the same passage to allow accurate kinetic comparisons. All experiments were performed *without* prior acid loading, which had been used previously to maximally activate Na/H exchange before introducing experimental conditions. Thus, these studies were conducted under conditions that more closely approximate the native physiological environment. ^{22}[Na] flux solution was introduced only to the apical compartment of the permeable supports. The total amount of Na taken up at each extracellular concentration was corrected for the specific activity of ^{22}[Na] in each solution; values are presented as mM Na taken up per Transwell over the 10-min flux. Sodium fluxes across the apical membrane were measured after a 10 min-pretreatment with CPT-cAMP (50 μM), thapsigargin (50 ng/ml), or PMA (50 nM), *without prior acid loading.* To initiate the flux, media were removed and Transwells were dipped in isotonic choline wash (150 mM choline-Cl, 15 mM HEPES, pH 7.4). Transwells were placed into dishes with nonradioactive flux solutions of varying sodium concentrations and an identical flux solution with 1 μCi/ml ^{22}[Na] was added to the apical side. Flux solution consisted of (in mM): 2 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 1 ouabain, 15 HEPES, pH 7.4, 1–90 NaCl and 150–139 choline-Cl to balance increased NaCl. To inhibit NHE2, 30 μM HOE-642 was included in the flux solution. We previously determined the dose-response curves for HOE-642 and DMA inhibition of NHE2 and NHE3 in C2N2 and C2N3 cells (McSwine et al., 1998). At the concentrations used here, HOE-642 inhibits NHE2, whereas DMA inhibits both NHE2 and NHE3. At 30 μM HOE-642, NHE2 was inhibited by ≈87% in C2N2 cells and NHE3 was inhibited by only ≈13% in C2N3 cells. In contrast, 500 μM DMA inhibits both NHE2 and NHE3 (and NHE1) in the C2 cells. Fluxes were 10 min in duration. NHE2 and NHE3 fluxes (in their respective cell line) were measured at 2, 5, 10, and 15 min for the lowest and highest Na concentrations used. In these cells and under conditions used in this study, Na uptake was linear at 10 min (*data not shown*). Fluxes were terminated by removal of flux solution and four rapid washes (less than 10 sec) in ice-cold HEPES buffered saline. Transwells were allowed to dry, and the membranes cut out and solubilized. Intracellular ^{22}[Na] was quantified by liquid scintillation spectrometry.

Table 1. Effect of CPT-cAMP on C2/N2 Kinetics

Control						
[Na] mM	**5**	**10**	**20**	**40**	**60**	**90**
Influx/10min/well						
Average	**3.45**	**7.50**	**14.25**	**21.44**	**24.36**	**25.70**
STDev	0.37	0.48	0.21	0.39	1.06	1.23
[Na]/flux	**1.45**	**1.33**	**1.40**	**1.87**	**2.46**	**3.50**
AveDev	0.27	0.39	0.16	0.32	0.75	0.99
CPT-cAMP (50 nM)						
Na mM	**5**	**10**	**20**	**40**	**60**	**90**
Influx/10min/well						
Average	**1.57**	**3.22**	**5.70**	**9.65**	**12.45**	**13.64**
STDev	0.09	0.06	0.43	0.59	0.73	0.81
[Na]/flux	**3.19**	**3.10**	**3.51**	**4.15**	**4.82**	**6.60**
AveDev	0.06	0.04	0.35	0.44	0.62	0.65

STDev = standard deviation based on sample.
AveDev = average of absolute deviations of data points from their mean.

Data Analysis

Results were analyzed by Hanes-Woolf plot ([Na] *vs.* [Na]/v) with least squares fit regression line analysis to calculate and compare maximal velocity (V_{max}) and sodium affinity constants (K_{Na}) under control and experimental conditions. This method of analysis provided more reliable and consistent kinetic constants than other basic velocity equations. The Hanes-Woolf equation can be represented as $[S]/v = (1/V_{max})[S] + K_m/V_{max}$. A plot of [S] *vs.* [S]/*v* is linear with a slope of $1/V_{max}$. When $[S]/v = 0$, the intercept on the [S] axis equals $-K_m$.

Results

Effect of Increased Intracellular cAMP on NHE2 and NHE3 Fluxes

Our previous study (McSwine et al., 1998) had indicated that an increase in intracellular cAMP significantly decreased activities of both isoforms. To define the kinetics of this phenomenon, we examined the effect of this second messenger on the activity of each isoform over a range of extracellular Na concentrations. Minimum second messenger concentrations exhibiting significant effect on one of the exchangers was used for all subsequent studies. Dose responses for CPT-cAMP and thapsigargin on both NHE2 and NHE3 are shown in Fig. 1. The effect of increasing intracellular cAMP ($[Na]_o$ *vs.* $[Na]_o$/flux) is presented in Tables 1 and 2 and is plotted in Fig. 2. Each panel in Fig. 2 indicates the specific apical uptake activity of NHE2 (*A*) or NHE3 (*B*) by Hanes-Woolf plot, without (control) and with the permeable cAMP analogue, 8-CPT-cAMP. At 50 μM, V_{max} for NHE2 was significantly *decreased* (24.4 ± 1.6 nmoles/10 min/Transwell *vs.* 39.4 ± 2.6 for control, $n = 6$, $P < 0.01$). NHE2 K_{Na} increased (68.2 ± 7.2 mM Na with cAMP *vs.*

Table 2. Effect of CPT-cAMP on C2/N3 Kinetics

Control						
[Na] mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10min/well						
Average	**1.02**	**1.94**	**3.52**	**6.67**	**11.17**	**13.97**
STDev	0.31	0.70	1.09	2.08	5.02	5.19
[Na]/flux	**1.03**	**1.10**	**1.49**	**1.58**	**1.99**	**3.08**
AveDev	0.65	0.28	0.33	0.35	0.63	0.81
CPT-cAMP (50 nM)						
Na mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10 min/well						
Average	**0.42**	**0.79**	**1.51**	**3.56**	**5.14**	**5.28**
STDev	0.15	0.32	0.48	0.23	0.73	1.83
[Na]/flux	**2.59**	**2.74**	**3.49**	**2.98**	**3.93**	**8.05**
AveDev	0.65	0.77	0.78	0.73	0.39	1.97

41.4 ± 2.0 mM Na for control, $n = 6$, $0.1 < P < 0.05$). For NHE3, V_{max} was also substantially *decreased* by cAMP (7.7 ± 1.7 with cAMP *vs.* 19.3 ± 1.6 for control, $n = 6$, $P < 0.01$) cells. However, there was no statistically significant change in NHE3 K_{Na} (17.4 ± 0.2 mM Na *vs.* 23.2 ± 0.8 in control cells).

Effect of Increased Intracellular Ca^{++} on NHE2 and NHE3 Fluxes

Treatment with thapsigargin increases intracellular calcium by blocking microsomal Ca^{++} ATPase, activating several signal transduction pathways. This reagent may also stimulate mitogen-activated protein kinase (MAPK) independently of protein kinase C, via Src and Raf-1 (Chao et al., 1997). The concentration of thapsigargin chosen (50 ng/ml) was based on dose-response curves shown in Fig. 1, *C* and *D*. Tables 3 (NHE2) and 4 (NHE3) show average flux activity and Na/v of each isoform. Under nonacid loaded conditions, the V_{max} of NHE2 (Fig. 3*A*) was *decreased* (27.0 ± 3.0 nmoles/10 min/Transwell *vs.* 49.5 ± 3.8 for control, $n = 5$, $P < 0.05$). NHE2 K_{Na} was changed only slightly (73.0 ± 3.1 *vs.* 58.4 ± 4.0 mM Na for control, $n = 5$, $P < 0.01$). In contrast, only V_{max} of NHE3 (Fig. 3*B*) was affected, decreasing significantly from 11.9 ± 1.8 mM Na in control to 4.4 ± 0.4 mM Na with elevated $[Ca^{++}]_i$ ($n = 5$, $P < 0.05$). There was no significant change in NHE3 K_{Na} (17.7 ± 3.8 with thapsigargin *vs.* 20.2 ± 4.6 mM Na in control, $n = 5$).

Effect of Phorbol Ester on NHE3 Activity

We had determined previously (McSwine et al., 1998) that PMA activation of protein kinase C does not alter NHE2 activity in the C2 cells; therefore it was not further tested in these studies. However, NHE3 basal activity was inhibited by 50 nM PMA (Table 5). In this case, both V_{max} and K_{Na} were affected (Fig. 4). V_{max} was inhibited by about 60% (12.9 ± 0.6 nmoles/10 min/Transwell *vs.* 20.9 ± 1.7 in control, $n = 5$, $P < 0.01$). Sodium affinity was doubled with a K_{Na} of 45.0 ± 1.6 mM Na *vs.* 24.3 ± 1.6 mM Na under control conditions ($n = 5$, $P < 0.01$).

Discussion

The present studies suggest that effects of the second messengers cAMP, calcium, and protein kinase C on NHE2 and NHE3 activities involve different mechanisms. Prior studies using intracellular pH measurements have suggested the involvement of endosomal recycling, proton modifier sites and other mechanisms. However, intracellular pH measurements may provide an incomplete picture because they are dependent on both carrier activity and the intracellular buffering capacity. The latter is sensitive to many variables, including cell volume and localized pH changes, both of which can influence calculations of an exchanger's activities. In a carefully controlled study of NHE3 in OK cells, Krayer-Pawlowska (1991) reported that buffering capacity in cells tested under identical conditions varied with pH_i attained; at low pH_i, buffering capacity and Na/H exchange rates were greater than at higher pH_i. Therefore, depending on the pH_i generated by acid loading, NHE activity rates (to rectify pH_i) would not necessarily be a direct reflection of the effects of the signal transduction pathway under study. Additionally, contributions of other factors to pH rectification cannot be entirely dismissed. In contrast, unidirectional apical to cell ^{22}[Na] influx, with appropriate amiloride analogue inhibitor, measures the activity of each NHE directly, allowing more accurate determination of isoform kinetics.

Although the NHE mutant fibroblasts provided a useful model system to characterize these isoforms when first cloned, they do not express regulatory systems unique to intestinal epithelia. Therefore, they are not good systems to study regulation of the brush border NHEs. The C2 cells are polarized and, unlike fibroblasts, differentiate like intestinal epithelia, expressing many proteins characteristic of enterocytes. The C2bbe line represents a homogeneous subset of the Caco-2 population. While it is difficult to compare activity measurements from different studies due to the variety of techniques used, important distinctions exist between the Caco-2 and C2 cells. Acid loading is not required to demonstrate apical membrane NHE activity in wild type (which expresses endogenous NHE2) or transfected C2 cells. In addition, modulation of apical membrane NHE activity in C2 cells by a variety of second messenger pathways appears to be similar to responses reported for native intestinal brush borders. In contrast, some of

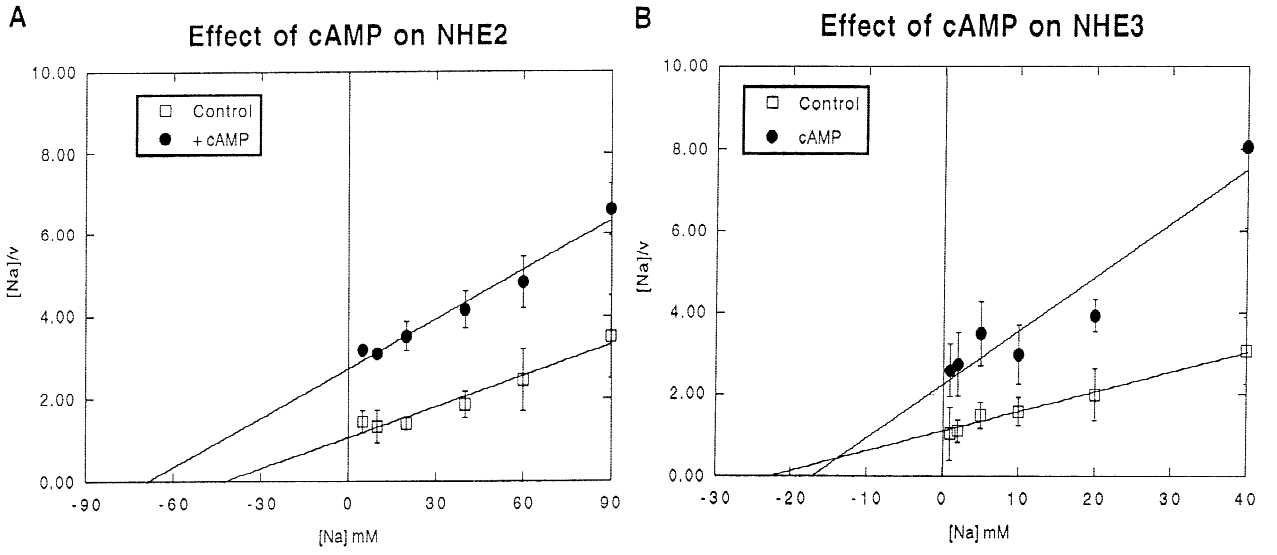

Fig. 2. The effect of CPT-cAMP on NHE2 and NHE3. The two panels show Hanes-Woolf interpretation of non-acid loaded, brush border, unilateral Na influx rates by NHE2 (*A*), and NHE3 (*B*), with (●) and without (❑) the cell permeable cAMP analogue, 8-CPT-cAMP (50 μM). Points are derived from 6 experiments. Cell lines (in duplicate) were paired in each experiment for similar passage, etc. Generation of the plot was by Kaleidagraph™, with least square best-fit line extension drawn to intercept with the *x* axis, which equals $-K_{Na}$. The *y* intercept equals K_{Na}/V_{max}.

Table 3. Effect of Thapsigargin on C2/N2 Kinetics

Control						
[Na] mM	**5**	**10**	**20**	**40**	**60**	**90**
Influx/10min/well						
Average	**3.00**	**6.75**	**14.37**	**22.27**	**24.02**	**27.13**
STDev	0.07	0.37	0.86	0.75	2.08	2.04
[Na]/flux	**1.67**	**1.48**	**1.40**	**1.80**	**2.51**	**3.33**
AveDev	0.03	0.07	0.06	0.05	0.16	0.18
Thapsigargin (50 μg/ml)						
Na mM	**5**	**10**	**20**	**40**	**60**	**90**
Influx/10min/well						
Average	**1.59**	**3.06**	**5.74**	**9.93**	**11.74**	**14.31**
STDev	0.03	0.11	0.56	0.94	0.98	1.16
[Na]/flux	**3.15**	**3.28**	**3.51**	**4.06**	**5.14**	**6.32**
AveDev	0.05	0.08	0.25	0.31	0.30	0.395

Table 4. Effect of Thapsigargin on C2/N3 Kinetics

Control						
[Na] mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10min/well						
Average	**0.89**	**1.37**	**1.76**	**3.27**	**6.39**	**8.08**
STDev	0.04	0.08	0.07	0.23	0.23	0.85
[Na]/flux	**1.12**	**1.45**	**2.84**	**3.06**	**3.13**	**4.95**
AveDev	0.02	0.06	0.06	0.18	0.19	0.70
Thapsigargin (50 μg/ml)						
[Na] mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10min/well						
Average	**0.41**	**0.56**	**0.85**	**1.28**	**2.89**	**3.55**
STDev	0.03	0.22	0.06	0.47	0.49	0.27
[Na]/flux	**2.44**	**3.57**	**5.89**	**7.82**	**6.94**	**11.28**
AveDev	0.02	0.16	0.04	0.35	0.41	0.21

these agents either do not modulate activity or elicit different responses in fibroblasts or the heterogeneous Caco-2 population. The kinetic values reported here are derived from changes in basal activities, without the non-physiological activation of the NHEs by acid loading. We believe that the conditions we have used approximate normal cellular responses *in vivo.* For example, PKA activation does not inhibit NHE3 transfected into the Caco-2 parental cell line (Yun et al., 1997), while it does inhibit both NHE2 and NHE3 in the C2 cells. The latter reflects regulation observed *in vivo.* Differences between the two cell lines may be the result of expression of certain accessory proteins by the C2 cells. In the case of NHE3, only V_{max} was affected, suggesting that the number of active exchangers in the plasma membrane was reduced. This could be accomplished by inhibiting recycling endosomal traffic, as suggested by Kurashima et al. (1998) #1575, or by inactivation of those exchangers in the plasma membrane. The latter explanation is consistent with current studies of the NHE3 protein kinase A response regulatory proteins. We cannot assume that where we observed no changes in K_{Na} that the exchanger itself was not allosterically altered to affect function. Furthermore, we cannot discriminate inactivation of the available exchanger molecules by second messengers (e.g., by PKA induced phosphorylation) from indirect effects mediated by regulatory proteins. Weinman et al. in a series of studies (Weinman, Steplock

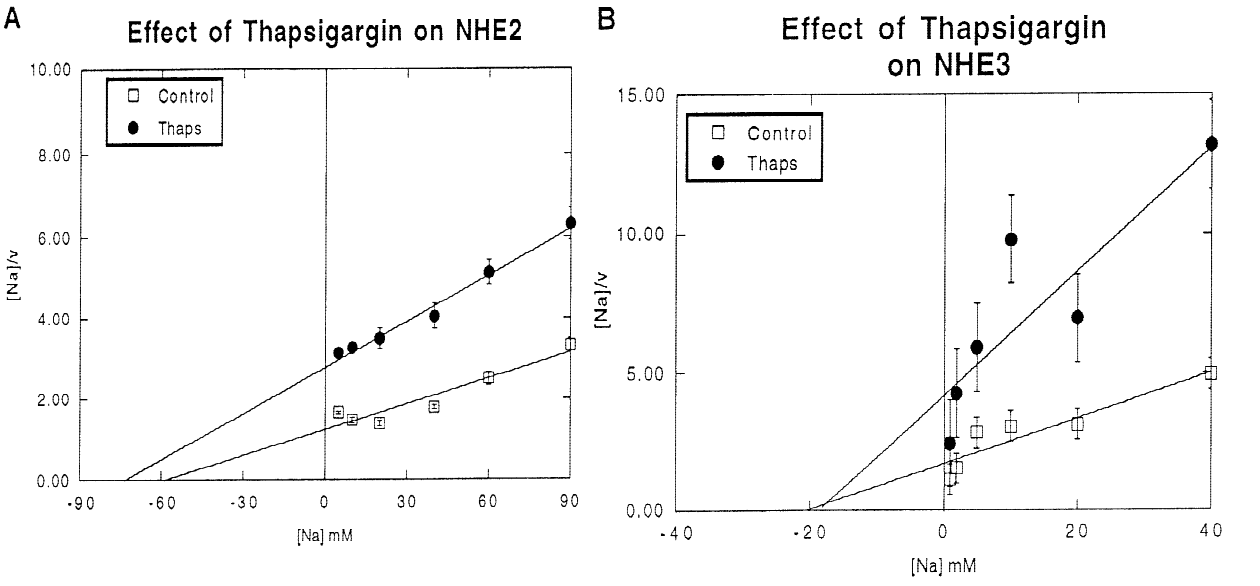

Fig. 3. The effect of increased intracellular Ca^{++} on NHE2 and NHE3 activities. Conditions of each experiment were identical to those in Fig. 2. Not preactivated by acid loading, NHE2 (*A*) and NHE3 (*B*) activities are shown as in Fig. 2, with (●) and without (❑) thapsigargin (50 ng/ml).

Table 5. Effect of PMA on C2/N3 Kinetics

Control						
[Na] mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10min/well						
Average	**0.97**	**1.85**	**3.25**	**6.32**	**10.71**	**14.25**
STDev	0.24	0.52	0.92	1.61	3.64	3.71
[Na]/flux	**1.07**	**1.14**	**1.61**	**1.65**	**2.01**	**2.95**
AveDev	0.17	0.21	0.30	0.28	0.43	0.62
PMA (50 nM)						
Na mM	**1**	**2**	**5**	**10**	**20**	**40**
Influx/10min/well						
Average	**0.36**	**0.56**	**1.14**	**2.16**	**4.47**	**6.23**
STDev	0.03	0.01	0.08	0.14	0.23	0.27
[Na]/flux	**2.82**	**3.54**	**4.39**	**4.64**	**4.49**	**6.43**
AveDev	0.18	0.04	0.20	0.22	0.16	0.21

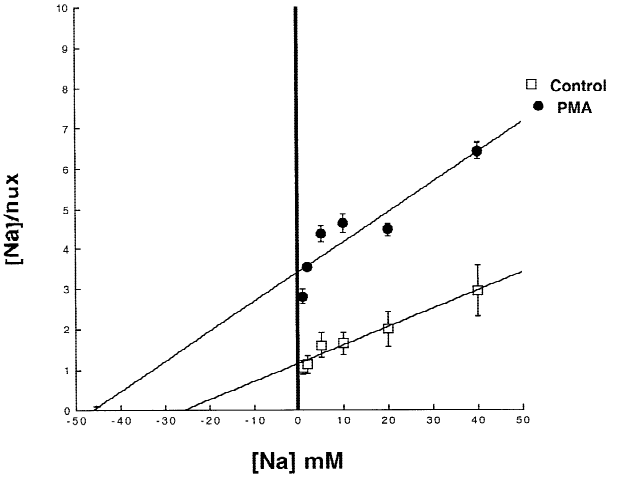

Fig. 4. Effect of activated protein kinase C on NHE3. C2/N3 cells were examined in the presence (●) or absence (❑) of 50 nM PMA. Hanes-Woolf plots of the influx data are shown as before, with concentrations of extracellular Na on the x axis; best fit linear regression analysis line was extended to intercept the *x* axis, indicating the $-K_{Na}$.

& Shenolikar, 1993; Weinman et al., 1995; Weinman et al., 1998) have shown that inhibition of renal brush border NHE3 by PKA requires the regulatory protein, NHERF. Yun et al. (1997) demonstrated that transfection of NHERF or another regulator protein, E3KARP, into cells that previously showed no cAMP inhibition of NHE3 restored that response. Neither of these proteins are endogenously expressed in Caco-2 cells (Yun et al., 1997). PKA regulation of NHE3 in the C2 cells strongly suggests the presence of one of these regulators, or one as yet unidentified with similar properties. Recent studies of NHERF and E3KARP (in PS120 and polarized OK cells) (Lamprecht, Weinman & Yun, 1998; Yun et al., 1998) suggested that they serve to physically link NHE3 to the cytoskeleton and PKA, facilitating the phosphorylation (and inactivation) of NHE3 at serine 605 (Kurashima et al., 1997). In contrast, inhibition of NHE2 by cAMP was distinguished by a small, but significant, effect on the affinity of this exchanger for Na as well as reduced V_{max}, suggesting modifications of sodium binding site as well as reduction of active exchangers on the plasma membrane.

Previously, we reported no effect on NHE2 in these cells by PMA-induced stimulation of PKC (McSwine et al., 1998). Phorbol ester inhibition of NHE3, however,

doubled K_{Na} and reduced V_{max}, by about 60%. Janecki et al. (1998) reported that endosomal recycling contributed to approximately half of the PKC induced inhibition of NHE3. This is consistent with our data and suggests the existence of another layer of regulation, which has altered Na binding affinity to the NHE3 isoform.

Thapsigargin induces increased intracellular [Ca^{++}], a condition common to a number of signal transduction pathways known to modulate sodium absorption in the intestine and specifically inhibit NHE3. Ca^{++} bound calmodulin (CaM) activates CaM-dependent protein kinases, inhibiting intestinal NHE3 in rabbit ileal brush borders (Cohen et al., 1990). In contrast, endothelin bound ET_B receptor activates NHE3 in OK cells by mechanisms which include increased intracellular [Ca^{++}] and activated Ca-calmodulin (CaM) kinase (Chu et al., 1996). However, while an increase in $[Ca^{++}]_i$ is required, it is not sufficient by itself and tyrosine kinases are the second essential component of endothelin regulation. In the C2 cells, the thapsigargin-induced $[Ca^{++}]_i$ increase resulted in a decrease in the initial rates of both NHE2 and NHE3 activities. Inhibition of NHE2 involved changes in both V_{max} and K_{Na}. In contrast, only the V_{max} of NHE3 was affected. Thapsigargin may also activate MAP kinase (via Src and Raf-1 kinases) independently of PKC or Ca^{++} influx (Chao et al., 1997). Yamaji et al. (1995) reported that the Src family of kinases is involved in the response of NHE3 to chronic acidosis. Recently, D'Souza et al. (1998) and Kurashima et al. (1998) demonstrated that a portion of NHE3 is present in recycling endosomes. Blocking phosphatidylinositol 3-kinase (a member of the Src family and a critical component of the vesicular traffic pathway) inhibited the NHE3 response to low internal pH by inhibiting the exocytosis step. Src kinases may, therefore, be involved in both negative and positive regulation of NHE3 in response to a variety of cellular conditions, perhaps by controlling the rate of endosomal recycling.

In this study, we have directly measured Na/H exchange by measuring Na uptake and not acidifying the cell to artificially prestimulate the NHEs. If the sole factor regulating NHE1, NHE2 and NHE3 is second messenger induced alterations in pH_i, then the effect on all three should be identical. In fact, CPT-cAMP has no effect on and thapsigargin and PMA increase basolateral NHE1 activity (McSwine et al., 1998), while PMA has no effect on NHE2, but inhibits NHE3. Clearly, each isoform responds to second messengers according to its own set of regulators and pH_i is not the sole determinant.

In summary, we have characterized by kinetic analysis the regulation of NHE2 and NHE3 by second messengers integral to a number of signal transduction pathways. This regulation closely parallels responses previously seen *in vivo*, establishing the utility of this enteroccytelike cell line as a model system for analysis of these two intestinal epithelial exchangers. This system facilitates our understanding of how a specific signal transduction pathway controls the responses of each isoform, as well as the role each plays in normal and abnormal gut physiology.

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