Differential expression and regulation of Na\(^+\)/H\(^+\) exchanger isoforms in rabbit parietal and mucous cells

HEIDI ROSSMANN, THORSTEN SONNENTAG, ALEXANDER HEINZMANN, BARBARA SEIDLER, OLIVER BACHMANN, DOROTHEE VIEILLARD-BARON, MICHAEL GREGOR, AND URSULA SEIDLER

First Department of Medicine, Eberhard-Karls University Tübingen, D-72076 Tübingen, Germany

Received 21 July 2000; accepted in final form 16 March 2001

Several Na\(^+\)/H\(^+\) exchanger (NHE) isoforms are expressed in the stomach, and NHE1 and NHE2 knockout mice display gastric mucosal atrophy. This study investigated the cellular distribution of the NHE isoforms NHE1, NHE2, NHE3, and NHE4 in rabbit gastric epithelial cells and their regulation by intracellular pH (pH\(_i\)), hyperosmolarity, and an increase in cAMP. Semiqualitative RT-PCR and Northern blot experiments showed high NHE1 and NHE2 mRNA levels in mucous cells and high NHE4 mRNA levels in parietal and chief cells. Fluorescence optical measurements in cultured rabbit parietal and mucous cells using the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein and NHE isoform-specific inhibitors demonstrated that both cell types, intracellular acidification activates NHE1 and NHE2, whereas hyperosmolarity activates NHE1 and NHE4. The relative contribution of the different isoforms to pH\(_i\) and hyperosmolarity-activated Na\(^+\)/H\(^+\) exchange in the different cell types paralleled their relative expression levels. cAMP elevation also stimulated NHE4, whereas an increase in osmolarity above a certain threshold further increased NHE1 and not NHE4 activity. We conclude that in rabbit gastric epithelium, NHE1 and NHE4 regulate cell volume and NHE1 and NHE2 regulate pH. The high NHE1 and NHE2 expression levels in mucous cells may reflect their special need for pH\(_i\) regulation during high gastric acidity. NHE4 is likely involved in volume regulation during acid secretion.

Na\(^+\)/H\(^+\) exchanger isoform 1; Na\(^+\)/H\(^+\) exchanger isoform 2; Na\(^+\)/H\(^+\) exchanger isoform 4; stomach; intracellular pH regulation; intracellular pH; volume regulation

Address for reprint requests and other correspondence: U. Seidler, Abteilung Innere Medizin I, Eberhard-Karls Universität Tübingen, Otfried-Müller Str. 10, D-72076 Tübingen, Germany (E-mail: ursula.seidler@uni-tuebingen.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
emphasis on their role in gastric physiology, we investigated 1) the relative mRNA expression levels of NHE1, NHE2, NHE3, and NHE4 in the different cell types of the rabbit gastric mucosa and 2) the activation of the different NHE isoforms by low pH, hyperosmolality, and cAMP in cultured rabbit parietal and mucous cells.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all reagents were obtained from Sigma-Aldrich and Fluka (Deisenhofen, Germany) or Merck (Darmstadt, Germany) at tissue culture grade, molecular biology grade, or the highest grade available.

Rabbit gastric cell purification for molecular biology studies. Parietal, chief, and mucous cells were purified from rabbit gastric mucosa, and the homogeneity of the three cell fractions was assessed by light microscopy after staining cytospin preparations as described previously (27, 28). The mucous cell fraction consists of 90–95% periodic acid-Schiff-granule-positive cells, whereas the parietal cell fraction shows a purity of 95–98% and the chief cell population contains <2% contaminating cells. These findings were confirmed by the expression level of the H⁺–K⁺-ATPase in the different cell fractions as determined by Northern blot analysis (see Ref. 24 for data).

RNA isolation and Northern blot analysis. Isolation of total and purified poly(A)⁺ RNA and Northern blot analysis were carried out as described previously (11, 12, 24). Membranes were probed (see Fig. 1) with rabbit NHE1 (5’-untranslated region and nt 1–1524 of coding sequence), rabbit NHE2 (nt 1628–2954 of coding sequence and 3’ region and nt 1–1524 of coding sequence), rabbit NHE2 (nt 174–2032 of coding sequence), rabbit NHE3 (nt 1183–2496 of coding sequence), rabbit NHE4 (nt 272–2151 of coding sequence), rat NHE2 (nt 1628–2954 of coding sequence and 3’-untranslated region), rat NHE3 (nt 1185–2496 of coding sequence), rat NHE4 (nt 1–1524 of coding sequence), rat NHE4 (nt 1185–2496 of coding sequence), G448 NHE isoforms in gastric mucosa, and the homogeneity of the three cell types.

Primers for cloning cDNA fragments and semiquantitative RT-PCR experiments.

Table 1. Primers used for probe generation and semiquantitative RT-PCR experiments

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>PCR Product, bp</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH forward</td>
<td>ACGGAACGGTCAAGGCTGAGA</td>
<td>371</td>
<td>53°C</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>GGCGTGGACCTGGTCATGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H⁺–K⁺-ATPase forward</td>
<td>TGGTCTGACCTCTCTACTCCTCC</td>
<td>405</td>
<td>70°C</td>
</tr>
<tr>
<td>H⁺–K⁺-ATPase reverse</td>
<td>GGGGAGATGAGATTGTTTCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primers for cloning cDNA fragments were used as controls for Northern blot analysis. All fragments were from rabbit. Annealing took place for 60 s for all primers. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) GenBank (GB) accession no. was L23961; rabbit H⁺–K⁺-ATPase (α-subunit) GB accession no. was X64694; rabbit Na⁺/H⁺ exchanger isoform 1 (NHE1) GB accession no. was X59935; rabbit NHE2 GB accession no. was L13733; rabbit NHE3 accession no. was M87007; rabbit NHE4 (partial sequence) was from Z. Wang and G. Shull, unpublished data; rabbit histone 3.3a GB accession no. was X51897. *See Ref. 24 for data.

Semiquantitative RT-PCR. Semiquantitative PCR was carried out as described previously (9, 24). Homologous primers for rabbit NHE1, NHE2, NHE3, NHE4, GAPDH, and histone 3.3a were deduced from published sequence information or after sequencing an appropriate cDNA fragment (Table 1). The identity of the NHE1, NHE2, NHE3, and NHE4 amplimers was confirmed by restriction analysis (see Fig. 1C). For semiquantitative PCR, the products were separated on an agarose gel, and the optical density of the ethidium bromide-stained bands was measured using the ImageMaster VDS system and software (Amersham Pharmacia, Freiburg, Germany). The amplification efficiency of the gene of interest and histone 3.3a was determined by calculating the slope after semilogarithmic plotting of the values against the cycle number (see Fig. 2A). The virtual relationship integrated optical density (ODI) of the studied gene vs. ODI of histone 3.3a was calculated (see Fig. 2B). To compare NHE1, NHE2, NHE3, and NHE4 expression levels, the ODI values of the different PCR products were corrected according to their length.

Purification and culture of rabbit parietal and mucous cells. Cell culture was adapted from the method of Chew et al. (10) as we (1, 24) have described in detail previously. To assess the functional integrity, the ability of the parietal cells to respond to secretory stimuli was periodically determined by measurement of [¹⁴C]aminopyrine (AP; Amersham Pharmacia) uptake into the cells as described by Chew et al. (10). Mucous cells were evaluated optically only. For fluorescence measurements, mucous cells were selected that had settled in groups of three or more and in which large mucous granules could be visualized under the microscope.

Fluorescence microscopy for determination of pH. pH measurements are described elsewhere in detail (1). Cultured cells were loaded with 5 μM 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM (Molecular Probes, Leiden, The Netherlands) and incubated for 30 min in buffer A (120 mM NaCl, 14 mM HEPES, 7 mM Tris, 3 mM KH₂PO₄, 1.2 mM CaCl₂, 1.2 mM MgSO₄, and 20 mM glucose, pH 7.4 gassed with O₂), then alternately excited at 440 ± 10 and 490 ± 10 nm at a rate of 100/s. Emission wavelength was 530 nm. At the end of each experiment, the 440 nm-to-490 nm ratio was calibrated to pH with clamping pHi to extracellular pH (pHi) using the high K⁺-nigericin method as described previously (1). Cellular acidification was achieved by an ammonium prepulse [2–15 min with 40 mM NH₄Cl or (NH₄)₂SO₄].
Determination of the intrinsic buffering capacity. Intrinsic buffering capacity was determined as previously described by Boyarsky et al. (Ref. 7; see Ref. 24 for values).

Statistics. Results are given as means ± SE. Proton fluxes were calculated by performing linear regression analysis on individual pH$i$ traces during the first 1 to 2 min of stimulation (linear phase). Unless otherwise indicated, Student’s t-test was used for paired samples and ANOVA was used for multiple comparisons.

RESULTS

Molecular characterization of NHE isoforms expressed in rabbit gastric mucosa and their distribution in different epithelial cell types. As detected by Northern blot analysis and RT-PCR (Fig. 1) NHE1, NHE2, and NHE4 are expressed in rabbit gastric mucosa, whereas NHE3 is not. A 4.8- and 4.4-kb mRNA could

---

Fig. 1. A: high-stringency Northern blot analysis of ~2 µg twice-purified polyA⁺ RNA from rabbit kidney cortex, colonic mucosa, gastric mucosa, parietal cell fraction, and mucus cell fraction, probed with ³²P-labeled homologous Na⁺/H⁺ exchanger (NHE) isoforms NHE1 and NHE3 cDNA fragments, as described in MATERIALS AND METHODS. Hybridization of the filter with a cDNA fragment of the H⁺-K⁺-ATPase a-subunit ensures the quality of the cell separation. Rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe confirms loading of intact RNA in all lanes. Exposure times: 16 h for NHE1 and NHE3 (short exposure), 3 days for NHE3 (long exposure), 3 h for H⁺-K⁺-ATPase, and 6 h for GAPDH. B: high-stringency Northern blot analysis of ~20 µg once-purified polyA⁺ RNA from rabbit parietal and mucus cell fractions, probed with ³²P-labeled homologous NHE1, NHE2, and NHE4 cDNA fragments, as described in MATERIALS AND METHODS. Exposure times: 12 h for NHE1, 40 h for NHE2, and 17 h for NHE4. C: amplification and restriction analysis of NHE1, NHE2, and NHE4 cDNA fragments. As expected, the NHE1 fragment (left) is cut by Ava I (lane 1: 232 and 124 bp) but not by Smal I (lane 2). The NHE2 (right) fragment is cut by Hinc II (lane 6: 113 and 67 bp), but not by Sau96 I (lane 5), which should cut a nonspecifically amplified NHE4 PCR product. Conversely, NHE4 (right) is cut by Hae III (lane 2), but not by Hind II (lane 1), which should cut a nonspecifically amplified NHE2 PCR fragment. Lanes 3 and 7 (right): undigested PCR products. Lanes 3 (left) and 4 (right): molecular weight standards. Even after 40 cycles, no NHE3 band was amplified from rabbit gastric mucosa.
Role of NHE1, NHE2, and NHE4 in parietal and mucous cell pH$_i$ recovery. To evaluate the physiological significance of the different NHE isoforms in parietal and mucous cells, we evaluated their contribution to the Na$^+$/H$^+$ exchange-mediated recovery from an intracellular acid load. Cultured parietal and mucous cells were acidified by an ammonium prepulse to approximately pH 6.4, and pH$_i$ recovery was measured fluorometrically without and with inhibitors in concentrations that selectively inhibit NHE1, NHE2, or NHE1, NHE2, NHE3, and NHE4 (5, 25). Parietal cell resting pH$_i$ in a HEPES-O$_2$ buffer was 7.24 ± 0.03, which corresponds to previous measurements (19, 21), whereas mucous cell resting pH$_i$ was significantly higher than parietal cell pH$_i$ (7.43 ± 0.04). When acidified to pH$_i$ of ~6.4, parietal cells recovered with an initial proton efflux rate of 21 ± 1.1 mM/min (Fig. 3, A and B). Of this proton efflux, 80% was inhibited by 1 μM HOE-642 (very specific for NHE1 inhibition at this low concentration), and almost all of the residual flux was inhibited by 25 or 50 μM HOE-642, which also inhibits NHE2 (25). There was no significant difference between 25 or 50 μM HOE-642 (data not shown), indicating that either concentration is suitable for full NHE2 inhibition but not NHE4 inhibition. The residual proton efflux under 500 μM dimethylamiloride (DMA) (which also inhibits NHE4; Ref. 5) was not significantly different from that under 25 μM HOE-642, indicating that NHE1 and NHE2, but not NHE4, mediate pH$_i$ recovery from an intracellular acid load.

In mucous cells, initial proton efflux rates after pH$_i$ 6.4 were 65 ± 16 mM/min and therefore significantly higher than in parietal cells (Fig. 3, C and D). HOE-642 at 1 μM inhibited only about two-thirds of this proton efflux, whereas 25 and 50 μM HOE-642 inhibited 87% and 91%, which were not significantly different from that inhibited by 500 μM DMA (92%). These results demonstrate that 1) a comparable acid load stimulates a significantly higher Na$^+$/H$^+$ exchange-mediated proton efflux rate in mucous cells than in parietal cells, and 2) this proton efflux is predominantly mediated by NHE1 in both cell types, with the rest being mediated by NHE2.

Kapus et al. (17) observed different pH$_i$/pH$_o$ relationships in NHE1-, NHE2-, and NHE3-transfected activator protein-1 (AP-1) cells and a relative shift of the pH$_o$ dependence of acid-induced proton efflux to more alkaline values compared with those of NHE1- and NHE3-transfected cells. Therefore, we investigated if the relationship of NHE1- to NHE2-mediated proton efflux changes with an increase in external and internal pH. Figure 4 shows the pH$_i$ curves (Fig. 4A) and proton efflux rates (Fig. 4B) during a change in the perfusate of cultured parietal cells from pH 7.4 (pH$_o$ 7.2) to pH 8. Interestingly, 41% of the ensuing intracellular alkalinization was inhibited by 500 μM DMA and therefore mediated by Na$^+$/H$^+$ exchange, whereas the other 59% was likely due to proton or base conductances. Under these experimental conditions, NHE1 and NHE2 contributed similarly to the Na$^+$/H$^+$ ex-
change-mediated alkalinization, and again NHE4 did not contribute. This is comparable to an 80% NHE1-20% NHE2 distribution when parietal cells were acidified to pH$_i$ 6.4 and pH$_o$ 7.4. Thus the percentage of proton flux mediated by NHE1 and NHE2 in a cell type expressing both isoforms is indeed dependent on pH$_i$ and pH$_o$. Of note, total Na$^+$/H$^+$ exchange rates were 1.6 mM/min in the former and 20 mM/min in the latter condition.

Activation of parietal and mucous cell NHE1, NHE2, and NHE4 by hyperosmolarity. The two basic and distinct cellular functions of Na$^+$/H$^+$ exchange are pH$_i$ and volume regulation. Therefore, we next investigated the activation of the different NHE isoforms in

**Fig. 2.** Semiquantitative RT-PCR analysis of rabbit NHE1, NHE2, and NHE4 expressed in gastric epithelial cell types. A: parallel curves for NHE1 (356 bp) and histone 3.3a (523 bp), NHE2 (180 bp) and histone 3.3a, and NHE4 (270 bp) and histone 3.3a amplified from rabbit gastric mucosa are shown. The ratio of the gene of interest to histone 3.3a was determined during the exponential phase of both reactions as described previously (see Ref. 24). The ethidium bromide-stained bands, which were analyzed, are shown below each diagram. ODI, integrated optical density; R, amplification efficiency; n cycles indicates the number of PCR cycles, as do the numbers given for the stained bands. NHE3 could not be amplified within 40 cycles. B: NHE1 vs. histone 3.3a, NHE2 vs. histone 3.3a, and NHE4 vs. histone 3.3a fragments were amplified from rabbit gastric mucosa, parietal, mucous, and chief cells. The ratio of the gene of interest to histone 3.3a, representing the relative expression level of the studied gene, was plotted as a bar graph for the studied tissues (n = 3).
parietal and mucous cells by hyperosmolarity. Exposure of cultured parietal (A and B) and mucous cells (C and D) by the ammonium pulse technique in the presence and absence of HOE-642 or dimethylamino-ride (DMA). HOE-642 at 1 μM (this concentration is selective for NHE1) inhibits 80% of the Na+/H+ exchange rate during pH recovery in parietal cells and 62% in mucous cells. HOE-642 at 50 μM (at this concentration NHE1 and NHE2 are inhibited) abolished the remaining proton flux in parietal and mucous cells. TMA, tetramethylammonium; n.s., not significant.

underlying proton influx, is 1.65 ± 0.03 mM/min. HOE-642 at 1 μM inhibited 55% of the hyperosmolarity-induced proton efflux, and 25 and 50 μM HOE-642 had only a marginal further inhibitory effect, whereas 500 μM DMA, which also inhibits NHE4, completely prevented alkalinization (Fig. 5, B and D). In mucous cells, hyperosmolarity also caused a DMA-sensitive cellular alkalinization by 0.25 ± 0.06 pH units, and the proton efflux rate during the initial linear phase of alkalinization was 1.35 ± 0.12 mM/min. Of this proton efflux

parietal and mucous cells by hyperosmolarity. Exposure of cultured parietal cells to 400 mosmol/kgH2O caused a rapid pHi increase of 0.21 ± 0.03 pH units (Fig. 5A). DMA (500 μM) completely inhibited proton efflux and unmasked a slow acidification after exposure to 400 mosmol/kgH2O, possibly due to anion exchange activation, which is expected to have only a relatively small effect on pHi at the given low pHi and in the absence of CO2-HCO3−. Thus DMA-sensitive proton efflux, calculated on the basis of this
Fig. 5. pH trace (A–C: parietal cells; E–G: mucous cells) and initial proton efflux rates (D: parietal cells; H: mucous cells) after addition of hyperosmolar solution to cultured parietal and mucous cells. A and D: exposure of cultured parietal cells to 400 mosmol/kgH₂O caused a rapid pH increase. B and D: HOE-642 at 1 µM (this concentration is selective for NHE1) inhibits 55% of the Na⁺/H⁺ exchange rate; 25 or 50 µM HOE-642 (concentrations selective for NHE1 and NHE2) inhibits an additional 10%. The remaining flux rate is inhibited by 500 µM DMA (C and D) and represents NHE4 activity. E and H: the same type of experiments performed in cultured mucous cells yielded qualitatively similar results. HOE-642 at 1 µM inhibits 65% of the Na⁺/H⁺ exchange rate (F and H); 50 µM HOE-642 inhibits an additional 9%, and 500 µM DMA (G and H) inhibits the remaining flux rate (26%).
rate, 65% was inhibited by 1 µM HOE-642, 9% by 50 µM HOE-642, and the residual flux by 500 µM DMA (Fig. 5, E–H).

These results demonstrate that the hyperosmolarity-induced Na\(^+\)/H\(^+\) exchange activation in both parietal and mucous cells is predominantly due to NHE1 and NHE4. In the two cell types, the relative contribution of NHE1 and NHE4 to hyperosmolarity-induced Na\(^+\)/H\(^+\) exchange paralleled their relative expression levels, i.e., a higher NHE4 contribution in parietal cells, which also express more NHE4 than mucous cells. NHE2 contribution to hyperosmolarity-activated Na\(^+\)/H\(^+\) exchange was present but minimal both in parietal and mucous cells. These findings demonstrate that hyperosmolarity is able to activate NHE2, consistent with previous results (17) in NHE2-transfected cell lines, but that hyperosmolarity-induced NHE2 activation is marginal in gastric epithelial cells.

Effect of increasing osmolar strength on NHE1, NHE2, NHE3, and NHE4 activity. In hepatocytes, increasing osmolar strength sequentially recruits additional volume-regulatory mechanisms (40). We therefore wondered if a similar situation existed for NHE1 and NHE4 activation in parietal cells. The perfusate of BCECF-loaded cultured parietal cells was changed from 300 to 350, 400, or 500 mosmol/kgH\(_2\)O, and the resulting pH\(_i\) increase was measured. Figure 6 demonstrates the parietal cell proton efflux rates due to Na\(^+\)/H\(^+\) exchange stimulation on exposure to 350, 400, and 500 mosmol/kgH\(_2\)O. We were surprised by the results: total DMA-sensitive proton efflux stimulated by 350 mosmol/kgH\(_2\)O was 1.4 mM/min, and 0.82 mM/min or 60% was due to NHE4 activation. Higher osmolar strengths increased overall Na\(^+\)/H\(^+\) exchange rates dramatically to 3.42 mM/min at 500 mosmol/kgH\(_2\)O. Surprisingly, the increase in Na\(^+\)/H\(^+\) exchange rates was largely due to an increase in NHE1 activity, which increased from 0.46 mM/min at 350 mosmol/kgH\(_2\)O to 2.31 mM/min at 500 mosmol/kgH\(_2\)O, whereas NHE4 activity increased from 0.82 mM/min at 350 mosmol/kgH\(_2\)O to 1.13 mM/min at 500 mosmol/kgH\(_2\)O. Hyperosmolarity-induced NHE2 activity was minimal at all osmolar strengths. Thus the ratio of NHE1 to NHE4 activity changed from 0.56 at 350 mosmol/kgH\(_2\)O to 1.5 at 400 mosmol/kgH\(_2\)O and to 2.24 at 500 mosmol/kgH\(_2\)O. These results suggest that both NHE isoforms are already activated in parietal cells at fairly modest increases in osmolar strength, where NHE4 is the predominant active isoform, but that NHE1 activity can increase far more dramatically than NHE4 activity at higher osmolarities.

Activation of NHE4 by cAMP. We (1) have previously reported that acid secretagogues differentially activate the NHE isoforms in cultured rabbit parietal cells, with forskolin- or histamine-induced Na\(^+\)/H\(^+\) exchange being mediated predominantly (55 and 65%) by NHE4. In this study, we found that parietal cell Na\(^+\)/H\(^+\) exchange rates stimulated by 400 mosmol/kgH\(_2\)O were somewhat higher than those previously measured under forskolin or histamine stimulation (1) but that the percentage of NHE4-mediated Na\(^+\)/H\(^+\) exchange was lower (~40%). Volume measurements demonstrated that 400 mosmol/kgH\(_2\)O causes only a slightly larger cell shrinkage than forskolin (31) but that forskolin and hyperosmolarity do not cause a stronger cell shrinkage than hyperosmolarity alone (Sonnentag, unpublished results). We therefore wondered if forskolin might activate NHE4 independently of cell shrinkage.

To further explore this possibility, we measured the hyperosmolarity-induced proton efflux rate in the absence and after sequential NHE1, and NHE1 and NHE2, inhibition in the presence and absence of forskolin (Fig. 7). We found that, in the presence of forskolin, hyperosmolarity consistently stimulated a higher proton efflux rate in both the absence and the presence of 1 and 25 µM HOE-642 than did hyperosmolarity alone. This suggests that an intracellular increase in cAMP levels has a distinct stimulatory effect on NHE4 that is not explained by cAMP-mediated cell shrinkage.

DISCUSSION

The present study was undertaken to explore the cellular distribution of the NHE isoforms expressed in rabbit gastric mucosa and to investigate whether the different isoforms may have different modes of regulation and potential physiological significance in the stomach.

A previous study (14) demonstrated that NHE1 is strongly expressed in the gastric mucosa compared with other segments of the gastrointestinal tract and the kidney. This study demonstrates particularly high NHE1 expression levels in gastric mucous cells. NHE1 is thought to be involved in pH\(_i\) and volume homeostasis and to be activated during cellular proliferation and
migration (38). Several studies suggest that protons enter the surface cells during periods of strong luminal acidification, and both Kiviluoto et al. (18) and we (30, 33) have demonstrated that Na\(^+\)/H\(^+\) exchange is one of the homeostatic mechanisms involved in the maintenance of a near-neutral pH\(_i\) in the amphibian gastric mucosa. Also, evidence (14) from the duodenum demonstrates that NHE1 is involved in HCO\(_3^\)-generation. Thus the exposure of gastric mucous cells to the high luminal proton concentration, their secretion of HCO\(_3^\)-, their exposure to highly anisotonic luminal fluids during meals, and their migration throughout most of their short life span may all necessitate high NHE1 activity. We found an extraordinarily high Na\(^+\)/H\(^+\) exchange rate in cultured mucous cells in response to an intracellular acid load, and two-thirds of this rate was due to NHE1 activity. On the other hand, hyperosmolarity elicited no stronger NHE1 activation in

Fig. 7. pH\(_i\) trace (A–C) and hyperosmolarity-induced initial proton efflux rates (D–F) under control conditions (A and D), after inhibition of NHE1 (1 μM HOE-642, B and E), and after inhibition of NHE1 and NHE2 (25 μM HOE-642, C and F) in the presence and absence of forskolin (Forsk) in cultured parietal cells. In the presence of forskolin, hyperosmolarity caused a higher proton efflux rate in the presence and absence of 1 and 25 μM HOE-642; n = 3–7. Because the aim of these experiments was to test whether a difference existed between forskolin and hyperosmolarity vs. hyperosmolarity alone, independent of the presence of HOE-642, all flux rates were compared with Wilcoxon's rank test for paired samples and P was found to be <0.01.
mucous than in parietal cells, despite the vastly different NHE1 expression levels. Therefore, we speculate that the very high NHE1 expression in gastric mucous cells may be related primarily to their necessity to extrude protons into the interstitium during HCO₃⁻ secretion and to prevent intracellular acidosis during periods of high luminal acid concentration. The fact that the NHE1 knockout mouse displays gastric but not intestinal histopathology (2) also suggests that this defect may not so much reduce their proliferative capacity (because this should have similarly severe effects in the rapidly proliferating small intestinal cells) but may weaken the gastric barrier to acid.

The acid-activated Na⁺/H⁺ exchange rate not inhibited by 1 μM HOE-642 (and therefore not mediated by NHE1) was inhibited by 25 or 50 μM HOE-642 and therefore was most likely due to NHE2. Both the percentage of and the absolute value for acid-activated Na⁺/H⁺ exchange rate sensitive to 50 μM but not 1 μM HOE-642 was far higher in mucous than in parietal cells. These findings correspond very well with the relative expression levels for NHE2 in mucous and parietal cells and make it highly likely that the acid-activated Na⁺/H⁺ exchange activity inhibited by 50 μM but not 1 μM HOE-642 is due to NHE2.

On the basis of high colonic expression levels and evidence for an apical location, the physiological role of NHE2 has been discussed as an alternative Na⁺ absorption mechanism in intestine and kidney (30, 34, 36, 37, 44). Surprisingly, NHE2-deficient mice show no intestinal or renal abnormalities and no diarrhea or electrolyte imbalance but do display severe gastric mucosal atrophy with total reduction of mucosal thickness and a particularly severe reduction of parietal and chief cells, corresponding to a complete loss of the acid secretory capacity by the age of 2–3 mo (26). Speculations as to the underlying mechanism for these gastric changes focused on NHE2 as an important player in parietal cell volume regulation and on a potential role of NHE2 in the mucosal protection mediated by the surface cells (26). Our data show that NHE2 expression is fairly low and NHE2 activation by hyperosmolality is minimal in parietal cells, making the first of the two hypotheses very unlikely. NHE2 is highly expressed in mucous cells and is activated in these cells by low pH but only minimally by hyperosmolality, thus strengthening the hypothesis that NHE2 may be involved in mucosal protection by the surface cells. When Na⁺/H⁺ exchange was studied in NHE2-transfected AP-1 cells, a remarkable feature was that NHE2 activity was strongly increased by raising the pHᵢ (42).

In isolated frog gastric mucosa, we (30 and unpublished observations) have previously observed that raising interstitial buffering capacity and thus interstitial pH caused an enhanced Na⁺/H⁺ exchange-mediated basolateral proton extrusion, increasing epithelial pHᵢ. On the basis of these findings, Schultheis et al. (26) speculated that NHE2 may be the NHE isoform that mediates Na⁺/H⁺ exchange activation by an increase in interstitial HCO₃⁻ concentration, which occurs during acid secretion. Indeed, we found in this study that, whereas overall Na⁺/H⁺-mediated acid extrusion rates decrease with increasing pHᵢ and pHₒ, the percentage of NHE1- to NHE2-mediated acid extrusion decreases with increasing pHᵢ and pHₒ. It is therefore feasible that NHE2 performs a substantial part of acid extrusion when the intracellular pH of gastric epithelial cells is near neutral and the interstitial pH high.

This hypothesis would be based on the assumption that gastric NHE2 is located in the basolateral membrane. Our attempts to localize NHE2 in rabbit stomach have failed so far, and no data are available on NHE2 localization in the stomach of other species. If we assume an apical location, as has been described in kidney (8) and intestine (13), its activity would result in proton secretion into the lumen. Possibly, the high carbonic anhydrase II expression levels in parietal cells require an alternative acid secretion mechanism in situations when the proton pump is not activated, and this explains the preferential degeneration of parietal cells in NHE2-knockout mice. However, we have never observed acid secretion in omeprazole-inhibited stripped mouse stomach in the Ussing chamber, a preparation that exhibits high agonist-induced acid secretory rates (I. Blumenstein and U. Seidler, unpublished observations).

Within the gastrointestinal tract, NHE4 is exclusively expressed in the stomach, and our results demonstrate that parietal cells have markedly higher NHE4 expression levels than surface cells. Interestingly, despite high expression levels, NHE4 did not contribute to the Na⁺/H⁺ exchange-mediated pHᵢ recovery from an intracellular acid load, demonstrating that NHE4 is not activated by low pHᵢ. On the other hand, the hyperosmolality-induced Na⁺/H⁺ exchange activity was in a significant way due to NHE4, and the relative contribution of NHE4 to hyperosmolality-induced Na⁺/H⁺ exchange activity paralleled the relative NHE4 expression levels in both parietal and mucous cells. These data demonstrate for the first time that hyperosmolality activates NHE4 in a cell type with endogenous NHE4 expression. These data explain why Bookstein et al. (5, 6) only observed Na⁺/H⁺ exchange activity in NHE4 transfected Na⁺/H⁺ exchange-deficient fibroblasts during hyperosmolar culture conditions and demonstrate that hyperosmolality conditions are not a prerequisite for NHE4 expression, just for activity. We then wondered whether parietal cells sequentially recruit NHE1 and NHE4 with increasing degrees of hyperosmolality, as has been shown (40) to occur in hepatocytes with Na⁺/H⁺ exchange, Na⁺-K⁺-2Cl⁻ cotransport, and Na⁺ conductance. However, we found that NHE4 becomes activated at very moderate osmolar strength, where it is the predominant isoform, and that it is NHE1, not NHE4, that can dramatically increase its flux rate with increasing osmolar strength. Thus the percentage of NHE4- to NHE1-mediated Na⁺ uptake shifts dramatically with increasing osmolar strength from a 2:1 relationship at 350 mosmol/kgH₂O to a 1:2 relationship at 500 mosmol/kgH₂O.
Because we (1) previously observed that the contribution of NHE4 to histamine- or forskolin-induced Na\(^+\)/H\(^+\) exchange activity in cultured rabbit parietal cells was significantly higher (>50% of total Na\(^+\)/H\(^+\) exchange activity) than the contribution to hyperosmolality-induced Na\(^+\)/H\(^+\) exchange activity in this study (~40%) but that a medium change to 400 mosmol/kgH\(_2\)O and stimulation by forskolin result in a similar degree of cellular shrinkage (31), we wondered if cAMP per se stimulates NHE4. After finding that forskolin plus hyperosmolality does not result in a stronger degree of parietal cell shrinkage than hyperosmolality alone (Sonnentag, unpublished observations), we tested the effect of simultaneous application of forskolin and hyperosmolality on NHE4 activation. We found that the simultaneous application of forskolin and a hyperosmolar medium resulted in a significantly higher Na\(^+\)/H\(^+\) exchange rate than hyperosmolality alone. A similar difference was observed in the absence of inhibitors and in the presence of 1 and 25 \(\mu\)M HOE-642, suggesting that the potentiating effect of forskolin was due to enhanced NHE4 activity (although we did not perform enough experiments to allow statistical evaluation for each inhibitor separately). The data suggest that hyperosmolality and cAMP are independent and/or additive activators of NHE4. In cultured rabbit parietal cells, rapid and pronounced cellular volume loss occurs during cAMP-mediated stimulation of acid secretion, followed by rapid volume recovery that is to a large part mediated by Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) exchange (31). In contrast to many other cell types, acid-secreting parietal cells do not regulate volume via Na\(^+\)/H\(^+\) exchange activity in this study (~40%), but that a medium change to 400 mosmol/kgH\(_2\)O and stimulation by forskolin result in a similar degree of cellular shrinkage (31), we wondered if cAMP per se stimulates NHE4. After finding that forskolin plus hyperosmolality does not result in a stronger degree of parietal cell shrinkage than hyperosmolality alone (Sonnentag, unpublished observations), we tested the effect of simultaneous application of forskolin and hyperosmolality on NHE4 activation. We found that the simultaneous application of forskolin and a hyperosmolar medium resulted in a significantly higher Na\(^+\)/H\(^+\) exchange rate than hyperosmolality alone. A similar difference was observed in the absence of inhibitors and in the presence of 1 and 25 \(\mu\)M HOE-642, suggesting that the potentiating effect of forskolin was due to enhanced NHE4 activity (although we did not perform enough experiments to allow statistical evaluation for each inhibitor separately). The data suggest that hyperosmolality and cAMP are independent and/or additive activators of NHE4. In cultured rabbit parietal cells, rapid and pronounced cellular volume loss occurs during cAMP-mediated stimulation of acid secretion, followed by rapid volume recovery that is to a large part mediated by Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) exchange (31). In contrast to many other cell types, acid-secreting parietal cells do not regulate volume via Na\(^+\)/K\(^+\)-2Cl\(^-\)-cotransporter (31), and one reason for the strong NHE4 expression in parietal cells may be the necessity to recruit another volume regulatory mechanism besides NHE1 during episodes of cellular shrinkage. The fact that cAMP activates NHE4 may enable parietal cells to simultaneously activate both the apical secretory mechanisms and the basolateral homeostatic mechanisms during cAMP-mediated stimulation of acid secretion.

In summary, this report demonstrates that gastric epithelial cells express the NHE isoforms NHE1, NHE2, and NHE4, with particularly high expression levels for NHE1 and NHE2 in mucous cells and high NHE4 expression levels in parietal and chief cells. Because gastric mucous and parietal cells can be purified and cultured, we were for the first time able to functionally study native NHE2- and NHE4-expressing cells. Surprisingly, acid-induced Na\(^+\)/H\(^+\) exchange activation was mediated by NHE1 and NHE2 in both cell types, with minimal NHE4 contribution. On the other hand, hyperosmolality-induced Na\(^+\)/H\(^+\) exchange activation was mediated by NHE1 and NHE4 in both cell types, with minimal NHE4 contribution. On the other hand, hyperosmolality-induced Na\(^+\)/H\(^+\) exchange activation was mediated by NHE1 and NHE4, and the expression levels paralleled the relative contribution of these isoforms to pH\(_i\)-controlled and hyperosmolality-induced Na\(^+\)/H\(^+\) exchange. NHE4 was activated both by hyperosmolality and cAMP. We therefore conclude that in the gastric epithelium NHE1 and NHE2 regulate pH\(_i\), whereas NHE1 and NHE4 regulate cell volume.

We thank Perikles Kosmidis for technical help, Wolf-Christian Siegel for help with primary cell culture and fluorometric experiments, John Orłowski, Chen Ming Tse, Chris Yun, Mark Donowitz, Zuo Wang, Gary Shull, and Andreas Pfeifer for supplying NHE cDNA fragments, and Richard Wahl for the use of the isotope laboratory.

This work was supported in part by Deutsche Forschungsgemeinschaft Grants Se-46091.9-4 and Se 460/2-5, Eberhard Karls University Tübingen Fortu¨ ne Program Grant Nr.137 (F-1281038), Bundesministerium für Bildung und Forschung Grant Fo-01KS9602, and the Tübingen Interdisciplinary Center for Clinical Research.

This work includes experiments performed by T. Sonnentag and A. Heinzmans toward fulfillment of the requirements for their doctoral theses.

REFERENCES


14. Jacob P, Christiani S, Rossmann H, Lamprecht G, Viollard-Baron D, Muller R, Gregor M, and Seidler U. Role of Na\(^+\)/HCO\(_3\) cotransporter NBC1, Na\(^+\)/H\(^+\) exchanger NHE1, and...


