Demonstration of a functional apical sodium hydrogen exchanger in isolated rat gastric glands

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Kirchhoff, Philipp, Carsten A. Wagner, Florian Gaetzschmann, Klaus Radebold, and John P. Geibel. Demonstration of a functional apical sodium hydrogen exchanger in isolated rat gastric glands. Am J Physiol Gastrointest Liver Physiol 285: G1242–G1248, 2003.—Previous studies have shown that gastric glands express at least sodium-hydrogen exchanger (NHE) isoforms 1–4. Our aim was to study NHE-3 localization in rat parietal cells and to investigate the functional activity of an apical membrane NHE-3 isoform in parietal cells of rats. Western blot analysis and immunohistochemistry showed expression of NHE-3 in rat stomach colocaling the protein in parietal cells together with the β-subunit of the H⁺-K⁺-ATPase. Functional studies in luminally perfused gastric glands demonstrated the presence of an apical NHE isoform sensitive to low concentrations of 5-ethylisopropyl amiloride (EIPA). Intracellular pH measurements in parietal cells conducted in omeprazole-pre-treated superfused gastric glands showed an Na⁺-dependent proton extrusion pathway that was inhibited both by low concentrations of EIPA and by the NHE-3 specific inhibitor S3226. This pathway for proton extrusion had a higher activity in resting glands and was inhibited on stimulation of histamine-induced H⁺-K⁺-ATPase proton extrusion. We conclude that the NHE-3 isoform located on the apical membrane of parietal cells offers an additional pathway for proton secretion under resting conditions. Furthermore, the gastric NHE-3 appears to work under resting conditions and inactivates during periods of H⁺-K⁺-ATPase activity.

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CLASSICALLY, GASTRIC ACID secretion is thought to occur when the H⁺-K⁺-ATPase activates by trafficking to the apical surface of the parietal cell and begins to secrete protons into the secretory canniciuli. The protons then combine with secreted Cl⁻ to form concentrated HCl, resulting in a luminal pH of ~1. This concentrated acid is then extruded from the lumen of the gland and, after passing through a mucus gel layer at the apex of the gland, results in a final secreted acid solution that has a pH of ~4. This process of gastric acid secretion is under both hormonal and neuronal control (13, 20, 23, 24). It has, however, remained only poorly understood what occurs at the apical surface of parietal cells in the gland under resting conditions. If the parietal cells would be completely inactivated following the removal of the H⁺-K⁺-ATPase from the apical surface, it would stop proton secretion into the lumen and, as a result, fail to secrete fluid and protons from the gland to the apex of the gland. Thus a lack of secretion during the resting phase may lead to a collapse of the mucous gel layer at the orifice of the gland into the gland lumen. Thus we hypothesized that there could be an alternative pathway in the gland that activates during the nonstimulated phase of gastric acid secretion to continue a steady efflux of protons out of the gland toward the mucous, thereby maintaining a pore through this mucous gel layer. The active secretion of additional protons in nonstimulated cells or in cells treated with a proton pump inhibitor drugs could be explained with this additional acid extrusion mechanism. In fact, recent reports (8, 16) allude to the fact that more and more patients are becoming resistant to proton pump inhibitor drugs resulting in “breakthrough” experiencing increased periods of non-omeprazole-sensitive acid secretion (7, 8, 25). This phenomenon of acid breakthrough, which is defined as gastric pH <4.0 continuously for >60 min, occurs despite maximal pharmacological inhibition of H⁺-K⁺-ATPase activity. Tachyphylaxis has been ruled out as a possible cause for this condition (8, 16).

Sodium hydrogen exchanger (NHE) activity is found in virtually all prokaryotic and eukaryotic cells, catalyzing the electroneutral exchange of hydrogen for sodium (4, 12). In mammals, at least eight distinct isoforms have been identified on a molecular level (4, 6). These various NHE isoforms are involved in the regulation of intracellular pH (pHi) and cell volume and aid in modulating extracellular fluid Na⁺ concentration. Factors regulating their activity include hormones, intracellular cAMP, and Ca²⁺ concentrations and acute or chronic extracellular acidification (4).

In the stomach, NHE activity was first reported by Paradiso et al. (14, 15). More recent work identified at...
least isoforms 1–4 in mucous, endocrine, and parietal cells of the rabbit, rat, and mouse stomach (2, 17, 19, 22). Whereas the role of the NHE-1, -2, and -4 isoforms in the stomach have been addressed in several studies (2, 19, 21), the role and localization of the NHE-3 isoform has not been examined in detail. The present study was designed to investigate specifically the role of NHE-3 and the possibility of an apical functional NHE isoform in parietal cells of freshly isolated rat gastric glands and to investigate whether this transport protein is normally active under resting conditions.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats 150–250 g (Charles River Laboratories) were housed in climate- and humidity-controlled, light-cycled rooms, fed standard chow with free access to water, and handled according to the humane practices of animal care established by the Yale Animal Care and Use Committee. Before experiments, animals were starved for 12 h with free access to water.

Membrane preparation and Western blot analysis. After kidneys and stomachs were removed from Sprague-Dawley rats, gastric glands were harvested with a macrodissection to remove the muscularis and then suspended in an ice-cold K-HEPES buffer (in mM: 200 mannitol, 80 K-HEPES, 41 KOH, pH 7.5) with pepstatin, leupeptin, K-EDTA, and PMSF added as protease inhibitors. To obtain crude membrane fractions, tissues were homogenized and centrifuged at 1,000 g for 10 min; the supernatant was collected and centrifuged at 100,000 g for 60 min. The pellet containing the plasma membranes was resuspended in homogenization buffer and stored at −80 °C until use. Fifty micrograms of membrane proteins were solubilized in sample loading buffer and separated on a 8% polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes, with unspecific protein binding blocked by PBS containing 5% nonfat dry milk. The membranes were incubated with either a polyclonal anti-NHE-3 antibody 1314 (1:5,000, friendly gift from Dr. O. Moe, Dallas (3)) for 2 h at room temperature. The membranes were washed three times, blocked for 1 h, and incubated for 1 h at room temperature with the secondary goat anti-rabbit antibody 1:5,000 linked to alkaline phosphatase (Promega). The protein signal was detected with the CDP Star chemiluminescence system (Roche Diagnostics).

Immunohistochemistry. Male Sprague-Dawley rats (200–250 g) were anesthetized with ketamine and perfused through the left ventricle with PBS followed by paraformaldehyde-lysine-periodate (PLP) fixative (11). Stomachs were removed, cleaned from food residues, and fixed overnight at 4°C by immersion in PLP. Stomachs were washed three times with PBS, and semithin sections were cut at a thickness of 1 μm after cryoprotection with 2.3 M sucrose and 50% polyvinylpyrrolidone in PBS for at least 12 h. Immunostaining was carried out as described previously (26). Sections were incubated with 1% SDS for 5 min, washed three times with PBS, and incubated with PBS containing 1% bovine serum albumin for 15 min before the primary antibody. The primary antibodies (mouse monoclonal anti-human β-gastric F-ATPase (Affinity Bioreagents), goat polyclonal, affinity-purified anti-mouse NHE-1 (COOH terminal; Santa Cruz Biotechnology), and rabbit polyclonal anti-rat NHE-3 [antibody 1568 kindly provided by Dr. O. Moe, Dallas (3)] or a commercial affinity-purified antibody from Chemicon was diluted 1:1,000, 1:100, or 1:50, respectively, in PBS and applied overnight at 4°C. Sections were then washed twice for 5 min with high-NaCl PBS (PBS + 2.7% NaCl) and once with PBS and then incubated with the secondary antibodies (donkey anti-rabbit Alexa 546, donkey anti-goat Alexa 488, donkey anti-mouse Alexa 488; Molecular Probes) and 4,6-diamidino-2-phenylindole (Sigma) for nuclear staining at dilutions of 1:1,000, 1:400, 1:200, and 1:50, respectively, for 1 h at room temperature. Sections were washed twice with high-NaCl PBS and once with PBS before mounting with VectaMount (Vector Laboratories). The specimens were viewed with a Nikon E–800 microscope or a Leica SP1 ultraviolet confocal laser scanning microscope.

Isolation of gastric glands. After the removal of the stomach, it was opened longitudinally and the corpus and antrum were isolated and sliced into 0.5-cm² sections and washed with cold Ringer solution to remove residual food particles. The tissues were transferred to the stage of a dissecting microscope. Individual glands were isolated using a hand-dissection technique as described previously (5, 18, 27, 28). After isolation, individual isolated glands were either allowed to adhere to coverslips precoated with Cell-Tak (Collaborative Research) or were fixed between a series of concentric glass micropipettes to allow both apical and basolateral perfusion using a technique similar to that previously described (27, 28). For the superfusion studies, the individual hand-dissected glands were transferred to the stage of an inverted microscope and attached to a glass coverslip using the biological adhesive Cell-Tak (Collaborative Research).

Perfusion of isolated gastric gland lumens. Individual hand-dissected gastric glands were transferred to the stage on an inverted microscope and attached to a series of concentric glass micropipettes. Perfusion was established by cannulation of the proximal and distal end of the gland so that the apical and/or basolateral perfusates could be changed using a technique that we previously described in detail (27, 28). This exchange technique allowed us to directly control the concentration of ions on either membrane independently as well as to add hormones or inhibitors directly to either membrane surface. In addition to controlling the perfusion pressure and flow rate, we continuously monitored the lumen of the gland with the imaging system and could confirm that flow was maintained during the course of the experiment.

Digital imaging for pHi measurements. Isolated gastric glands were incubated in a HEPES-buffered Ringer solution containing 10 μM BCECF-AM (Molecular Probes) for 10 min as described previously (5). After being loaded, the chamber was flushed with Ringer solution to remove deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Olympus IX70; Olympus), which was used in the epifluorescence mode with a ×40 objective. BCECF was successively excited at 440 ± 10 and 490 ± 10 nm, and the resultant fluorescent signal was monitored at 535 ± 10 nm using an intensified charge-coupled device camera. Individual regions of interest were outlined and simultaneously monitored during the course of the study. In both the perfused and superfused glands, a minimum of seven cells or regions were selected per gland. All data including the individual images for both the 440- and 490-nm wavelengths were recorded to the hard disk, which allowed us to return to the individual images after the experiment for further analysis.

Intensity ratio data (490/440) were converted to pH values using the high-K+/nigericin calibration technique. Data are expressed as change in pH per minute, and percentages were calculated using Gauss’ law of error propagation and tested for significance using Student’s t-test.
All studies were carried out in the nominal absence of \( \text{HCO}_3^-/\text{H}_2\text{CO}_3 \), and acidification was accomplished using the standard ammonium chloride prepulse technique (20 mM) (27, 28). After acidification, glands were bathed either in a standard HEPES or a zero Na\(^+\)/H\(_2\)CO\(_3\)Ringer solution.

All chemicals were obtained from Sigma; 5-ethylisopropyl amiloride (EIPA) was from Molecular Probes. The NHE-3 specific inhibitor S3226 was a friendly gift from Drs. G. Punter and H. J. Lang (Aventis; Frankfurt, Germany). Omeprazole was a kind gift of Dr. K. Andersson (AstraZeneca; Mölndal, Sweden).

RESULTS

Western blot analysis. Our results confirm the presence of NHE-3 protein in rat gastric mucosa (Fig. 1). Controls were taken from rat kidney, which expresses high amounts of NHE-3 protein. Identical molecular weight bands were found in the protein isolated from stomach and kidney.

Immunocytochemistry. Figure 2 shows staining for the NHE-3 protein, demonstrating a selective parietal cell-specific staining pattern (A and D). Figure 2, B and E, shows co-staining for the \( \beta \)-subunit of the gastric H\(^+\)-K\(^+\)-ATPase, which localizes specifically to the gastric parietal cells. Overlays of both images demonstrate colocalization of both proteins in the same cells, identifying these cells as parietal cells. Immunohistochem-
istry, however, did not allow safe conclusions as to the apical localization of NHE-3 in parietal cells. As also demonstrated in Fig. 3, A and C, NHE-3 (red) and the β-subunit of the gastric H^+-K^+-ATPase (green) colocalize in parietal cells. Immunolabeling of the same sections with antibodies against the NHE-1 isoform (red) together with the β-subunit of the gastric H^+-K^+-ATPase (green) revealed that NHE-1 could be only detected in cells negative for NHE-3 and the H^+-K^+-ATPase (Fig. 3, B and D). NHE-1-positive cells were smaller than the neighboring parietal cells and found only in the neck region of the gastric gland.

Perfusion of gastric gland lumen. We determined that in perfused nonstimulated gastric glands from rats that had been fasted for 12 h before the experiment, bilateral removal of Na^+ resulted in a rapid and reversible acidification of parietal cells to ~6.60 ± 0.02 (n = 5 animals, 3 glands per animal, 6 cells per gland; see Fig. 4A). After this acidification, addition of Na^+ to the lumen resulted in a rapid pH_i alkalization of parietal cells that was inhibitable with the NHE activity inhibitor EIPA (10 μM) added to the luminal perfusate (control: 0.26 ± 0.01 ΔpH/min, EIPA: 0.02 ± 0.01 ΔpH/min; see Fig. 4, B and D). Luminal EIPA, however, had no influence on the basolateral Na^+-dependent pH_i recovery (Fig. 4, B and D). In a separate series of experiments, perfused glands were pretreated with histamine (10^-7 M) and the H^+-K^+-ATPase inhibitor omeprazole (100 μM) before exposure to the bilateral Na^+-free solution (see solution compositions in Table 1). Under these conditions, there was minimal Na^+-dependent pH_i alkalization from the lumen (0.03 ± 0.01 ΔpH/min; see Fig. 4, C and D). These data are consistent with the presence of an apical NHE on the luminal side of gastric parietal cells with a sensitivity against EIPA similar to NHE-3 (9, 12).

pH_i measurements in superfused gastric glands. Results are based on investigations using at least three animals per experimental group and monitoring at least eight parietal cells in three different glands. In control experiments, in the presence of omeprazole (100 μM) without histamine, the pH_i recovery rate was 0.18 ± 0.01 pH units/min after a change from the sodium-free bath to sodium-containing HEPES solution (see Fig. 5, A and B). When 10 μM amiloride was added to the bath, we found no significant change in the recovery rate following Na^+ readdition, which suggests the involvement of an amiloride-insensitive apical NHE-3 (0.21 ± 0.02 pH units/min) and is consistent with the data obtained in the luminally perfused glands. We followed this series of studies by adding the NHE-3-specific inhibitor HOE S3226 (100 nM) together with 10 μM amiloride and found that the Na^+-dependent recovery was almost completely reduced by 92.9 ± 13.0% under these conditions (0.02 ± 0.002 pH units/min). Addition of 10 μM EIPA, which has been
Fig. 4. Intracellular pH (pH$_i$) measurements in single parietal cells in isolated luminally perfused rat gastric glands. A: original pH$_i$ tracing of a single parietal cell, demonstrating apical and basolateral Na$^+$/H$^+$ exchange activities on selective basolateral or lumenal Na$^+$/H$^+$ removal and addition. Removal of Na$^+$ from both bath and lumenal perfusate led to intracellular acidification, addition of only lumenal Na$^+$ alkalinized the pH$_i$ of the parietal cells, and basolateral Na$^+$-dependent alkalinization was not affected by luminal EIPA. C: effect of stimulation of gastric acid secretion by histamine (10$^{-7}$ M) on the luminal Na$^+$/H$^+$-dependent pH$_i$ alkalinization. To unmask all NHE activity, H$^+$/K$^+$-ATPase activity was inhibited by omeprazole. Stimulation of parietal cells with histamine led to the complete inhibition of the luminal but not basolateral NHE activity. D: bar graph summarizing the effects of separate luminal and basolateral removals and addition of Na$^+$ on pH$_i$, the inhibition of the apical Na$^+$/H$^+$ exchange activity with EIPA (10$^{-5}$ M), and the effect of histamine on the luminal Na$^+$/H$^+$-dependent pH$_i$ recovery.

Table 1. Composition of solutions

<table>
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<th>Standard HEPES, mM</th>
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<th>Na$^+$-free HEPES + NH$_4$Cl, mM</th>
<th>High K$^+$ calibration, mM</th>
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<td>1</td>
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</tr>
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<td>7.4</td>
<td>6.0, 7.0, 8.0</td>
</tr>
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</table>

N-methyl-D-glucamine (NMDG) was titrated with HCl. All solutions were titrated to pH 7.4 at 37°C using either NaOH or KOH. NMDG was titrated with HCl. 5-Ethylisopropyl amiloride and HOE S3226 were added to the solutions from a stock solution dissolved in DMSO or water, respectively. Omeprazole and/or histamine were added directly to the individual solutions above. For the nigericin solution, nigericin was added from an ethanol stock solution to the high-K solution, and the pH of the solution was adjusted to 6.5, 7.0, and 7.5 for each study.

Fig. 5. pH$_i$ measurements in single parietal cells in isolated superfused rat gastric glands. A: original pH$_i$ tracings demonstrating the effects of several NHE inhibitors [amiloride (10$^{-5}$ M), EIPA (10$^{-5}$ M), Hoe 3326 (100 nM)] and histamine (100 nM) on Na$^+$/H$^+$-dependent pH$_i$ recovery after an acid load (NH$_4$Cl prepulse, not shown as part of the tracing). B: bar graph summarizing the effects of NHE inhibitors and histamine on Na$^+$/H$^+$-dependent pH$_i$ recovery. Amiloride inhibiting the basolateral NHE-1 isoform had no effect on pH$_i$ recovery rates, indicating that the Na$^+$/H$^+$-dependent pH$_i$ recovery was not due to NHE-1. EIPA inhibiting NHE-1 to -3 at this concentration completely inhibited pH$_i$ recovery. The NHE-3 specific inhibitor Hoe 3326 inhibited ~50% of the pH$_i$ recovery, demonstrating that NHE-3 contributes substantially to overall NHE activity. Histamine reduced the amiloride-insensitive NHE activity by ~50%.
shown at that concentration to inhibit NHE-1 to -3 isoforms and inhibited the Na$^+$-dependent recovery by 94.3 ± 8.4% (0.01 ± 0.001 pH units/min).

We then examined the effects of activation of gastric acid secretion by histamine (in the presence of omeprazole and amiloride) on the activity of the Na$^+$-dependent pH$\text{I}$ recovery. Activation of parietal cells by pre-incubation with histamine (10$^{-7}$ M) resulted in a greatly decreased Na$^+$-dependent recovery by 62.3 ± 6.2% (0.079 ± 0.001 pH units/min), which is a similar result to that found in the perfused preparation. These results suggest a small Na$^+$-dependent component that is not histamine inhibitable, consistent with the presence of several NHE isoforms differentially regulated.

**DISCUSSION**

The results of our study give evidence that the rat parietal cell not only has a functional NHE isoform at the basolateral membrane (1) but in addition has a functional NHE-3 protein that appears to be localized to the apical pole of the cell in the same region as the H$^+$-K$^+$-ATPase. The idea of multiple isoforms for NHE being present in the stomach was recently proposed following a molecular characterization of parietal cell preparations (2, 19). It has been shown that the NHE isoforms NHE-1, -2, and -4 contribute to different cellular functions in gastric parietal and mucous cells, such as pH$\text{I}$ regulation or cell-volume regulation (2, 19). Loss of the NHE-2 isoform in mouse stomach is associated with severe alterations of parietal cell function and morphology (21). We have now for the first time demonstrated in a native tissue preparation that 1) parietal cells have a functional apical NHE protein, 2) the apical NHE isoform is functional in the resting cells and can act as a proton extrusion mechanism from these cells when they are at rest, 3) the NHE activity is downregulated by histamine, and 4) the NHE-activity has pharmacological characteristics consistent with the NHE-3 isoform. These data may also explain how the gland is capable of maintaining a large proton gradient at the luminal surface of the gland even while the glands are in a nonstimulated state. We have also been able to demonstrate that during histamine stimulation, the cells appear to inactivate this apical proton efflux pathway and use the more potent H$^+$-K$^+$-ATPase to maintain the proton efflux that is responsible for driving luminal pH to ~1, which leads to concentrated acid efflux from the lumen of the gland into the interior of the stomach. Inactivation of the apical NHE isoform during active gastric acid secretion may even protect the parietal cell from a reversal of the Na$^+$+/H$^+$-exchange mechanism leading to proton influx into the cell. The identification of NHE-3 along with the recent description of Na$^+$-$\text{K}^+$-$2\text{Cl}^-$ cotransporter (NKCC1) activity in the rat gastric gland (10) and its localization to the parietal cell identifies novel additional electrolyte pathways that may be involved in maintaining the intra- and extracellular environment of both resting and stimulated glands. These transport proteins may act to maintain a continuous secretion at rest and, at the same time, permit the gland to efflux a small amount of protons from the lumen of the gland, which could prevent the mucous gel layer from collapsing into the gland and plugging the orifice of the gland. When taken with the recently identified NKCC1 protein in the parietal cell, this may explain the reduced levels of gastric acid in guinea pigs treated with bumetanide (1), because the apical efflux of Cl$^-$ could be reduced under these conditions.

With our observations of inactivation in the presence of histamine, these data could also explain some previous findings in patients experiencing gastric acid secretion breakthrough in the presence of H$\text{2}$ receptor and H$^+$-$\text{K}^+$-ATPase inhibitors. This clinical observation might be explained by a model in which basal acid secretion occurs through NHE activity despite complete inhibition of the classic acid secretory mechanisms, with the problem only being exacerbated in the presence of H$\text{2}$ blockers because histamine would be unable to downregulate NHE activity under these conditions. Further experiments, however, will be needed to clarify whether NHE activity may account for additional non-H$^+$-$\text{K}^+$-ATPase acid secretion in these patients.

In summary, we showed the presence of NHE-3 in rat stomach parietal cells, and we identified a functional NHE on the luminal side of rat gastric parietal cells, that has a pharmacological profile consistent with NHE-3 and inactivates on stimulation of parietal cells with histamine. The identification of the apical NHE-3 protein may aid in effectively developing additional therapies for acid secretory disease and provide a potential mechanism for drug resistance to classic proton pump inhibitors.

**DISCLOSURES**

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**REFERENCES**


