Different acid secretagogues activate different Na\(^+\)/H\(^+\) exchanger isoforms in rabbit parietal cells

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Bachmann, O., T. Sonnentag, W.-K. Siegel, G. Lamprecht, A. Weichert, M. Gregor, and U. Seidler. Different acid secretagogues activate different Na\(^+\)/H\(^+\) exchanger isoforms in rabbit parietal cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1085–G1093, 1998.—Rabbit parietal cells express three Na\(^+\)/H\(^+\) exchanger isoforms (NHE1, NHE2, and NHE4). We investigated the effects of carbachol, histamine, and forskolin on Na\(^+\)/H\(^+\) exchange activity and acid formation in cultured rabbit parietal cells and tested the effect of NHE isoform-specific inhibition on agonist-induced Na\(^+\)/H\(^+\) exchange. Carbachol (10\(^{-4}\) M) was the weakest acid secretagogue but caused the strongest Na\(^+\)/H\(^+\) exchange activation, which was completely blocked by 1 µM HOE-642 (selective for NHE1); histamine (10\(^{-4}\) M) and forskolin (10\(^{-5}\) M) were stronger stimulants of \(^{14}C\)aminopyrine accumulation but weaker stimulants of Na\(^+\)/H\(^+\) exchange activity. HOE-642 (1 µM) reduced forskolin-stimulated Na\(^+\)/H\(^+\) exchange activity by 35%, and 25 µM HOE-642 (inhibits NHE1 and -2) inhibited an additional 13%, but 500 µM dimethyl amiloride (inhibits NHE1, -2, and -4) caused complete inhibition. The presence of 5% CO\(_2\)-HCO\(_3\) markedly reduced agonist-stimulated H\(^+\) efflux rates, suggesting that the anion exchanger is also activated. Hyperosmolarity also activated Na\(^+\)/H\(^+\) exchange. Our data suggest that, in rabbit parietal cells, Ca\(^{2+}\)-dependent stimulation causes a selective activation of NHE1, whereas cAMP-dependent stimulation activates NHE1, NHE2, and more strongly NHE4. Because intracellular pH (pHi) did not change in the presence of CO\(_2\)-HCO\(_3\) and concomitant activation of Na\(^+\)/H\(^+\) and anion exchange is one of the volume regulatory mechanisms, we speculate that the physiological significance of secretagogue-induced Na\(^+\)/H\(^+\) exchange activation may not be related to pH, but to volume regulation during acid secretion.

NHE1; NHE2; NHE4; stomach; acid secretion; histamine; forskolin; carbachol; intracellular pH; volume regulation

THE INVESTIGATION OF ION homeostasis and intracellular pH (pHi) control in the gastric parietal cells during acid secretion has yielded controversial results (7, 14, 15, 27, 30). Investigators have all found an increase in the anion flux rate via the basolateral anion exchanger, although the mode of activation has remained obscure (7, 15, 18, 27), and many have also observed a slight increase in the pHi during secretagogue stimulation of the parietal cells (14, 15, 18). It has remained unclear whether this increase was due to the action of the H\(^+\)-K\(^-\)-ATPase per se or activation of an Na\(^+\)/H\(^+\) exchanger (15, 18, 27). Also, the functional significance, if any, of this pH increase is controversial (7, 15, 18, 27). Thus it is still largely unclear how the parietal cell accomplishes ion, pH, and volume homeostasis during acid formation.

Several years ago, a method was developed by Chew and co-workers (6) that allowed the maintenance of isolated rabbit parietal cells in culture for a period of up to 2 wk with preserved H\(^+\)-K\(^-\)-ATPase expression and a better response to secretagogues than seen in freshly isolated cells. Saccomani et al. (21) demonstrated that cultured parietal cells display a polarized membrane arrangement. We have therefore speculated that parietal cells maintained in culture may be an interesting model to study possible changes in pH, and ion transport processes in response to hormonal stimulation. In this study, we examine the effects of secretagogues and hyperosmolarity on parietal cell Na\(^+\)/H\(^+\) exchange activity and pH, and investigate which of the three different Na\(^+\)/H\(^+\) exchanger isoforms expressed in rabbit parietal cells (namely, NHE1, NHE2, and NHE4) are activated by the respective secretagogues.

MATERIALS AND METHODS

Materials. 4-Iso propyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE-642) was prepared by Hoehst (Frankfurt, Germany). 3-(Cyano methyl)-2-methyl-8-(phenylmethoxy)-imidazo-[1,2a]-pyridine (Sch-28080) was from Schering (Berlin, Germany). Collagenase was from Worthington (Freehold, NJ). Pronase E was from Merck (Darmstadt, Germany). BSA (cell culture grade) was from Paesel und Lorei (Frankfurt, Germany). \(^{14}C\)aminopyrine was from Amersham (Braunschweig, Germany). A 1:1 mixture of Ham’s F-12 and DMEM (with HEPES and L-glutamine and without HCO\(_3\)\(_{-}\)), HEPES, Tris, epidermal growth factor (EGF) (murine; cell culture grade), histamine, carbachol, insulin, transferrin, sodium selenite, hydrocortisone, gentamicin, novobiocin, and gentamicin were from Sigma (Deisenhofen, Germany); ranitidine was from Glaxo Wellcome (Bad Oldesloe, Germany), and Nycodenz was from Nycomed (Oslo, Norway). Matrigel was from Becton Dickinson (Bedford, MA). 2’,7’-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was from Molecular Probes (Leiden, The Netherlands). Antibody HK12.18 (against the cytoplasmatic domain of hog H\(^+\)-K\(^-\)-ATPase) was a generous gift from Dr. Adam Smolka (University of South Carolina, Charleston, SC). All other chemicals were either from Sigma or from Merck at tissue culture grade or the highest grade available.

Isolation and culture of rabbit parietal cells. Cells were isolated as described previously (25–27) with minor differences as described below. Cell culture was adapted from the method published by Chew et al. (6). Rabbit gastric cells were enzymatically dispersed after high-pressure perfusion of the stomach in situ as previously described (25, 27). Elutriation
was performed using a Beckman J M 6-C centrifuge with a JE-5.0 quick-assembly rotor. The elutriation buffer was composed of (in mM) 140 NaCl, 14 HEPES, 7 Tris, 3 K2HPO4, 1.2 CaCl2, 1.2 MgSO4, and 20 glucose and 1 g/l BSA, 0.5 mM dithiothreitol (DTT), and 10 mg/l gentamicin, pH 7.4. The cell suspension was loaded into the 5-ml small chamber, and cells were elutriated at a constant rotor speed of 1,750 rpm in four fractions with flow rates of 15, 30, 35, and 65 ml/min. Fraction 4, primarily consisting of parietal and chief cells, was then loaded on top of a Nycodenz step gradient (2:1, 1:1, and 1:2 dilution of Nycodenz-elutriation buffer) and centrifuged at 1,000 g for 8 min without brake. The second band (thick yellow) was aspirated and diluted to 20 ml in culture medium (DMEM-Ham's F-12 medium containing 2 g/l albumin, 800 nM insulin, 5 mg/l transferrin, 5 µg/l sodium selenite, 10 nM hydrocortisone, 8 nM EGF, 5 mg/l gentamicin, 50 mg/l novobioin, and 100 mg/l gentamicin). The suspension was then washed, and 250,000 cells/dish were placed onto 22-mm coverslips (for fluorescence studies) or directly into 35-mm culture dishes (for aminopyrine uptake studies) that were coated with 50 µl of Matrigel (diluted 1:7 in ice-cold H2O). Cells were cultured in a humidified incubator at 37°C in air. The medium was changed every day.

Immunofluorescence. Immunofluorescence studies were carried out as described by Soroka et al. (28) with minor differences. Briefly, cultured cells were incubated for 45 min in buffer B (114.4 mM NaCl, 5.4 mM KCl, 5 mM Na2HPO4, 1 mM NaH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 2 g/l BSA, 10 mM glucose, 0.5 mM DTT, 1 mM pyruvate, and 10 mM HEPES, pH 7.4) ± 1 mM histamine, washed with buffer C (60 mM PIPES, 5 mM EGTA, and 2 mM MgCl2, pH 6.8, prewarmed to 37°C), and fixed for 15 min at room temperature with 3% freshly prepared parafomaldehyde in buffer C. After cells were washed twice with PBS, they were permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed twice again with PBS. Antibody HK-12.18 was applied at 1:25 dilution in 0.1% BSA-PBS (pH 7.15) for 3 h. Cells were incubated for 30 min with FITC-labeled goat anti-mouse IgG as second antibody and then photographed using an AGFA RS-1000 film and 3-min exposure.

Fluorescence microscopy for determination of pHi. Cultured cells were incubated with 5 µM BCECF-AM (Molecular Probes) in buffer (in mM: 140 NaCl, 14 HEPES, 7 Tris, 3 K2HPO4, 1.2 CaCl2, 1.2 MgSO4, and 20 glucose) for 30 min at 37°C. Cells were then washed free of BCECF-AM and incubated for 30 min at 37°C in buffer and washed again, and the coverslips were mounted in a custom-made perfusion chamber that was placed on the heated stage of an inverted fluorescence microscope (Nikon Diaphot-TMD). Preheated and continuously gassed solutions were connected by a mani-
fold to the chamber, allowing rapid fluid changes without interruption of flow. Cells were alternately excited at 440 ± 10 nm and 490 ± 10 nm at a rate of 100/s. Emitted light from an individual group of cells was collected through a 510-nm dichroic mirror, a 530-nm long-pass filter, and an adjustable diaphragm and recorded by a photomultiplier. Data acquisition (1 ratio values) and processing were performed using the software provided by the manufacturer (Photon Technologies, Wedel, Germany) except for the excitation shutter, which was controlled by a custom-made program that allowed changes between continuous and intermittent (to reduce photobleaching) illumination during the experiment. At the end of each experiment, the 440 nm-to-490 nm ratio was calibrated to pHi after clamping pH i to extracellular pH using the high K⁺-nigericin method (100 mM potassium gluconate, 40 mM KCl, 14 mM HEPES, 7 mM Tris, 3 mM KH₂PO₄, 2 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 20 mM glucose, and 10 μM nigericin, pH 6.6 or pH 7.4). Background fluorescence was found to be negligible and was not corrected for.

Determination of the intrinsic buffering capacity. Intrinsic buffering capacity (βi) was determined as previously described by Boyarsky et al. (2, 3). Typical pH₁-dependent intrinsic buffering curves with a βi value of 70 mM/pH unit at pH₁ of 6.6, 38 mM/pH unit at 7.0, and 21 mM/pH unit at 7.4 were obtained and were used to calculate H⁺ efflux rates for each given pH₁. The shape of the curve resembles those of many other cell types and is not shown.

[¹⁴C]aminopyrine uptake. [¹⁴C]aminopyrine uptake was studied as described by Chew and Hersey (5), with minor differences. After cells were washed twice and preincubated for 60 min in buffer B, cells were incubated with 1 ml of buffer B plus 0.05 μCi [¹⁴C]aminopyrine for 30 min. Agonists were then added for 45 min. Next, the supernatant was discharged, and cells were washed twice with buffer B and then lysed with 1 ml 3% Triton X-100, 500 μl of which were counted in a beta counter.

Statistics. Results are given as means ± SE. H⁺ fluxes were calculated by performing linear regression analysis on individual pH₁ traces during the first 1–2 min of stimulation (linear phase). ANOVA was used for multiple comparisons.

RESULTS

Validation of the parietal cell culture. Before the start of the experiments in this study, a number of experiments were performed to validate the parietal cell culture. Immunocytochemical staining of H⁺-K⁺-ATPase revealed that almost all cells in the culture express this protein even after 7 days of culture (Fig. 1). Cell number was determined over the length of the culture. We observed a dramatic increase in basal [¹⁴C]aminopyrine uptake between day zero and day one, whereas agonist-stimulated [¹⁴C]aminopyrine uptake remained similar; therefore, the ratio of [¹⁴C] uptake between stimulated and unstimulated cells declined sharply (Fig. 2). We then used acridine orange to stain the acidic membrane compartments in the parietal cells and observed that, in contrast to freshly isolated cells, in which the secretory membranes are collapsed, quite a few parietal cells display red vesicle structures that shift their color to green under omeprazole treatment (data not shown). Thus the reason for the high basal [¹⁴C]aminopyrine uptake is spontaneous acid formation in many of the cultured cells. Histamine treatment resulted in the appearance of more or enlargement of preexisting acidic compartments.

Determination of acid secretion by [¹⁴C]aminopyrine uptake after stimulation with different agonists. To demonstrate appropriate acid secretory responsiveness and to determine differences in potency of the agonists of acid formation used in this study, [¹⁴C]aminopyrine uptake was measured on stimulation and compared with a control that was pretreated with ranitidine to prevent effects from potential endogenous histamine release. Carbachol (10⁻⁴ M) stimulated [¹⁴C]aminopyrine uptake 2.6 ± 0.6-fold (n = 5), 10⁻⁵ M histamine stimulated uptake 4.0 ± 0.7-fold, and 10⁻⁵ M forskolin stimulated uptake 3.9 ± 0.5-fold compared with control. The difference between [¹⁴C]aminopyrine uptake values in control incubated in the presence and absence of 10⁻⁵ M ranitidine was not significant, indicating that contamination with histamine-releasing cells is small.

Effects of agonists of acid secretion on pH₁ and Na⁺ / H⁺ exchange activity. The resting pH₁ in clusters of four to six cultured parietal cells, measured microfluorometrically with the fluorescent dye BCECF, was found to be 7.16 ± 0.02 pH units over the whole time course of the experiments. Carbachol, which was the weakest agonist for [¹⁴C]aminopyrine uptake, caused a consistent increase in parietal cell resting pH₁ of 0.2 ± 0.03 pH units (Fig. 3A), which was inhibited by the concomitant addition of 10⁻⁶ M atropine or intracellular...
lar Ca$^{2+}$ chelation by preincubation with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM (data not shown). Forskolin and histamine, which were both stronger agonists for [$^{14}$C]aminopyrine uptake, also caused increases of parietal cell pH$_i$ of 0.15 ± 0.02 and 0.13 ± 0.03 pH units (P < 0.001, n = 5–7) (Fig. 3A). Omeprazole pretreatment caused rapid cellular acidification after stimulation, but the pH$_i$ increase was unchanged (Fig. 4A). Sch-28080 also did not influence the pH$_i$ increase. On the other hand, Na$^+$ removal completely abolished the response to all secretagogues, and 100 µM dimethyl amiloride (DMA) completely inhibited the response to carbachol but not to histamine and forskolin (data not shown), whereas 500 µM DMA completely inhibited the pH$_i$ increase seen with any secretagogue (data not shown). This suggests that Na$^+/H^+$ exchanger activation was the underlying mechanism for the pH$_i$ rise with all tested secretagogues. To quantify Na$^+/H^+$ exchange activation, we determined the H$^+$ efflux rate during the initial 1–2 min after stimulation.}

**Fig. 3.** A: all 3 secretagogues (forskolin, histamine, and carbachol) caused a significant increase in parietal cell intracellular pH (pH$_i$) in a HEPES-buffered, O$_2$-gassed perfusate. B: 1 µM HOE-642 (specific for the Na$^+/H^+$ exchanger isoform NHE1 in this concentration) inhibited these pH$_i$ increases in a variable fashion. C: initial secretagogue-induced H$^+$ efflux rates as a measurement of Na$^+/H^+$ exchanger activation and effect of 1 µM HOE-642 and 500 µM dimethyl amiloride (DMA) on these rates. ns, Not significant.
secretagogue stimulation (linear pH\textsubscript{i} increase) (Figs. 3C and 4B and Table 1).

In the presence of 5% CO\textsubscript{2}-HCO\textsubscript{3} -, the parietal cell resting pH\textsubscript{i} was 7.05 ± 0.04, and the effect of all three agonists on pH\textsubscript{i} was either markedly reduced or insignificant. Because this marked reduction of the effect of the secretagogue on pH\textsubscript{i} could not be solely explained by the intracellular buffer capacity, which is ~30–40% higher in the presence of 5%CO\textsubscript{2}-HCO\textsubscript{3} - in the observed resting cell pH\textsubscript{i} range, we determined the initial H\textsuperscript{+} flux rates because, unlike pH\textsubscript{i} changes, the H\textsuperscript{+} flux rates are independent of the intracellular buffer capacity. We found that, in the presence of 5% CO\textsubscript{2}-HCO\textsubscript{3} -, carbachol elicited an initial H\textsuperscript{+} efflux rate of −0.006 ± 0.14 mM/min (n = 3), forskolin of 0.069 ± 0.03 mM/min (n = 4), and histamine of 0.11 ± 0.07 (n = 3). These data demonstrate that the H\textsuperscript{+} efflux rates are much lower in the absence than in the presence of 5% CO\textsubscript{2}-HCO\textsubscript{3} - and suggest that secretagogue stimulation of parietal cells in CO\textsubscript{2}-HCO\textsubscript{3} - activates an acid-loading mechanism as well, most likely the anion exchanger.

Effects of NHE-specific inhibition on the agonist-mediated pH\textsubscript{i} rise. The differential sensitivities of the carbachol-, histamine-, and forskolin-induced Na\textsuperscript{+}/H\textsuperscript{+} exchange activation to DMA suggested to us that different isoforms are likely stimulated by the different agonists. We therefore tested the effect of the NHE1-specific inhibitor HOE-642 on secretagogue-induced Na\textsuperscript{+}/H\textsuperscript{+} exchange activation. HOE-642 has been shown to block the different NHE subtypes in a strictly concentration-dependent manner, which made it a very useful compound for us to identify the NHE isoforms involved in secretagogue-induced Na\textsuperscript{+}/H\textsuperscript{+} exchanger activation. Preincubation with 1 µM HOE-642, a concentration that will fully inhibit NHE1 in transfected fibroblasts (22), completely inhibited the carbachol-induced H\textsuperscript{+} efflux but only 28% and 35% of the histamine- and forskolin-induced flux rates, respectively (Fig. 3, B and C). Increasing the concentration of HOE-642 to 25 µM, a concentration that will fully inhibit NHE2 activity, caused an additional 13% inhibition of the initial H\textsuperscript{+} flux rate, but 500 µM DMA, which has been shown to inhibit NHE4 activity in transfected fibroblasts (1), caused complete inhibition (Fig. 5, B and C). Because only these three isoforms are expressed in rabbit parietal cells, the results suggest that Ca\textsuperscript{2+}-mediated stimulation causes a selective stimulation of the rabbit parietal cell NHE1, whereas cAMP-dependent stimulation activates predominantly NHE4 and to a lesser extent NHE1 and NHE2.

To further substantiate the hypothesis that forskolin activates NHE4, we tested the effect of cytochalasin D on forskolin-mediated Na\textsuperscript{+}/H\textsuperscript{+} exchange activation because this substance has been reported to substantially reduce acid-induced stimulation of NHE4 expressed in fibroblasts (1). A 5-min preincubation with 5 µM cytochalasin D reduced forskolin-induced Na\textsuperscript{+}/H\textsuperscript{+} exchange activation by ~66% (Fig. 6).

Effect of hyperosmolarity on parietal cell Na\textsuperscript{+}/H\textsuperscript{+} exchange. The absence of a secretagogue-induced pH\textsubscript{i} change in physiological CO\textsubscript{2}-HCO\textsubscript{3} - concentrations and the apparent activation of both Na\textsuperscript{+}/H\textsuperscript{+} and anion exchange by all secretagogues suggested to us that the physiological significance of agonist-induced Na\textsuperscript{+}/H\textsuperscript{+} exchanger activation in parietal cells could be a volume regulatory response after secretion-associated cell shrinkage. Hyperosmolarity activates Na\textsuperscript{+}/H\textsuperscript{+} exchange in many cell types (10, 11); therefore, the effect of hyperosmolarity on parietal cell Na\textsuperscript{+}/H\textsuperscript{+} exchange

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<th>Table 1. Proton efflux rates during the first minute of forskolin stimulation under different experimental conditions</th>
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<td>Forskolin only</td>
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Values are means ± SE; n = 4–9 experiments. HOE-642 and dimethyl amiloride (DMA) were added 10 min before, omeprazole and Sch-28080 30 min before, and cytochalasin D 5 min before forskolin stimulation. TMA-Cl, tetramethylammonium chloride; NS, not significant.
was tested. Figure 7 shows a rapid rise in pH1 induced by an increase in medium osmolarity of 100 mosM. This pH1 rise was absent in the absence of Na1 (data not shown) or in the presence of 500 µM DMA (Fig. 7B) but was partially preserved in the presence of 1 µM HOE-642 (data not shown), indicating that more than one isoform is involved.

DISCUSSION

The present study was undertaken to investigate whether the different NHE isoforms expressed in rabbit parietal cells show a differential response to acid secretagogues. We found that carbachol caused the strongest activation of Na+/H+ exchange, and its effect was completely blocked by atropine or intracellular Ca2+ chelation, indicating a typical muscarinic receptor, Ca2+-dependent signaling pathway. Also, the addition of 100 µM DMA or 1 µM HOE-642 completely inhibited the carbachol-induced Na+/H+ exchange activation. Because Scholz et al. (22) demonstrated a full inhibition of the initial rates of amiloride-sensitive 22Na+ influx by 1 µM HOE-642 in NHE1-transfected PS120 fibroblasts, a <20% inhibition in NHE2-transfected fibroblasts, and no inhibition in NHE3-transfected fibroblasts, this substance is particularly well suited to distinguish between NHE1 activation and that mediated by the other isoforms. The exquisite sensitivity of carbachol-mediated Na+/H+ exchange activation to HOE-642 suggests that Ca2+-mediated stimulation is exclusively mediated via the NHE1 isoform.

Histamine and forskolin induced a pH1 increase that was inhibited by 100 µM DMA and 1 µM HOE-642 to a minor extent only but was completely blocked by 500 µM DMA or the removal of Na+ from the medium, suggesting that other NHE isoforms are involved. Increasing the HOE-642 concentration to 25 µM completely inhibited the NHE2 isoform in transfected fibroblasts (22), which has also been reported to be stimulated by CAMP in transfected Na+/H+ exchanger-deficient Chinese hamster ovary cells (9). HOE-642 at 25 µM inhibited only an additional 13% of the forskolin-stimulated H+ flux rate, suggesting that the majority of CAMP-dependent Na+/H+ exchanger activation is mediated via the NHE4 isoform, since only these three isoforms are expressed in rabbit parietal cells (24).

Apart from its known amiloride resistance, little is known about the NHE4 isoform, since expression in transfected cells has been achieved by only two groups. In one laboratory (1) hyperosmolar medium was necessary to induce expression of NHE4, and in another (4) the application of stilbenes was necessary. DMA at 500...
μM had been inhibitory for NHE4, and preincubation with cytochalasin D reduced NHE4 exchange activity markedly (1). An inhibitory effect of cytochalasin D was found for both the total and the HOE-642-insensitive part of forskolin-stimulated Na⁺/H⁺ exchange activity in rabbit parietal cells; although this finding does not allow positive identification, it is at least consistent with the hypothesis that the HOE-642-insensitive part is due to NHE4 activation.

We had observed this relatively amiloride-resistant histamine-induced pHi increase in cultured parietal cells many years ago but had been unable to explain the findings reasonably (20). When Orłowska et al. (17) published the cloning of rat NHE3 and NHE4 and the expression of both isoforms in rat stomach, we speculated that the cAMP-mediated pHi increase may be due to an amiloride-resistant NHE isoform. We obtained the cDNAs or PCR-cloned cDNA fragments for the different NHE isoforms for rat and rabbit and found that, whereas all four NHE isoforms are expressed in rat stomach, albeit with very different distributions among the different cell types, NHE3 is expressed in rabbit stomach at extremely low abundance and only in surface cells, whereas the other three isoforms are strongly expressed (24). We have now quantified NHE1, -2, and -4 expression in the different rabbit gastric cell types using histone 3.3a as an internal standard and found that mucus cells express very high NHE1 and NHE2 levels and low NHE4 levels, whereas parietal cells display a more even distribution, with NHE1 and NHE2 expression slightly higher than NHE4 expression (unpublished results). Although expression levels do not accurately reflect protein abundance, they usually give a rough estimate of the expected function if the levels are not low, and this was not the case for NHE expression in the stomach. Thus it was somewhat surprising to us that we found relatively little contribution of the NHE2 isoform to the secretagogue-induced pHi increase, especially in light of the finding that the NHE2 knockout mouse shows severe gastric histopathology (23). This may be in part because it is clearly difficult to obtain true estimates of NHE2 function, since one has to use a window between different drug concentrations to estimate the NHE2 contribution to a given Na⁺/H⁺ exchange rate, and this can clearly underestimate its true activity. In fact, a concentration of 1 μM inhibits NHE2 activity in transfected fibroblasts by ~20% (22) and 25 μM may not fully inhibit NHE2 at physiological extracellular Na⁺ concentrations. Therefore, a few percentages of the Na⁺/H⁺ exchange activation that we attribute to NHE1 and NHE4 may be NHE2-mediated. However, it is also possible that the conditions of our experiments are not those under which NHE2 function is strongly activated in the parietal cell.

What could be the physiological role of NHE activation during acid secretion? In 1988, Muñoz et al. (15) demonstrated Na⁺/H⁺ exchange activity by forskolin in freshly isolated rabbit parietal cells and speculated that the resultant alkalization may be important for activation of the highly pHi-sensitive Cl⁻/HCO₃⁻ exchanger. Thus Na⁺/H⁺ exchange activation and the resultant pHi increase have been discussed as a signalling mechanism during acid secretion. We were not able to reproduce these findings in our study (27), and we contribute our failure to see forskolin-induced alkalization to our use of freshly isolated cells and because NHE4 may be particularly sensitive to the cytoskeletal derangement that is certain to occur during cell isolation (we did see a carbachol-induced Na⁺/H⁺ exchange activation in freshly isolated cells, although to a lesser extent than was seen in cultured cells (20)). Nevertheless, we and others had found secretagogue-induced Cl⁻/HCO₃⁻ activation in freshly isolated cells and gastric glands in the absence of significant Na⁺/H⁺ activation, demonstrating that a Na⁺/H⁺ exchanger-mediated pHi increase cannot be the only, or the most important, signal for Cl⁻/HCO₃⁻ exchange activation (7, 27).

Parietal cells in culture have been shown to display a polarized membrane arrangement (21), indicating that, although they do not fully resemble native parietal cells due to the lack of the appropriate basal lamina and...
neighboring cells, they have reformed a part of their cytoskeletal structure. In cultured parietal cells, each of the secretagogues tested in the study caused a significant increase in parietal cell pH in a HEPES-buffered medium but none caused a significant increase in a medium buffered with 5% CO2-HCO3- concentration of parietal cells. We therefore speculate that the secretagogue-induced Na+/H+ exchange activation during acid secretion does not lie in its capability to raise pH.

A dual activation of Na+/H+ and Cl-/HCO3- exchange is one of the mechanisms for regulatory volume increase. A volume increase has been shown to be solely due to the higher buffer capacity in the presence of CO2-HCO3- but none caused a significant increase in a medium buffered with 5% CO2-HCO3-. This cannot be due to the higher buffer capacity in the presence of CO2-HCO3- but suggests that an acid-loading mechanism, most likely the Cl-/HCO3- exchanger, is concomitantly activated. This suggests that the physiological role of Na+/H+ exchanger activation during acid secretion does not lie in its capability to raise pH.

Interestingly, Na+/H+ exchange activation was not proportional to the intensity of secretagogue stimulation of acid formation. If cell shrinkage were simply a result of activation of the ion transport processes directly involved in HCl transport across the canalicu-
lar membrane, i.e., apical K+ and Cl- channels and the H+-K+-ATPase, this would be expected. However, different basolateral K+ channels are activated by Ca2+- and cAMP-dependent agonists, and this activation could result in cell shrinkage, irrespective of the effect on acid secretion. However, if cell shrinkage were the primary stimulus for Na+/H+ exchange activation, then one would expect that all three tested agonists result in an NH2 isoform activation that is proportional to the agonist-induced volume loss, which, surprisingly to us, was not the case. The same holds true for any other Na+/H+ activation, which is secondary to the activation of another ion transporter during acid secretion, i.e., Na+-K+-ATPase activation secondary to K+ channel opening with resultant demand for intracellular Na+. This is not compatible with the very different effect of HOE-642 on Na+/H+ exchange activation by the tested secretagogues and suggests a primary, and differentially regulated, activation of the different NHE isoforms expressed in rabbit parietal cells by the different secretagogues. The data suggest that Na+/H+ exchange activation during stimulation of acid secretion is directly activated by the respective agonist and that different signal transduction pathways result in the activation of a different set of NHE isoforms. Thus, it would appear that secretagogue stimulation of parietal cells results in a primary activation of the different ion transport pathways that are required both for the initiation of the secretory process and for the homoeostatic processes necessary to maintain it. The observation that muscarinic and histaminergic stimulation results in a differential activation of Na+/H+ exchanger isoforms suggests that, in regard to Na+/H+ exchange activation, a potentiated cellular response may not only be the result of a positive cooperation at the level of the intracellular signaling cascade but may also be due to the recruitment of several different transport proteins.

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