The calcium-sensing receptor acts as a modulator of gastric acid secretion in freshly isolated human gastric glands

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Gastric acid secretion by parietal cells is under the control of both neuronal regulation via the vagus nerve and release of acetylcholine and under the control of endocrine and paracrine factors including gastrin and histamine. Histamine is released from neighboring enterochromaffin-like cells (ECL) and triggers an intracellular signaling cascade in parietal cells leading to the insertion of H+–K+-ATPases from tubulovesicular structures into the luminal membrane, where acid secretion takes place (17). The exposure to histamine also causes a simultaneous rise in intracellular Ca2+ ([Ca2+]i), which has served as an additional marker for activated acid secretion (2, 4).

In addition to these classic pathways regulating gastric acid secretion, the calcium-sensing receptor (CaSR) has been identified in rat gastric parietal cells (2, 10). The CaSR is activated by divalent cations, Ca2+ and Mg2+, the trivalent cation Gd3+, and by substrates like spermine. Its sensitivity to these ligands is modulated by L-amino acids and pH (5, 6, 18). Activation of CaSR in rat parietal cells induced an increase in Ca2+ concentrations ([Ca2+]i) (2, 8), suggesting that CaSRs could be involved in the regulation of gastric acid secretion. Indeed, further experiments using freshly isolated rat gastric glands demonstrated that the CaSR leads to a stimulation of histamine-induced H+–K+-ATPase activity (8). On the other hand, inactivation of CaSRs by a reduction of extracellular divalent cations prevented the histamine-mediated stimulation of H+–K+-ATPase activity. Taken together, these results suggested that the CaSR represents a novel receptor in the stomach that may modulate the histamine-induced stimulation of gastric acid secretion (8, 10).

The investigation of human parietal cells and the regulation of acid secretion has been hampered in the past by the lack of appropriate human cell models as most cell lines lose their responsiveness to physiological stimuli for acid secretion or alter their morphology or the expression of key proteins involved in ion transport and acid secretion (17). The use of freshly isolated human gastric glands may therefore be useful to investigate some aspects of short-term regulation and basic properties of ion transport and acid secretion. Some attempts have been made in the past to use gastric glands obtained from biopsy samples and measure several parameters linked to parietal cell activity (14).

To this end, we tested if fresh tissue samples obtained from stomach surgery could be used to isolate intact human gastric glands and if these glands were viable for physiological experiments. Furthermore, we examined whether a CaSR-dependent

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pathway modulating acid secretion via \( H^+\text{-}K^+\text{-ATPase} \) is present in human parietal cells. Our results demonstrate that freshly isolated gastric glands are viable and express functional \( H^+\text{-}K^+\text{-ATPases} \) stimulated by histamine. Expression of the CaSR was shown by immunohistochemistry, and this receptor modulates \( H^+\text{-}K^+\text{-ATPase} \) activity in human parietal cells.

**MATERIALS AND METHODS**

**Patients.** Over a period of 8 mo, samples of gastric tissue were obtained from a total of 29 patients. Patients underwent the Roux-en-Y gastric bypass operation for morbid obesity (7), and small samples of gastric tissue (including mucosal and muscle layers) from the gastrojejunostomy were collected. Gastric tissue originated from the fundus of the stomach. Samples were collected from 8 male and 21 female patients. Male patients had an average age of 48.6 ± 2.6 yr with an average body mass index of 44.4 ± 2.6. Female patients were 39.9 ± 2.2 yr old and had a body mass index of 44.3 ± 1.2. Because of obesity, many of the patients suffered from metabolic syndrome (31.0%). Drugs altering gastric acid secretion (proton pump inhibitors, \( H_2 \) receptor antagonists) were discontinued 1 wk before the surgery.

Informed consent was obtained from all patients, and the study was approved by the local Ethics committee.

**Isolation of gastric glands and digital imaging for intracellular \( pH \) and \( Ca^{2+} \).** Gastric tissue was stored for transport in ice-cold MEM solution (GIBCO; Langley, OK). Tissue was then transferred to the stage of a dissecting microscope and sliced into 0.5-cm square sections. Individual glands were isolated using a hand dissection technique as described previously (8, 13) at a temperature of about 10°C. After isolation, the glands were transferred to cocultures pre-coated with adhesive Cell-Tak (BD Cell-Tak Cell and Tissue Adhesion, BD Biosciences) and mounted in a thermostatically controlled chamber maintained at 37°C on an inverted microscope (Zeiss Axiovert 200) equipped with a video-imaging system for the duration of the experiment. Isolated gastric glands were loaded with 10 \( \mu \)M of the pH-sensitive dye 2,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF) AM (Molecular Probes; Eugene, OR) for 10 min in HEPES-buffered Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 32.2 mM HEPES, and 5 mM glucose; pH 7.4) at 37°C. After the glands were loaded, the chamber was flushed with HEPES-buffered Ringer solution to remove nonesterified dye. Measurements were performed in the epifluorescence mode with a \( \times 40/1.30 \) oil-immersion objective on an inverted microscope. BCECF was successively excited at 440 nm and 495 nm from a monochromator light source, and the resultant fluorescence signal was monitored at 535 nm using an intensified charge-coupled device camera. Data points were acquired every 7 s. Resulting 495- to 440-nm intensity ratio data were converted to intracellular \( pH \) (pHi) values using the high-K\(^+\)/Nigericin calibration technique (19). Over the pH range of 6.3–7.8, fluorescence varied in a linear fashion with extracellular pH. Data are expressed as changes in \( pH \) (\( \Delta pH \)) per minute. Acid extrusion was monitored in the absence of bicarbonate as intracellular alkalization after the removal of \( Na^+ \) from the bath and using the \( Na^+\)Cl prepulse technique, which caused reproducible and sustained intracellular acidification. Alkalization rates (\( \Delta pH/\text{min} \)) for the calculation of \( Na^+\)-independent \( pH \) recovery (\( H^+\text{-}K^+\text{-ATPase activity} \)) and \( Na^+\)-dependent \( pH \) recovery (\( Na^+\text{/H}^+ \) exchanger activity) rates were measured in ranges of \( pH \) of 6.50–6.70 and 6.75–6.90, respectively.

To measure \( Ca^{2+} \), gastric glands were loaded with 10 \( \mu \)M of the \( Ca^{2+}\)-sensing dye fura-2 AM (Molecular Probes) in the chamber for 20 min at room temperature. To eliminate residual nonesterified dye from the bath, glands were superfused with standard HEPES-buffered Ringer solution for 2 min. Fura-2 was excited with light of 340/380-nm wavelengths. \( [Ca^{2+}]_t \) was calculated from the ratio of fluorescence at excitation of 340/380 nm using the following equation as described previously: \( Ca^{2+}_t = \frac{(R - R_{\text{min}}) / (R_{\text{max}} - R)}{F_{\text{max}}} \times F_{\text{max}} \times K_a \), where \( R \) is the measured ratio of emitted light, \( R_{\text{min}} \) is, \( R_{\text{max}} \) is, \( F_{\text{max}} \) is the fluorescence at 380 nm with 2 mM \( Ca^{2+} \) bath solution, \( F_{\text{max}} \) is the fluorescence at 380 nm with 0 mM \( Ca^{2+} \) bath solution, and the dissociation constant \( K_a \) = 225 nM for fura-2-calcium binding (9).

All chemicals used were obtained from Sigma and Molecular Probes. Omeprazole was a kind gift from Astra Hassle (Mölndal, Sweden).

Activation of acid secretion via histamine and inhibition by omeprazole was induced by preincubation of the glands for 10 min before the experiment combined with BCECF. All data are summarized as means ± SE and were analyzed by grouping measurements at baseline values and during experimental periods. Significance was determined using an unpaired Student’s t-test with \( P < 0.05 \) considered to be statistically significant.

**Immunohistochemistry.** Human stomach samples were washed several times with PBS and fixed by immersion with paraformaldehyde-periodate fixative (16) overnight at 4°C. Stomachs were washed three times with PBS, and thin sections were cut at a thickness of 5 \( \mu \)m after cryoprotection with 2.3 M sucrose in PBS for at least 12 h. Immunostaining was carried out as described previously (13). Sections were incubated with 1% SDS for 5 min, washed three times with PBS, and incubated with PBS containing 1% BSA for 15 min before incubation with the primary antibody. The primary antibodies [mouse monoclonal anti-pig \( \beta \)-gastrin \( H^+\text{-}K^+\text{-ATPase} \) (Affinity Bioreagents), rabbit polyclonal anti-pig \( \alpha \)-gastric \( H^+\text{-}K^+\text{-ATPase} \) (Chemicon), rabbit polyclonal affinity-purified anti-CaSR against amino acids 12–27 of rat CaSR (Affinity Bioreagents)] were diluted 1:2,000, 1:1,000, and 1:50, respectively, in PBS and applied overnight at 4°C. In addition, rabbit polyclonal antibody was generated to a maltose-binding fusion protein (MBP) of the entire extracellular domain of the rat CaSR (residues 1–642). The rabbit anti-rat CaSR-\( \beta \)-galactosidase antibody was affinity purified using the MBP-CaSR protein (AminoLink Plus Immobilization Kit, Pierce) and used at a dilution of 1:50. Peptide protection experiments were performed by incubating the affinity-purified antibody at 1:50 with the immunizing peptide (0.8 \( \mu \)g/ml) in PBS for 1 h at room temperature and directly applying the dilution after centrifugation to remove precipitates. Stomach sections were then washed twice for 5 min with high-NaCl-PBS (PBS + 2.7% NaCl) and once with PBS and incubated with secondary antibodies [donkey anti-rabbit Alexa 546 and donkey anti-mouse Alexa 488 (Molecular Probes)] at a dilution of 1:1,000 and 1:200, respectively, for 1 h at room temperature. Sections were washed twice with high-NaCl-PBS and once with PBS before being mounted with VectaMount (Vector Laboratories; Burlingame, CA). Specimens were viewed with a Leica SP1 UV CLSM confocal microscope, and pictures were processed using Adobe Photoshop.

**RESULTS**

Freshly isolated human gastric glands are suitable for functional experiments. In the first series of experiments, we tested whether the freshly isolated human gastric glands were suitable for functional experiments investigating regulation of \( H^+\text{-}H^+\text{-ATPase} \) activity. To this end, immunohistochemistry was performed on the tissue samples obtained to examine expression of both subunits of gastric \( H^+\text{-}K^+\text{-ATPase} \). Immunohistochemistry demonstrated that both \( \alpha \)- and \( \beta \)-subunits could be detected, and thus acid-secretory parietal cells were present in samples obtained from the antral part of the human stomach (Fig. 1). \( pH \) measurements of single parietal cells within freshly isolated gastric glands were used to measure \( H^+\text{-}K^+\text{-ATPase} \) activity. The activity of the pump was calculated from the rate of alkalization of \( pH \) (\( \Delta pH/\text{min} \)) after acidification using the \( Na^+\)Cl prepulse technique in the absence of sodium and bicarbonate. \( H^+ \) extrusion under these
Fig. 1. Immunolocalization of α- and β-subunits of gastric H⁺-K⁺-ATPase in the human stomach. Samples of the human stomach obtained from the antral part were stained with specific antibodies against the α-subunit (A and B) and β-subunit (C) of gastric H⁺-K⁺-ATPase. The distribution and localization was specific only to a subset of cells along the gastric gland (Fig. 2, A and B). After exposure of the glands to histamine (100 μM), the rate of Na⁺-independent pH₇ recovery represented H⁺-K⁺-ATPase activity, glands were preincubated with 100 μM of the specific inhibitor of gastric H⁺-K⁺-ATPase omeprazole for 10 min before the experiment and were exposed to histamine (100 μM) as described above. Concomitantly, the rate of Na⁺-independent pH₇ recovery rate (0.0094 ± 0.002 pH units/min) and reduced it to the same level as seen in glands not exposed to histamine (n = 65 parietal cells from 3 glands from 4 patients; Fig. 2, C and D). Thus the freshly isolated human gastric glands contained functional parietal cells that showed H⁺-K⁺-ATPase activity that could be stimulated with the physiological agonist histamine and inhibited by the specific blocker omeprazole.

Expression of the CaSR in human parietal cells. Immunohistochemistry using two different antibodies directed against different epitopes of the CaSR demonstrated staining of the basolateral side of a subset of cells along the gastric gland (Fig. 3A). No specific signal for CaSR was observed with preimmune serum (Fig. 3F), after peptide protection with the immunizing peptide (Fig. 3G), or with application of only the secondary antibody (Fig. 3H). To test whether the CaSR was expressed in parietal cells, double labeling for the CaSR and the β-subunit of gastric H⁺-K⁺-ATPase was performed in samples obtained from patients that fasted for at least 12 h before the operation. Colocalization of both the CaSR and the β-subunit of gastric H⁺-K⁺-ATPase was observed, demonstrating expression of the CaSR in human gastric parietal cells (Fig. 3). Higher magnification pictures showed that the CaSR and the β-subunit of gastric H⁺-K⁺-ATPase β-subunit do not localize to the same compartment of resting parietal cells, with the H⁺-K⁺-ATPase β-subunit residing in intracellular structures, consistent with its localization in tubulovesicular structures (Fig. 3, D and E).

Modulation of histamine-induced stimulation of H⁺-K⁺-ATPase activity by CaSR in human gastric glands. To examine the effect of the CaSR on the activity of gastric H⁺-K⁺-ATPase and its stimulation by histamine, we reduced the concentration of total divalent cations from 1 mM Mg²⁺ and 1.2 mM Ca²⁺ in control solution to only 0.1 mM Ca²⁺ and 0 mM Mg²⁺, a concentration of divalent cations leaving the CaSR inactive. Glands were preincubated in this low-divalent cation solution for 10 min before the experiment and were stimulated with 100 μM histamine as described above. Conditions of low extracellular divalent cations abolished histo-
mine-induced alkalinization (0.015 ± 0.003 pH units/min, n = 61 parietal cells from 9 glands from 9 patients; Fig. 4C). In contrast, stimulation of the CaSR with the trivalent cation Gd3+ (100 μM) in low-divalent cation solution (100 μM Ca2+, 0 mM Mg2+) stimulated H+-K+-ATPase activity even in the absence of histamine (Na+-independent pHi recovery: 0.075 ± 0.004 pH units/min, n = 68 parietal cells from 8 glands from 6 patients; Fig. 4B). This stimulatory effect was also seen when gastric glands were preincubated with histamine in low-divalent cation solution (100 μM Ca2+, 0 mM Mg2+) and Gd3+ applied directly during the phase of Na+-independent alkalinization. Gd3+ induced an immediate increase in the alkalinization rate (data not shown), suggesting a rapid activation of H+ extrusion. Similarly, increasing extracellular Ca2+ to 5 mM in the presence of histamine stimulated H+-K+-ATPase activity above levels seen at 1 mM extracellular Ca2+ (n = 101 parietal cells from 6 glands from 4 patients). These data suggest that also the physiological ligand Ca2+ can stimulate the CaSR and increase H+-K+-ATPase activity.

To rule out that histamine released from neighbouring ECL cells mediated the effect of Gd3+ on parietal cells, gastric glands were preincubated with the H2 receptor inhibitor cimetidine (100 μM) and stimulated with Gd3+. The Na+-independent pHi recovery rate was not altered by incubation with cimetidine, and Gd3+ was still effective in stimulating realalkalinization (0.095 ± 0.01 pH units/min, n = 34 parietal cells from 4 glands from 2 patients; Fig. 5).

Furthermore, to examine if Gd3+-induced stimulation of the Na+-independent pHi recovery rate was due H+-K+-ATPase activity, gastric glands were preincubated for 10 min with the H+-K+-ATPase inhibitor omeprazole (100 μM), which almost completely abolished intracellular alkalinization (0.011 ± 0.001 pH units/min, n = 58 parietal cells from 6 glands from 4 patients; Fig. 5), demonstrating that the CaSR stimulated H+-K+-ATPase activity.

CaSR activation leads to increases in Ca2+, CaSR activation has been shown to induce increases in [Ca2+]; in rat gastric parietal cells and in a number of other tissue preparations and cell culture lines (2, 3, 8). Therefore, we tested whether activation of CaSR by Gd3+ increased Ca2+. Activation of the CaSR with Gd3+ (100 μM) increased Ca2+ even under conditions of low extracellular divalent cations (Fig. 6).

**DISCUSSION**

Gastric acid secretion involves a complex process of either neuronal or paracrine stimulatory pathways converging in the insertion of H+-K+-ATPases into the luminal membrane of acid-secreting parietal cells (for a review, see Ref. 20). Besides the classic routes of activation through acetyl choline, gastrin, and histamine, a number of metabolic factors (including serum calcium and protein) or amino acid-rich diets influence gastric acid secretion via only partly characterized pathways. The recent identification of the CaSR in gastric tissue and its localization to parietal cells has raised the question as to its function in these specialized cells and its potential role as a metabolic sensor (10). We (2, 8) have previously shown that the CaSR is functionally active in rat parietal cells and is able to modulate gastric acid secretion via stimulation of H+-K+-ATPase activity. In the present study, we tested whether freshly isolated human gastric glands could be used for functional studies using a modification of the techniques that we had previously developed for rat and mouse isolated gastric glands. Our results demonstrate that freshly isolated human glands expressed both subunits of gastric H+-K+-ATPase and secrete acid in response to histamine and that acid secretion is sensitive to the specific inhibitor omeprazole.

We also demonstrated that the CaSR is expressed in human gastric parietal cells and is functionally active. Stimulation of the CaSR by increased concentrations of divalent or trivalent ions led to enhanced proton extrusion via omeprazole-sensitive H+-K+-ATPase. A reduction of extracellular divalent cations resulted in a reduction, or, in the case of histamine, an inactivation, of histamine-induced H+-K+-ATPase activity. Thus enhanced CaSR activity can modulate H+-K+-ATPase activity
Fig. 3. Localization of the calcium-sensing receptor (CaSR) in the human stomach. Human stomach samples obtained from fasted patients were used to localize the CaSR. A: staining of a subset of cells was observed with an antiserum raised against CaSR (red). B: staining against the H⁺-K⁺-ATPase β-subunit to identify acid-secreting parietal cells (green). C: overlay of stainings against CaSR (red) and the H⁺-K⁺-ATPase β-subunit (green) demonstrates expression of both proteins in the same cells. D and E: high-magnification pictures showing that the CaSR (red) is localized to a different subcellular compartment than the H⁺-K⁺-ATPase β-subunit (green) in resting parietal cells. F–H: overlay of stainings against the H⁺-K⁺-ATPase β-subunit (green) and with the use of preimmune serum against the CaSR (F), preincubation of anti-CaSR with the immunizing peptide (G), or omission of the anti-CaSR antibody (all in red) and use of only the secondary antibody (H), which demonstrate that no signal similar to the CaSR could be seen. Magnification: ×400 in A–C and F–H, ×600 in D, and ×800 in E.
in both the absence and presence of the potent secretagogue histamine. However, it remains to be established whether the CaSR provides a pathway for stimulation or regulation of gastric acid secretion independent from the classic route via histamine or acetylcholine in vivo. Activation of the CaSR was associated with a rise in $\text{Ca}^{2+}$, an event that has been linked to activation of $\text{H}^+\text{-K}^+\text{-ATPases}$. A direct correlation in $\text{Ca}^{2+}$ levels and CaSR-mediated regulation of $\text{H}^+\text{-K}^+\text{-ATPase}$ activity requires further investigation.

The CaSR has also been shown to be sensitive to changes in extracellular pH and to be allosterically sensitized by L-amino acids shifting the activation curve for divalent cations to the left (5, 6, 18). Both high extracellular $\text{Ca}^{2+}$ and L-amino acids have been shown to stimulate gastric acid secretion through only poorly understood mechanisms. We have recently shown that L-amino acids can stimulate gastric $\text{H}^+\text{-K}^+\text{-ATPase}$ activity. *Significant difference between experimental treatments and control; #significant difference between 1 mM $\text{Ca}^{2+}$ and 5 mM $\text{Ca}^{2+}$.

Fig. 4. Acid secretion is modulated by the CaSR. A: reduction of extracellular cations from 1 mM $\text{Ca}^{2+}$ and 1.2 mM $\text{Mg}^{2+}$ to 100 $\mu$M $\text{Ca}^{2+}$ and 0 mM $\text{Mg}^{2+}$, respectively, abolished the stimulatory effect of histamine on intracellular alkalinization ($\text{H}^+\text{-K}^+\text{-ATPase activity}$) ($n = 61$ cells from 9 glands from 9 patients). B: addition of the divalent cation receptor agonist Gd$^{3+}$ (100 $\mu$M) even in a low cation-containing solution induced an increase of the rate of alkalinization in both the presence or absence of histamine ($n = 68$ cells from 8 glands from 6 patients). C: bar graph summarizing the effects of low and high divalent cations and Gd$^{3+}$ on $\text{H}^+\text{-K}^+\text{-ATPase}$ activity. *Significant difference between experimental treatments and control; #significant difference between 1 mM $\text{Ca}^{2+}$ + 100 $\mu$M histamine and 5 mM $\text{Ca}^{2+}$ + 100 $\mu$M histamine.

Fig. 5. CaSR stimulates $\text{H}^+\text{-K}^+\text{-ATPase}$ activity and does not require $\text{H}_2$ receptors. Inhibition of $\text{H}^+\text{-K}^+\text{-ATPase}$ activity with the specific inhibitor omeprazole (100 $\mu$M) abolished the stimulatory effect of Gd$^{3+}$ on the rate of pH recovery, demonstrating that Gd$^{3+}$/CaSR activates $\text{H}^+$ extrusion via $\text{H}^+\text{-K}^+\text{-ATPase}$ ($n = 58$ cells from 6 glands from 4 patients). Blockade of $\text{H}_2$ histamine receptors with cimetidine (100 $\mu$M) did not influence the Gd$^{3+}$/stimulated pH recovery, ruling out the involvement of histamine in the effect on $\text{H}^+\text{-K}^+\text{-ATPase}$ activity ($n = 34$ cells from 4 glands from 2 patients).

Fig. 6. Activation of the CaSR by Gd$^{3+}$ increases the intracellular calcium concentration ([Ca$^{2+}$]). A: Original tracing of [Ca$^{2+}$], measurements using fura-2 in a single parietal cell, showing that exposure to Gd$^{3+}$ (100 $\mu$M) led to a sustained increase in [Ca$^{2+}$]. B: bar graph summarizing the measurement in the absence (48.8 ± 2.3 nM intracellular Ca$^{2+}$) and presence (77.6 ± 4.4 nM intracellular Ca$^{2+}$) of Gd$^{3+}$ ($n = 27$ cells from 3 glands from 2 patients).
ity in isolated rat gastric glands by a dual mechanism (1, 12). At low concentrations, this appears to involve the uptake of amino acids by amino acid transporters, whereas at higher concentrations, the CaSR appears to be involved (1, 12). Thus, under these conditions, the CaSR could be acting as a metabolic sensor through which several metabolic pathways could modulate gastric acid secretion (11).

In conclusion, our data show the viability of freshly isolated human gastric glands for investigation of human gastric acid secretion, the identification of the CaSR in parietal cells, and the ability of the CaSR to directly modulate acid secretion independently from secretagogues.

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