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

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Dufner, Matthias M., Philipp Kirchhoff, Christine Remy, Patricia Hafner, Markus K. Müller, Sam X. Cheng, Lie-Qi Tang, Steven C. Hebert, John P. Geibel, and Carsten A. Wagner. The calcium-sensing receptor acts as a modulator of gastric acid secretion in freshly isolated human gastric glands. Am J Physiol Gastrointest Liver Physiol 289: G1084–G1090, 2005. —Gastric acid secretion is modulated by two distinct pathways: a neuronal pathway via the vagus nerve and release of acetylcholine and an endocrine pathway involving gastrin and histamine. Recently, we demonstrated that activation of H⁺-K⁺-ATPase activity in parietal cells in freshly isolated rat gastric glands is modulated by the calcium-sensing receptor (CaSR). Here, we investigated if the CaSR is functionally expressed in freshly isolated gastric glands from human patients undergoing surgery and if the CaSR is influencing histamine-induced activation of H⁺-K⁺-ATPase activity. In tissue samples obtained from patients, immunohistochemistry demonstrated the expression in parietal cells of both subunits of gastric H⁺-K⁺-ATPase and the CaSR. Functional experiments using the pH-sensitive dye 2’,7’-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein and measurement of intracellular pH changes allowed us to estimate the activity of H⁺-K⁺-ATPase in single freshly isolated human gastric glands. Under control conditions, H⁺-K⁺-ATPase activity was stimulated by histamine (100 μM) and inhibited by omeprazole (100 μM). Reduction of the extracellular divalent cation concentration (0 Mg²⁺, 100 μM Ca²⁺) inactivated the CaSR and reduced histamine-induced activation of H⁺-K⁺-ATPase activity. In contrast, activation of the CaSR with the trivalent cation Gd³⁺ caused activation of omeprazole-sensitive H⁺-K⁺-ATPase activity even in the absence of histamine and under conditions of low extracellular divalent cations. This stimulation was not due to release of histamine from neighbouring enterochromaffin-like cells as the stimulation persisted in the presence of the H₂ receptor antagonist cimetidine (100 μM). Furthermore, intracellular calcium measurements with fura-2 and fluo-4 showed that activation of the CaSR by Gd³⁺ led to a sustained increase in intracellular Ca²⁺ even under conditions of low extracellular divalent cations. These experiments demonstrate the presence of a functional CaSR in the human stomach and show that this receptor may modulate the activity of acid-secreting H⁺-K⁺-ATPase in parietal cells. Furthermore, our results show the viability of freshly isolated human gastric glands and may allow the use of this preparation for experiments investigating the physiological regulation and properties of human gastric glands in vitro.

GASTRIC ACID SECRETION by parietal cells is under the control of both neuronal regulation via the vagus nerve involving the 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⁺), 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⁺]) (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 activation of 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^+-K^+-ATPase is present in human parietal cells. Our results demonstrate that freshly isolated gastric glands are viable and express functional H^+-K^+-ATPases stimulated by histamine. Expression of the CaSR was shown by immunohistochemistry, and this receptor modulates H^+-K^+-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 ± 12. Because of obesity, many of the patients suffered from metabolic syndrome (31.0%). Drugs altering gastric acid secretion (proton pump inhibitors, H2 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 covescoated with adhesive Cell-Tak (BD Cell-Tak Cell and Tissue Adhesion, BD Biosciences) and mounted in a thermoregulated 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 μM 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 CaCl2, 1.2 mM MgCl2, 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 ×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 6.3–7.8, fluorescence varied in a linear fashion with extracellular pH. Data are expressed as changes in pH (ΔpH) 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 NH4Cl prepulse technique, which caused reproducible and sustained intracellular acidification. Alkalization rates (ΔpH/min) for the calculation of Na^+-independent pH recovery (H^+-K^+-ATPase activity) and Na^+-dependent pH recovery (Na^+/H^+ exchanger activity) 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 μM 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+}]i was calculated from the ratio of fluorescence at excitation of 340/380 nm using the following equation as described previously: 

\[ \text{Ca}^{2+}_{i} = \left[ \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \right] \times (F_{\text{max}}/F_{\text{min}}) \times K_d, \]

where R is the measured ratio of emitted light, R_{min} is, R_{max} is, F_{max} is the fluorescence at 380 nm with 2 mM Ca^{2+} bath solution, F_{min} is the fluorescence at 380 nm with 0 mM Ca^{2+} bath solution, and the dissociation constant (K_d) was 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 Hässle (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 grouped 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-lysine-periodate fixative (16) overnight at 4°C. stomachs were washed twice times with PBS, and thin sections were cut at a thickness of 5 μm 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 β-gastric H^+-K^+-ATPase (Affinity Bioreagents), rabbit polyclonal anti-pig α-gastric H^+-K^+-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–462 polyclonal antibody was affinity purified using the MBP-CaSR protein (Aminolink Plus Immobilization Trial 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 μg/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^+-H^+-ATPase activity. To this end, immunohistochemistry was performed on the tissue samples obtained to examine expression of both subunits of gastric H^+-K^+-ATPase. Immunohistochemistry demonstrated that both α- and β-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^+-K^+-ATPase activity. The activity of the pump was calculated from the rate of alkalization of pH (ΔpH/min) after acidification using the NH4Cl prepulse technique in the absence of sodium and bicarbonate. H^+ extrusion under these
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 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 Mg^{2+} and 1.2 mM Ca^{2+} in control solution to only 0.1 mM Ca^{2+} and 0 mM Mg^{2+}, 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 hista-
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 alkalinization (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-secretory 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 $\mathrm{Ca^{2+}}$, an event that has been linked to activation of H^+–K^+–ATPases. A direct correlation in $\mathrm{Ca^{2+}}$ levels and CaSR-mediated regulation of H^+–K^+–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 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 H^+–K^+–ATPase activity and does not require H2 receptors. Inhibition of H^+–K^+–ATPase activity with the specific inhibitor omeprazole (100 μM) abolished the stimulatory effect of Gd^{3+} on the rate of pH recovery, demonstrating that Gd^{3+} activates H^+ extrusion via H^+–K^+–ATPase (n = 58 cells from 6 glands from 4 patients). Blockade of H2 histamine receptors with cimetidine (100 μM) did not influence the Gd^{3+}-stimulated pH recovery, ruling out the involvement of histamine in the effect on H^+–K^+–ATPase activity (n = 34 cells from 4 glands from 2 patients).

**Fig. 4.** Acid secretion is modulated by the CaSR. A: reduction of extracellular cations from 1 mM Ca^{2+} and 1.2 mM Mg^{2+} to 100 μM Ca^{2+} and 0 mM Mg^{2+}, respectively, abolished the stimulatory effect of histamine on intracellular alkalinization (H^+–K^+–ATPase activity) (n = 61 cells from 9 glands from 9 patients). B: addition of the divalent cation receptor agonist Gd^{3+} (100 μM) 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 H^+–K^+–ATPase activity. *Significant difference between experimental treatments and control; #significant difference between 1 mM Ca^{2+} + 100 μM histamine and 5 mM Ca^{2+} + 100 μM histamine.

**Fig. 5.** CaSR stimulates H^+–K^+–ATPase activity and does not require H2 receptors. A: Original tracing of $[\mathrm{Ca^{2+}}]$ measurements using fura-2 in a single parietal cell, showing that exposure to Gd^{3+} (100 μM) led to a sustained increase in $[\mathrm{Ca^{2+}}]$. B: bar graph summarizing $[\mathrm{Ca^{2+}}]$ measurements 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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REFERENCES

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