Stimulatory Pathways of the Calcium-Sensing Receptor on Acid Secretion in Freshly Isolated Human Gastric Glands

Christine Remy^{1,2*}, Philipp Kirchhoff^{2*}, Patricia Hafner¹, Stephanie M. Busque¹, Markus K. Müller², John P. Geibel³ and Carsten A. Wagner¹

¹Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, ²Department of Visceral and Transplantation Surgery, University of Zürich, ³Departments of Cellular & Molecular Physiology and Surgery, Yale Medical School, New Haven, *C. Remy and P. Kirchhoff contributed equally to this study and share first authorship

Key Words

Gastric acid secretion • CaSR • Protein kinase C • Human

Abstract

Gastric acid secretion is not only stimulated via the classical known neuronal and hormonal pathways but also by the Ca²⁺-Sensing Receptor (CaSR) located at the basolateral membrane of the acid-secreting gastric parietal cell. Stimulation of CaSR with divalent cations or the potent agonist Gd³⁺ leads to activation of the H⁺/K⁺-ATPase and subsequently to gastric acid secretion. Here we investigated the intracellular mechanism(s) mediating the effects of the CaSR on H⁺/K⁺-ATPase activity in freshly isolated human gastric glands. Inhibition of heterotrimeric G-proteins (G_i and G_o) with pertussis toxin during stimulation of the CaSR with Gd³⁺ only partly reduced the observed stimulatory effect. A similar effect was observed with the PLC inhibitor U73122. The reduction of the H⁺/K⁺-ATPase activity measured after incubation of gastric glands with BAPTA-AM, a chelator of intracellular Ca²⁺, showed that intracellular Ca²⁺ plays an important role in the signalling cascade. TMB-8, a ER

Ca²⁺store release inhibitor, prevented the stimulation of H⁺/K⁺-ATPase activity. Also verapamil, an inhibitor of L-type Ca²⁺-channels reduced stimulation suggesting that both the release of intracellular Ca²⁺ from the ER as well as Ca²⁺ influx into the cell are involved in CaSR-mediated H⁺/K⁺-ATPase activation. Chelethrythrine, a general inhibitor of protein kinase C, and Gö 6976 which selectively inhibits Ca²⁺-dependent PKC_α and PKC_β-isozymes completely abolished the stimulatory effect of Gd³⁺. In contrast, Ro 31-8220, a selective inhibitor of the Ca²⁺-independent PKC_ε and PKC_δ isoforms reduced the stimulatory effect of Gd³⁺ only about 60 %. On the other hand, activation of PKC with DOG led to an activation of H⁺/K⁺-ATPase activity which was only about 60 % of the effect observed with Gd³⁺. Incubation of the parietal cells with PD 098059 to inhibit ERK1/2 MAP-kinases showed a significant reduction of the Gd³⁺ effect. Thus, in the human gastric parietal cell the CaSR is coupled to pertussis toxin sensitive heterotrimeric G-Proteins and requires calcium to enhance the activity of the proton-pump. PLC, ERK 1/2 MAP-kinases as well as Ca²⁺ dependent and Ca²⁺-independent PKC isoforms are part of the down-stream signalling cascade.

Copyright © 2007 S. Karger AG, Basel

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1015-8987/07/0194-0033\$23.50/0

Accessible online at:
www.karger.com/journals/net

Carsten A. Wagner

Institute of Physiology and Zurich Center for Integrative Human Physiology
University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich (Schweiz)
Tel. +41-1-63 50659, Fax. +41-1-63 56814
E-Mail Wagnerca@access.unizh.ch

Introduction

Stimulation of gastric acid secretion from parietal cells occurs after stimulation of a variety of neurotransmitter or hormone receptors such as for acetyl choline or histamine [1]. These receptors couple intracellularly to small G-proteins and activate multiple signal pathways including increases in intracellular Ca^{2+} and cAMP levels, activation of protein kinase C and ERK1/2 MAPK kinases [2].

In addition to these classic receptors, we and others have recently shown that the Calcium-sensing receptor (CaSR) is expressed in stomach and is also localized in acid-secretory parietal cells [3, 4]. In these cells, activation of the CaSR with Ca^{2+} or the trivalent cation Gadolinium (Gd^{3+}) stimulates H^+/K^+ -ATPase activity in the presence or absence of other stimuli [4, 5]. Moreover, activation of the CaSR leads to a rapid increase in intracellular Ca^{2+} concentrations in rat and human parietal cells [3-5]. Thus, the gastric parietal cell possesses a functionally active CaSR that is able to modulate H^+/K^+ -ATPase activity and gastric acid secretion.

Experiments with a number of CaSR transfected cell lines as well as with native tissues and cells expressing the CaSR endogenously have defined several intracellular signal cascades and molecules regulated by CaSR activation including heterotrimeric G-proteins, PLC, PLA, intracellular Ca^{2+} , protein kinases A and C, ERK1/2 kinases, and HETE [6, 7]. Thus, from these experiments it has become apparent that CaSR is linked to several distinct signal pathways in a cell and tissue specific manner which makes it necessary to examine these pathways in a cell-specific manner. Moreover, CaSR may even be coupled to distinct cascades in the same cell [7]. Therefore, to further understand the role of CaSR in the regulation of H^+/K^+ -ATPase activity in gastric parietal cells, we investigated the signal cascade involved in the stimulatory effect of CaSR on H^+/K^+ -ATPase activity. We used the previously characterized freshly isolated human gastric glands preparation and monitored changes in Na^+ -independent pH_i -recovery rates (i.e. alkalization rates) as a measure of H^+/K^+ -ATPase activity [4].

Our results show that the CaSR is coupled at least in part to pertussis toxin-sensitive small G-Proteins. Stimulation of H^+/K^+ -ATPase activity by the CaSR requires intact PLC, ERK1/2 MAP-kinases, and Ca^{2+} -dependent and -independent PKC isoforms.

Materials and Methods

Patients

Over a period of 10 months, samples of gastric tissue were obtained from a total of 48 patients. Patients underwent Roux-en-Y gastric bypass operation for morbid obesity [8] and small samples of gastric tissue (including mucosal and muscle layers) from the gastrojejunal anastomosis were collected. The gastric tissue originated from the fundus of the stomach. Samples were collected from 11 male and 31 female patients. Male patients had an average age of 43.2 ± 2.86 years with an average body mass index (BMI) of 42.8 ± 2.37 . Female patients were 41.3 ± 1.44 years old and had a BMI of 46.7 ± 1.22 . Drugs altering gastric acid secretion (proton pump inhibitors, H_2 -receptor antagonists) were discontinued one week prior to surgery.

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

Isolation of gastric glands, digital imaging for intracellular pH

Gastric tissue was stored for transport in ice-cold MEM solution (Gibco, Langley, OK, USA). The 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 [4, 9] at a temperature of about 4°C . After isolation, the glands were transferred to coverslips precoated with the bioadhesive Cell-Tak (BD Cell-Tak Cell and Tissue Adhesion, BD Biosciences) and then mounted in a thermostatically controlled chamber maintained at 37°C on an inverted microscope (Zeiss Axiovert 200) equipped with an 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 aceto-methyl ester (BCECF-AM) (Molecular Probes, Eugene, OR) for 10 min in a low Calcium HEPES-buffered Ringer solution (125 mM NaCl, 5 mM KCl, 0.1 mM CaCl_2 , 32.2mM HEPES, and 5 mM glucose, pH 7.4) at 37°C . After loading, the chamber was flushed with HEPES-buffered Ringer solution to remove non de-esterified dye. Measurements were performed in the epifluorescence mode with an 40x / 1.30 oil objective on an inverted microscope. BCECF was successively excited at 440 nm and 495 nm from a monochromator light source, the resultant fluorescent signal was monitored at 535 nm using an intensified charge-coupled device camera. Data points were acquired every 7 s. The resulting 495/440 intensity ratio data were converted to intracellular pH (pH_i) values using the high $\text{K}^+/\text{Nigericin}$ calibration technique [10]. Over the pH range of 6.3 - 7.8, fluorescence varied in a linear fashion with extracellular pH. Data were expressed as pH/min . Acid extrusion was monitored in the absence of bicarbonate as the rate of intracellular alkalization using the NH_4Cl -prepulse technique (6), which caused a reproducible and sustained intracellular acidification. Alkalization rates (pH_i/min) were used

Table 1. Summary of intracellular pH measurements. Data are provided as mean $\pm$ SEM. The number of single cells, glands, and patients is also given. * marks data significantly different from control, # marks data significantly different from gadolinium treated glands in the absence of any inhibitor. The number of cells, glands and patients is given.

Treatment	pH _i -recovery rate (Δ pH/min)	n (cells/glands/patients)
Control	0.021 $\pm$ 0.001	234 / 12 / 11
Gd ³⁺	0.074 $\pm$ 0.004*	344 / 24 / 18
Gd ³⁺ + PTX	0.030 $\pm$ 0.002*#	192 / 8 / 4
PTX	0.01 $\pm$ 0.0006*	147 / 5 / 3
Gd ³⁺ + U 73122	0.031 $\pm$ 0.002*#	106 / 7 / 6
U 73122	0.014 $\pm$ 0.012*	138 / 5 / 4
Gd ³⁺ + BAPTA	0.035 $\pm$ 0.002*#	101 / 6 / 4
Gd ³⁺ + TMB-8	0.028 $\pm$ 0.002#	76 / 5 / 3
TMB-8:	0.023 $\pm$ 0.003	70 / 5 / 3
Gd ³⁺ + verapamil	0.036 $\pm$ 0.004*#	92 / 6 / 3
verapamil	0.021 $\pm$ 0.002	90 / 5 / 3
Gd ³⁺ + TMB-8 + verapamil	0.019 $\pm$ 0.003#	38 / 3 / 2
Gd ³⁺ + Chelerythrine	0.022 $\pm$ 0.003#	94 / 6 / 4
Chelerythrine	0.014 $\pm$ 0.001*	189 / 11 / 3
DOG	0.045 $\pm$ 0.003*	138 / 7 / 3
Gd ³⁺ + Gö	0.018 $\pm$ 0.002#	71 / 5 / 3
Gö	0.028 $\pm$ 0.003	92 / 5 / 3
Gd ³⁺ + Ro-31-2880	0.040 $\pm$ 0.004*#	85 / 5 / 3
Ro-31-2880	0.016 $\pm$ 0.001	95 / 5 / 3
Gd ³⁺ + U73122 + Ro-31-2880	0.016 $\pm$ 0.002#	43 / 5 / 4
Gd ³⁺ + PD 098059	0.020 $\pm$ 0.001#	122 / 5 / 4
PD 098059	0.018 $\pm$ 0.001	103 / 5 / 3
Gd ³⁺ + Wortmannin	0.079 $\pm$ 0.004*	81 / 5 / 3
Wortmannin	0.030 $\pm$ 0.001	140 / 7 / 3

for the calculation of Na⁺-independent pH_i recovery (H⁺,K⁺-ATPase activity). Except for TMB-8, Gö6976, and Ro312880 (Calbiochem) all other chemicals used were obtained from Sigma and Molecular Probes. Activation of acid secretion via the CaSR using Gadolinium was induced by a preincubation of the glands for 10 min before the experiment combined with BCECF loading. The amount of calcium contained in all solutions was reduced to a minimum (100 μ M). All data are summarized as mean $\pm$ S.E. and were analyzed by grouping measurements at baseline values and during experimental periods. Significance was determined using the one-way ANOVA test with $p < 0.05$ considered to be statistically significant.

Results

The CaSR is stimulated by Gadolinium and is coupled to PTX-sensitive heterotrimeric G-Proteins

In the first series of experiments we performed intracellular pH-measurements in single parietal cells within

freshly isolated human gastric glands under control conditions with and without stimulation of the Calcium-Sensing Receptor (CaSR) with Gd³⁺ (100 M). All experiments were performed in the presence of low extracellular cations (100 μ M Ca²⁺, 0 Mg²⁺) in order to keep basal CaSR activity low. In the absence of any secretagogue stimulation only a low rate of intracellular pH_i alkalization was observed (0.021 $\pm$ 0.001 pH units/min). When glands were preincubated with Gd³⁺ (100 μ M) the rate of Na⁺-independent pH_i alkalization increased to 0.074 $\pm$ 0.004 (Figure 1). We have previously shown in the same preparation that this increased rate of pH_i-alkalinization is solely due to H⁺/K⁺-ATPase activity and completely abolished in the presence of its specific inhibitor omeprazole [4, 5]. Therefore, we used the rate of pH_i recovery to monitor activation of the CaSR and H⁺/K⁺-ATPase activity and to test the involvement of several pathways known to be activated by the CaSR in other cells and described previously to act on H⁺/K⁺-ATPase activity.

Previous studies on CaSR signalling in transfected

Fig. 1. Gadolinium is a potent stimulator of the CaSR. Single human gastric glands were isolated, loaded with the pH-sensitive dye BCECF to measure intracellular pH over single parietal cells and the pH_i recovery rate was calculated from the slope after an acid load using the NH_4Cl prepulse technique as described previously [4, 5, 9]. (A) Original tracing of an intracellular pH measurement under control conditions with low extracellular divalent cations ($100 \mu M Ca^{2+}$, $0 Mg^{2+}$). (B) Original tracing of an intracellular pH measurement in the presence of the potent CaSR-stimulator Gadolinium (Gd^{3+} , $100 \mu M$). (C) Bar graph, summarizing the data as mean $\pm$ SEM. marks significantly different from control.

and native cells demonstrated its coupling to heterotrimeric G-Proteins [6, 7, 11]. Therefore, we tested the involvement of pertussis toxin (PTX) sensitive heterotrimeric G-proteins. Preincubation of isolated gastric glands with PTX ($200 ng/ml$) for 10 min prior to the experiments abolished the stimulatory effect of Gd^{3+} to a large extent (0.030

Fig. 2. The CaSR is coupled to heterotrimeric G-Proteins and requires PLC activity. (A) Incubation of gastric glands with pertussis toxin (PTX, $200 ng/ml$) to inhibit G-proteins ($G_{\alpha 0}/G_{\alpha i}$ -subunits) reduced the stimulatory effect of Gd^{3+} almost completely. In control glands PTX also reduced the basal pH_i -recovery rate. (B) Incubation of gastric glands with the PLC inhibitor U73122 ($10 \mu M$) decreased the stimulatory effect of Gd^{3+} on pH_i -recovery by about 50 %. * marks significant difference between control and respective treatment, # marks differences between Gd^{3+} alone and pharmacological treatment.

± 0.002 pH units/min) (Figure 2A). Incubation of gastric glands with PTX without CaSR stimulation reduced the basal pH_i -recovery rate even further (0.01 ± 0.0006 pH units/min). Thus, the CaSR acts at least in part via PTX-sensitive heterotrimeric G-proteins (G_o/G_i).

Fig. 3. Ca^{2+} takes part in the signalling cascade of the CaSR. (A) Chelation of intracellular Ca^{2+} with BAPTA-AM (50 μM), greatly reduced the stimulatory effect of Gd^{3+} on the intracellular pH recovery rate. (B) TMB-8 (400 μM) blocking the release of Ca^{2+} from the ER, completely inhibited the stimulatory effect of Gd^{3+} without affecting the basal alkalinization rate. (C) Verapamil (Vera, 20 μM), to block membrane Ca^{2+} -channels, showed a clear reduction in the effect of Gd^{3+} . (D) Incubation of gastric glands with verapamil (20 μM) and TMB-8 (400 μM) together completely abolished the effect of Gd^{3+} on pH_i recovery. * marks significant

difference between control and respective treatment, # marks differences between Gd^{3+} alone and pharmacological treatment.

The CaSR acts via phospholipase C

It has been shown in other tissues that the CaSR stimulates phospholipase C (PLC) activity [7]. Isolated gastric glands were preincubated for 10 min with U73122 (10 μM) to inhibit PLC. The stimulatory effect of Gadolinium was almost completely prevented by the PLC inhibitor (0.031 ± 0.002 pH units/min). Similar to the observations with PTX, PLC inhibition also reduced the basal pH_i -recovery rate in the absence of Gd^{3+} (0.014 ± 0.012 pH units/min) (Figure 2B).

Activation of the gastric proton pump is Ca^{2+} dependent

Stimulation of CaSR in freshly isolated rat and human gastric glands has been shown to increase intracellular Ca^{2+} concentrations [3-5]. Thus, we investigated the role of intracellular Ca^{2+} in the stimulatory effect of CaSR activation on pH_i -recovery/ H^+/K^+ -ATPase activity. After chelation of intracellular Ca^{2+} with BAPTA-AM (50 μM), the effect of Gd^{3+} was reduced by about 60 %

(0.035 ± 0.002) (Figure 3A). We used several inhibitors to examine the source of Ca^{2+} . To examine if release of Ca^{2+} from ER stores is involved, we blocked the Ca^{2+} -release from the ER with TMB-8 (400 μM), which caused a reduction in the Gd^{3+} -induced alkalinization rate almost to control levels (0.028 ± 0.002 pH units/min) (Figure 3B). Further experiments with verapamil (Vera., 20 μM) to block Ca^{2+} -influx from the extracellular space caused a complete inhibition of the stimulated pH_i -recovery rate (0.036 ± 0.004 pH units/min) (Figure 3C). Treatment of gastric glands with verapamil or TMB-8 alone had no effect on the basal pH_i -recovery rates (verapamil: 0.021 ± 0.002 pH units/min; TMB-8: 0.023 ± 0.003 pH units/min). Treatment of the cells with both inhibitors TMB-8 and verapamil together completely prevented the stimulatory effect of Gadolinium (0.019 ± 0.003 pH units/min) (Figure 3D). Thus, stimulation of the CaSR induces a rise in intracellular Ca^{2+} which is essential for mediating the stimulatory effect on H^+/K^+ -ATPase activity. The rise in intracellular Ca^{2+} is most likely caused by both release

Fig. 4. Ca^{2+} - dependent and - independent PKC isoforms contribute to the stimulatory effect of the CaSR. (A) Chelerythrine (1 μM), a general PKC inhibitor, showed a significant reduction in the pH_i recovery rate during stimulation of the CaSR with Gd^{3+} . (B) Similarly, activation of protein kinase C with DOG (1 μM) showed a stimulatory effect which was, however, only about 60 % of the effect observed with Gd^{3+} . (C) Blockade of the Ca^{2+} -dependent PKC isoforms

PKC- and PKC- δ with Gö 6976 (1 μM) completely prevented the effect of Gd^{3+} . (D) In contrast, inhibition of the Ca^{2+} -independent PKC isoforms PKC- and PKC- ϵ with Ro-31-2880 (1 μM) reduced the stimulatory effect of Gd^{3+} by about 60 %. * marks significant difference between control and respective treatment, # marks differences between Gd^{3+} alone and pharmacological treatment.

from the ER and may also involve influx from the extracellular medium.

Different Protein kinase C isoforms are involved in the down-stream signal

Gastric parietal cells express different protein kinase C (PKC) isoforms which belong to three distinct subfamilies [12-14]. Among them, the Ca^{2+} -dependent PKC- and the Ca^{2+} -independent PKC- isoforms were detected in rabbit parietal cells on protein level [12-14]. Incubation of parietal cells with chelerythrine (1 μM), a pan-PKC inhibitor, resulted in a significant reduction of the H^+/K^+ -ATPase activity despite stimulation of the Calcium-Sensing receptor with Gadolinium (0.022 ± 0.003 pH units/min) (Figure 4A). Activation of PKC with DOG (1 μM) showed a stimulatory effect which was, however, only about 60 % of the effect observed with Gd^{3+} (0.045 ± 0.003 pH units/min) (Figure 4B). Next, we tested the involvement of the Ca^{2+} -dependent (PKC- α) and Ca^{2+} -independent (PKC- ϵ) PKC isoforms. Incubation

with Gö 6976 (1 μM), an inhibitor of Ca^{2+} -dependent PKC isoforms PKC- α , PKC- β 1, completely abolished the stimulatory effect of Gadolinium (0.018 ± 0.002 pH units/min) (Figure 4C). Ro-31-2880 (1 μM), an inhibitor of Ca^{2+} -independent PKC δ - and PKC- ϵ isoforms attenuated the stimulatory effect only partially (0.040 ± 0.004 pH units/min). Moreover, combination of Ro-31-2880 with the PLC inhibitor U73122 completely abolished the effect of Gd^{3+} on H^+/K^+ -ATPase activity (0.016 ± 0.002 pH units/min) (Figure 4D). All PKC inhibitors applied alone had no effect on basal pH_i -recovery rates (chelerythrine: 0.014 ± 0.001 pH units/min, 189 cells, 6 glands, 3 patients); Gö 6976: 0.028 ± 0.003 pH units/min; Ro-31-2880: (0.016 ± 0.001 pH units/min).

ERK1/2 -MAP-kinases take part in the signaling cascade of the CaSR

Activation of the CaSR has been shown to increase also ERK1/2 MAP-kinase activity [6, 7] and ERK1/2 activity is increased during ACh induced gastric acid se-

Fig. 5. ERK1/2 MAP-kinases participate in the signalling cascade of the CaSR. (A) PD098059 (20 μ M) which specifically inhibits ERK1/2 MAP-kinases completely prevented the stimulatory effect of Gd^{3+} . (B) In contrast, incubation of gastric glands with wortmannin to inhibit PI-3-Kinase was without effect on the stimulation by Gd^{3+} and did not alter basal pH_i -recovery rates. * marks significant difference between control and respective treatment, # marks differences between Gd^{3+} alone and pharmacological treatment.

cretion [15]. PD098059 (20 μ M) which inhibits specifically ERK1/2 MAP-kinases completely prevented the stimulatory effect of Gd^{3+} (0.020 ± 0.001 pH units/min). PD098059 alone had no effect on pH_i -recovery rates (0.018 ± 0.001 pH units/min) (Figure 5A).

Activation of the gastric H^+/K^+ -ATPase by the CaSR is PI-3-Kinase independent

Incubation of gastric parietal cells with wortmannin, a specific inhibitor of PI-3-kinases, did not show a significant reduction of H^+/K^+ -ATPase activity (0.079 ± 0.004 pH units/min). Treatment of parietal cells with the inhibitor alone had also no effect on the rate of alkalinization (0.03 ± 0.001 pH units/min, (Figure 5C).

Discussion

Regulation of gastric acid secretion involves a complex system of receptors, intracellular signal cascades, and effector proteins involved in trafficking and fusion of H^+/K^+ -ATPase containing vesicles or stimulation of ion channels [2]. Past work has gathered much information about the processes regulated by receptors for histamine, gastrin or acetyl choline, the so called "main substances" mediating the stimulation of gastric acid secretion [2]. More recent studies have shown that in addition to these well described receptors a Calcium-Sensing Receptor has been identified and is active in acid-secretory parietal cells [3-5]. Parietal cells express a CaSR on their basolateral side which when stimulated by divalent (Ca^{2+}) or trivalent cations (Gd^{3+}) results in an increase in intracellular Ca^{2+} and stimulated acid secretion [3-5]. Furthermore, activation of the CaSR leads to a stimulation of H^+/K^+ -ATPase activity in freshly isolated rat and human gastric glands in the absence and presence of other stimuli such as histamine [4,5]. The physiological role of the CaSR mediated increase in gastric acid secretion has not been

fully clarified to date. However, it has been shown that L-amino acids allosterically increase the affinity of divalent cations to the receptor [16, 17]. Protein-rich meals, in turn, stimulate gastric acid-secretion. Furthermore, systemic calcium balance is tightly linked to gastric acid-secretion, but the molecular link has not been fully established.

In the present study we examined the intracellular signalling cascade mediating the stimulatory effect(s) of the CaSR on H^+/K^+ -ATPase activity in freshly isolated human gastric glands. We have previously shown that this preparation contains a functional CaSR and that parietal cells are viable and contain an omeprazole-sensitive and histamine stimulated H^+/K^+ -ATPase [4].

The data presented here demonstrate that CaSR exerts its stimulatory effects on H^+/K^+ -ATPase activity via a complex secretory intracellular cascade (see model in Figure 6) that involves pertussis-toxin sensitive heterotrimeric G-proteins G_o and/or G_i . These G proteins

Fig. 6. Model of the stimulatory effect of the CaSR on H^+,K^+ -ATPase activity in gastric parietal cells. Proposed scheme for CaSR induced stimulation of H^+,K^+ -ATPase activity in gastric parietal cells. See discussion for details.

inhibit adenylate cyclases and reduce cAMP levels. In fact in rat colon crypts activation of CaSR reduces intracellular levels of cAMP and cGMP by stimulating their break-down by phosphodiesterases [18]. However, in our experiments PTX did not fully abolish the stimulation of H^+/K^+ -ATPase activity suggesting that other G-proteins may also be involved. This observation is in agreement with a number of studies showing that CaSR can couple to PTX-sensitive and insensitive heterotrimeric G-proteins in different cells [6, 7, 11]. Secondly, the CaSR stimulation requires an intact PLC activity and several Ca^{2+} -dependent and -independent protein kinase C isoforms. The involvement of similar PKC isoforms in basal and ACh stimulated acid-secretion has recently been described in a parietal cell culture model [12, 14]. The role of PKC is further corroborated by the fact that DOG, an activator of PKC, mimics the stimulatory effect of CaSR activation and that CaSR has been shown to signal via PKC in other cells [7, 11].

Activation of CaSR leads also to a rapid rise in intracellular Ca^{2+} in rat and human gastric parietal cells [3-5]. Thus, we further investigated the role and source of this intracellular Ca^{2+} rise. First, chelation of intracellular Ca^{2+} with BAPTA completely abolished the stimulatory effect of CaSR activation indicating that the rise in $[Ca^{2+}]_i$ plays an important role either in activating down-stream

signals such as Ca^{2+} -dependent PKC isoforms or may also play a role in other Ca^{2+} -dependent processes such as vesicle fusion and insertion of H^+/K^+ -ATPases into the membrane [1, 2]. Moreover, the data suggest that Ca^{2+} may be released from the ER and might also enter the cell via verapamil-sensitive L-type Ca^{2+} -channels. TMB-8, a blocker of ER Ca^{2+} -releasing channels prevented the CaSR induced stimulation of H^+/K^+ -ATPase activity. In the case of verapamil, the direct inhibitory interaction of verapamil with the H^+/K^+ -ATPase, several intracellular enzymes and accumulation in the membrane has been described [19-21]. Thus, we cannot rule out that the effect of verapamil on the stimulatory action of the CaSR could be unspecific and does not involve direct inhibition of Ca^{2+} -channels. Moreover, the contribution of Ca^{2+} influx from the extracellular space to the regulation of H^+/K^+ -ATPases has been described as being minor compared to the release from ER stores and the fact that TMB-8 fully prevented the stimulatory effect of CaSR activation is in agreement with this earlier observation [22].

In addition to PKC and intracellular Ca^{2+} , we also found evidence for the involvement of ERK1/2 MAP-kinases in the CaSR mediated stimulation of H^+/K^+ -ATPase activity. ERK1/2-kinases have been shown to be activated by the CaSR in other tissues [6] and mediate at

least in part the stimulatory effect of secretagogues like carbachol [15]. Interestingly, the activation of ERK1/2 MAP-kinases seems to involve both inhibitory as well as stimulatory pathways and is independent from intracellular Ca^{2+} [15]. This suggests that an intact ERK1/2 may be necessary for the stimulation of H^+/K^+ -ATPases by the CaSR either in a modulatory fashion or as a direct pathway. At the moment we have no direct evidence if ERK1/2 is down-stream of the PLC-PKC pathway or represents a separate pathway that plays a permissive role. Similarly, we have previously shown that the apical PTH receptor in the proximal tubule signals via the PLC-PKC pathway and converges with a cAMP-PKA dependent pathway in ERK1/2 stimulation [23].

In summary, activation of the CaSR in human gastric parietal cells leads to stimulation of H^+/K^+ -ATPase activity involving heterotrimeric PTX-sensitive and insensitive G-proteins, requires intact PLC and a rise in intra-

cellular Ca^{2+} , Ca^{2+} -dependent and -independent PKC isoforms, and intact ERK1/2 MAP-kinases. Further experiments will need to elucidate the direct targets of the signal cascade and determine the interaction with signal cascades activated by classic secretagogues like histamine or acetyl choline. The signal cascades described here could therefore be involved in enhancing or modulating signals controlling gastric acid secretion and help to explain the modulatory role of calcium in the control of gastric acid secretion [24].

Acknowledgements

This study was supported by a grant from the Hartmann Müller Stiftung to P. Kirchhoff. J Geibel was supported from grants from the National Institutes of Health (DK50230).

References

- Okamoto CT, Forte JG: Vesicular trafficking machinery the actin cytoskeleton and H^+/K^+ -ATPase recycling in the gastric parietal cell. *J Physiol* 2001;532:287-296.
- Yao X, Forte JG: Cell biology of acid secretion by the parietal cell. *Annu Rev Physiol* 2003;65:103-131.
- Cheng I, Qureshi I, Chattopadhyay N, Qureshi A, Butters RR, Hall AE, Cima RR, Rogers KV, Hebert SC, Geibel JP, Brown EM, Soybel DI: Expression of an extracellular calcium-sensing receptor in rat stomach. *Gastroenterology* 1999;116:118-126.
- Dufner MM, Kirchhoff P, Remy C, Hafner P, Muller MK, Cheng SX, Tang LQ, Hebert SC, Geibel JP, Wagner CA: The Calcium-Sensing Receptor (CaSR) acts as a modulator of gastric acid secretion in freshly isolated human gastric glands. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G1084-1090.
- Geibel JP, Wagner CA, Caroppo R, Qureshi I, Gloeckner J, Manuelidis L, Kirchhoff P, Radebold K: The stomach divalent ion-sensing receptor scar is a modulator of gastric acid secretion. *J Biol Chem* 2001;276:39549-39552.
- Hofer AM, Brown E M: Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol* 2003;4:530-538.
- Brown EM, MacLeod RJ: Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev* 2001;81:239-297.
- Fisher BL, Schauer P: Medical and surgical options in the treatment of severe obesity. *Am J Surg* 2002;184:9S-16S.
- Kirchhoff P, Wagner CA, Gaetzschnann F, Radebold K, Geibel JP: Demonstration of a functional apical sodium hydrogen exchanger in isolated rat gastric glands. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G1242-1248.
- Thomas JA, Buchsbaum, RN, Zimniak A, Racker E: Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 1979;18:2210-2218.

- 11 Ward DT: Calcium receptor-mediated intracellular signalling. *Cell Calcium* 2004;35:217-228.
- 12 Fahrman M, Kaufhold M, Rieg T, Seidler U: Different actions of protein kinase C isoforms alpha and epsilon on gastric acid secretion. *Br J Pharmacol* 2002;136:938-946.
- 13 Nandi J, Bosche MC, Levine RA: Effects of a phorbol ester and isoquinoline sulfonamides on rabbit parietal cell function. *J Pharmacol Exp Ther* 1996;279:97-105.
- 14 Chew CS, Zhou CJ, Parente JA, Jr: Ca^{2+} -independent protein kinase C isoforms may modulate parietal cell HCl secretion. *Am J Physiol* 1997;272:G246-256.
- 15 Nakamura K, Zhou CJ, Parente J, Chew CS: Parietal cell MAP kinases: multiple activation pathways. *Am J Physiol* 1996;271:G640-649.
- 16 Busque SM, Kerstetter JE, Geibel JP, Insogna K: L-type amino acids stimulate gastric acid secretion by activation of the calcium-sensing receptor in parietal cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G664-669.
- 17 Conigrave AD, Franks AH, Brown EM, Quinn SJ: L-amino acid sensing by the calcium-sensing receptor a general mechanism for coupling protein and calcium metabolism. *Eur J Clin Nutr* 2002;56:1072-1080.
- 18 Geibel J, Sritharan K, Geibel R, Geibel P, Persing JS, Seeger A, Roepke TK, Deichstetter M, Prinz C, Cheng SX, Martin D, Hebert SC: Calcium-sensing receptor abrogates secretagogue-induced increases in intestinal net fluid secretion by enhancing cyclic nucleotide destruction. *Proc Natl Acad Sci U S A* 2006;103:9390-9397.
- 19 Beil W, Bersimbaev RJ, Hannemann H, Sewing KF: Interaction of calcium channel antagonists with parietal cell acid production, adenylate cyclase, intracellular-free Ca^{2+} and $\text{H}^{+}/\text{K}^{+}$ -ATPase. *Pharmacology* 1990;40:8-20.
- 20 Nandi J, King RL, Kaplan DS, Levine RA: Mechanisms of gastric proton pump inhibition by calcium channel antagonists. *J Pharmacol Exp Ther* 1990;252:1102-1107.
- 21 Herling AW, Ljungstrom M: Effects of verapamil on gastric acid secretion in vitro and in vivo. *Eur J Pharmacol* 1988;156:341-350.
- 22 Negulescu PA, Machen TE: Ca transport by plasma membrane and intracellular stores of gastric cells. *Am J Physiol* 1993;264:C843-851.
- 23 Bacic D, Schulz N, Biber J, Kaissling B, Murer H, Wagner C A: Involvement of the MAPK-kinase pathway in the PTH mediated regulation of the proximal tubule type IIa $\text{Na}^{+}/\text{P}_{i}$ cotransporter in mouse kidney. *Pflügers Arch* 2003;446:52-60.
- 24 DelValle JLM, Yamada T: Gastric Secretion; in Yamada T (ed): *Textbook of Gastroenterology*. Philadelphia, JB Lippincott Company 1995, vol 1, pp 295-319.