Mechanisms of secretion-associated shrinkage and volume recovery in cultured rabbit parietal cells

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During acid secretion, parietal cells secrete 150–160 mM HCl into the secretory canaliculi. This process involves the movement of large quantities of electrolytes across the apical and basolateral cell membranes. We had previously observed a strong activation of the Na+/H+ exchange but no changes in intracellular pH (pHi) during secretagogue stimulation of cultured parietal cells (2). Since Na+/H+ exchange is also involved in cell volume regulation, we wondered whether parietal cells have a need for volume regulation during stimulation of acid secretion. Indeed, isolated rabbit parietal cells in culture displayed a rapid initial cell shrinkage in response to secretagogues, followed by a regulatory volume increase, which was largely dependent on Na+/H+ exchange (31). The mechanisms leading to the initial cell volume decrease as well as the relative importance of the different parietal cell Na+/H+ exchanger (NHE) isoforms in response to different secretagogues are, however, unknown.

We hypothesized that the observed volume loss after stimulation may occur via K+ and/or Cl− channels, which have been implicated in water and electrolyte efflux during stimulated secretion in various epithelia (3, 7, 23, 34, 35). K+ channels are present in the apical and basolateral membranes of the parietal cell (12, 14, 18, 32) and could potentially play a role in stimulation-associated cell shrinkage in both locations, which makes it important to differentiate between constitutively active and secretagogue-activated conductances. While the molecular identity of the basolateral K+ channels is largely unknown, there are good candidates for apical conductances mediating K+ recycling during H+–K+–ATPase activation (25, 26): KCNQ1, which is active at low pH (9, 14) and can be inhibited by the chromanol 293b (14), and different members of the inwardly rectifying K+ (Kir) family (12, 18, 22). Another possible pathway for secretion-mediated cell shrinkage are apical Cl− channel(s) enabling HCl formation (16, 21, 24). In addition, the function of the H+–K+–ATPase may or may not be necessary for full shrinkage.

In this study, we addressed the question whether K+ and Cl− channels, as well as the H+–K+–ATPase, are involved in the initial cell shrinkage during stimulation of acid secretion. To this end, cell volume was measured confocally in cultured rabbit parietal cells during pharmacological inhibition of the respective ion channels after cAMP-dependent as well as cholinergic stimulation. Furthermore, the relative contribution of the different NHE isoforms to the subsequent regulatory volume increase (RVI) was assessed.

Materials and Methods

Materials. The acetoxyethyl ester of BCECF/AM and calcine/AM were purchased from Molecular Probes (Leiden, The Netherlands). 4-Isopropyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE642) and trans-6-cyano-4-[N-ethylsulfonyl-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane (293b) were generous gifts from Sanofi-Aventis (Frankfurt, Germany). 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), charybdotoxin, forskolin, and carbachol were from Sigma (Deisenhofen, Germany). All other reagents were of cell culture grade or the highest grade available.

Parietal cell isolation and culture. Isolation of rabbit gastric parietal cell by enzymatic digestion, counterflow elutriation, and gradient centrifugation as well as cell culture were carried out following the method of Chew et al. (8) exactly as described previously by our laboratory (2, 27, 31). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
all functional experiments were performed on culture days 3 and 4. The experimental protocols were approved by the local authorities of animal welfare (Regierungspräsidium).

**Cytoplasmic volume measurements.** Parietal cells in primary culture do, despite the lack of contact to neighboring cells and the endocytosis of the apical membrane, partially reform their cytoskeleton (28), which is an important component of volume regulatory processes (19). Measuring cell volume changes during acid formation in these cells needs special techniques, since acid secretion does not occur into the extracellular space, but into intracellular vacuoles (31), and thus overall volume changes do not reflect cytoplasmic volume changes during stimulation of acid secretion. Therefore the concentration of the fluorescent dye calcein in defined regions of interest, rather than cell diameter, was used to quantify cell volume. Briefly, parietal cells cultured on coverslips were incubated with 3 μM calcein/AM for 30 min at 37°C in perfusion buffer (in mM: 118 NaCl, 22 NaHCO3, 14 HEPES, 7 Tris, 3 KH2PO4, 2 K2HPO4, 1.2 CaCl2, 1.2 MgSO4, and 20 glucose, pH 7.4, gassed with 5% CO2-95% O2), then washed twice and incubated another 30 min in buffer without dye. After the coverslip was mounted in a custom-made perfusion chamber, confocal x-z images were obtained every 10 s with an LSM 410 confocal microscope (Carl Zeiss, Oberkochen, Germany) using the 488-nm argon laser line. Background fluorescence was found to be negligible and was not corrected for. It is important to note that the parietal cells were selected on the basis of morphological criteria (typical fine granular structure under phase contrast, homogenous cytoplasmic dye distribution under fluorescence light, no large vacuoles existing in the resting state, small vacuoles present that enlarged during stimulation of acid secretion (in the control experiments without inhibitors)).

**14C aminopyrine uptake experiments.** Estimation of acid secretion was performed by measuring [14C]aminopyrine uptake as described previously (2). The apical membrane of cultured parietal cells forms large vacuoles into which the acid is secreted and where the weak base [14C]aminopyrine therefore accumulates. After preincubation with the respective inhibitors for 5 min, cells were stimulated by forskolin or carbachol for 45 min. The activity representing nonspecific accumulation (measured as the 10 μM omeprazole-independent [14C]aminopyrine uptake) was subtracted from each value, and stimulated acid secretion was estimated by forming the ratio of the results after secretagogue administration and basal [14C]aminopyrine uptake determined as the ranitidine (100 μM)-independent fraction.

**Microfluorometric determination of intracellular pH.** Fluorescence microscopy for determination of pH, was performed exactly as described previously (2, 27) using the fluorescent dye BCECF, by perfusing the cells with perfusion buffer containing secretagogues and inhibitors and subsequent calibration with the high-K+/nigericin method.

**Calculations and statistics.** After completion a volume measurement, three to four regions of interest within the cytoplasm of the cells not involving secretory vacuoles were selected, and the relative cumulative intensity was averaged and plotted against the time. Linear regression analysis was used to assess the changes in calcein concentration during the initial steep linear phase of the time course. Since the fluorescence signal is measured in x-z sections with a volume defined by the x-y resolution and the pinhole size, changes were expressed as voxel percent per second. For all experiments, statistical testing was carried out using Student’s t-test for independent samples or analysis of variance followed by Tukey’s honestly significant difference test where appropriate. Significance was defined as a P value below 0.05.

**RESULTS**

When cultured parietal cells are stimulated with cAMP-dependent or cholinergic compounds, a rapid cell volume loss occurs, which is indicative of their secretory activity (30). This initial shrinkage (corresponding to an increase in cytoplasmic calcium concentration) is followed by a somewhat slower RVI (corresponding to a decrease in cytoplasmic calcium concentration; Fig. 2, A and D), which we have previously shown to be Na+/H+ exchange dependent (31).

The time frame for maximal shrinkage is very rapid, and therefore we speculated that it may be associated with the earliest events that occur during the process of acid secretion. We therefore first investigated the potential role of K+ channel activation. The K+ channels inhibitor 293b inhibited both forskolin- and carbachol-stimulated acid formation (Fig. 1A)

**Fig. 1.** Stimulated acid formation during K+ and Cl− channel blockade. Compared with the ranitidine (100 μM)-treated control, forskolin (10 μM) and carbachol (100 μM) caused a 4- and 2.5-fold stimulation of [14C]aminopyrine uptake, respectively. Trans-6-cyano-4-[N-ethylsulfonyl]-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane (293b; A) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; B) concentration dependently inhibit [14C]aminopyrine uptake in cultured parietal cells (for inhibitor concentrations, see 1-ax) compared with the control (forskolin/carbachol only, n = 6–7 from 4 preparations in each group, means ± SE, **P < 0.01, ANOVA and Tukey’s honestly significant difference test (HSD)). In contrast, blocking Ca2+-activated K+ channels with charybdotoxin (ChTX, 100 nM; C) only caused a significant reduction of acid formation during cholineric (100 μM carbachol), but not during cAMP-dependent (10 μM forskolin) stimulation (n = 5–6 from 4 preparations in each group, means ± SE, **P < 0.01 vs. control, Student’s t-test for unpaired samples).
and in the same concentrations of 10 and 100 μM strongly inhibited the shrinkage we had observed with both forskolin and carbachol (Fig. 2, C and F). A regulatory volume increase still occurred subsequently (Fig. 2, B and E) but was correspondingly smaller in magnitude.

Since the KCNQ1 channel inhibitor 293b had less effect on carbachol-induced shrinkage than on forskolin-induced shrinkage, we asked the question whether K⁺ channels that are activated by cholinergic stimulation could additionally be involved. Intermediate-conductance K⁺ channels have been implicated in Ca²⁺-activated K⁺ secretion in colonic crypt cells (10) as well as K⁺ currents in a gastric epithelial cell line (15). A 50–100 nM concentration of charybdotoxin was reported to be effective for blocking these channels (10, 15), and 100 nM caused a significant decrease of [14C]aminopyrine uptake during carbachol, but not forskolin treatment (Fig. 1C). After preincubation with this substance, the forskolin-associated shrinkage in cultured parietal cells was unchanged, whereas the carbachol-associated shrinkage was significantly inhibited, pointing to a possible role of these channels in volume regulation after cholinergic stimulation (Fig. 3C).

To block K⁺ recycling via H⁺-K⁺-ATPase, we measured volume changes in the presence of SCH28080, which competitively binds to the K⁺ site of the enzyme (2). There was no effect of SCH28080 on cell volume under baseline conditions (Fig. 3, A and B). However, the initial cell shrinkage induced by cAMP-dependent and cholinergic stimulation was significantly inhibited (Fig. 3C). Thus, the K⁺ recycling process is also necessary for the maximal shrinkage caused by secretagogue application.

Cl⁻ secretion is also crucial for HCl formation and is thought to occur via NPPB-sensitive Cl⁻ channels (33). Although NPPB also interferes with some K⁺ channels (11), it is the only substance that likely inhibits the intracellular anion channels of the CLIC family that are the most probable conductances for apical Cl⁻ secretion (1, 24). We therefore examined the effect of NPPB on secretagogue-induced parietal cell shrinkage. NPPB, in the concentration necessary to inhibit [14C]aminopyrine accumulation (Fig. 1B), almost completely abolished the cell volume decrease observed after forskolin or carbachol (Fig. 4).

Following the initial shrinkage, a regulatory volume increase occurs, which is dependent on Na⁺/H⁺ exchange (31). Since the Na⁺/H⁺ exchanger isoforms expressed in the parietal cell membrane are differentially regulated by different secretagogues (2, 27), we first blocked all Na⁺/H⁺ exchanger isoforms expressed in rabbit parietal cells using 500 μM dimethylamiloride (DMA), which strongly reduced RVI after both forskolin- and carbachol-induced shrinkage (31). Interestingly, the 1 μM concentration of HOE642, which selectively inhibits NHE1 (2), eliminated the major part of the DMA-dependent RVI after forskolin and carbachol (Fig. 5). DMA (500 μM), which is the only reported substance with inhibitory potential
against NHE4 (5), almost completely blocked the regulatory volume increase (Fig. 5), while not affecting shrinkage (30).

To exclude that the inhibitors used in the experiments described above affect pH regulation, which could interfere with the interpretation of our results, we studied pH in cultured parietal cells using the same protocol as for the volume studies. Neither 293b (at a concentration of 10 and 100 μM) nor NPPB, charybdotoxin, or SCH2880 caused a significant pH change. A variety of other manipulations such as Cl− or Na+/H+ removal and application of omeprazole or stilbenes did so and were therefore not used in the present investigations. Furthermore, pH remained stable after subsequent stimulation with forskolin or carbachol (data not shown), which we already had previously observed in parietal cells in the presence of CO2/HCO3− (2).

**DISCUSSION**

Parietal cells in primary culture display strong volume changes during stimulation of acid formation. We have previously found that rabbit parietal cells use Na+/H+ exchange (likely in conjunction with one or several anion exchangers) as their predominant mechanism of volume recovery after shrinkage (31), which may in part explain the strong gastric phenotype of all knockout mouse strains for the different NHEs expressed in parietal cells (4, 13, 29). The mechanisms of the initial shrinkage, however, remained unexplored. In this paper, we show that the rapid stimulation-associated cell shrinkage is blocked by inhibition of secretagogue-activated K+ and Cl− channels. Inhibition of K+ recycling by the H+/K+ ATPase also resulted in a partial inhibition of stimulation-associated shrinkage. Furthermore, our results demonstrate the surprising finding that the NHE1 isoform is the predominant isoform that mediates the subsequent regulatory volume increase in rabbit parietal cells.

K+ and Cl− channel opening has been associated with cell shrinkage during stimulated secretion in various secretory epithelia, including the salivary gland (23), the trachea (7), the lacrimal gland (35), and the small and large intestine (3, 34). In

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**Fig. 3. Effect of SCH28080 and charybdotoxin on parietal cell stimulation-associated cell shrinkage.** The K+-competitive H+-K+ ATPase inhibitor SCH28080 (10 μM) did not cause volume changes in the resting state (A and B: representative tracings), but reduced forskolin-induced shrinkage by 36.8% and the carbachol-induced shrinkage by 68.7% (C). The K+ channel blocker charybdotoxin (100 nM) only caused a significant inhibition of the cell volume decrease after carbachol (100 μM, 0.022 ± 0.006 vs. 0.115 ± 0.02 voxel/s, P < 0.01), but not after forskolin (10 μM) stimulation (C, n = 5–6 from 3 preparations in each group, means ± SE, **P < 0.01 vs. control, ANOVA and Tukey’s HSD).

**Fig. 4. Secretagogue-induced parietal cell volume decrease is absent during Cl− channel inhibition.** The unspecific Cl− channel inhibitor NPPB (300 μM), which strongly reduces [3H]aminopyrine accumulation, did not lead to volume perturbations when added alone but causes a drastic reduction in calcine concentration change [92.8% for the forskolin (10 μM)- and 97.7% for the carbachol (100 μM)-induced shrinkage, A and B, representative tracings; C, cumulative results]. In fluorometric intracellular pH (pHi) measurements, no effect on other, pHi-regulating ion transporters was observed (not shown). Values are means ± SE; n = 5–7 from 4 preparations in each group. **P < 0.01 vs. control, Student’s t-test for unpaired samples.
Fig. 5. Contribution of the different Na⁺/H⁺ exchanger isoforms to the regulatory volume increase after stimulation in parietal cells. The NHE1-specific HOE642 concentration of 1 μM causes a significant reduction in the regulatory volume increase (RVI; measured as a decrease in dye concentration which was necessary for inhibition of acid secretion, n = 6–7 from 3–4 preparations in each group, means ± SE, *P < 0.05, **P < 0.01 vs. control, ANOVA and Tukey’s HSD).

parietal cells, K⁺ and Cl⁻ channels are needed for K⁺ recycling and HCl secretion, respectively (26). K⁺ channels have been functionally identified in both the apical and basolateral membrane of the parietal cells (26, 32). The apical channels are required for bringing K⁺ ions into the canalicular space for subsequent exchange against H⁺, while the basolateral K⁺ channels believed to maintain the electrochemical driving force for continuous Cl⁻ exit through the apical membrane. Ba²⁺, a nonselective blocker of K⁺ channels, causes substantial cell swelling already in resting parietal cells (31), indicating that Ba²⁺ inhibits constitutively active K⁺ channels. We therefore searched for K⁺ channel inhibitory compounds that would exclusively inhibit stimulation-associated K⁺ channels. The Hoechst compound 293b inhibits KCNQ1 and reversibly blocked acid secretion in isolated rabbit gastric glands with a half maximal inhibitory concentration of 3–20 μM (14). In our cellular system, 293b also inhibited both forskolin- and carbachol-induced [¹⁴C]aminopyrine uptake. In the same concentration which was necessary for inhibition of acid secretion, 293b caused no change in resting state cytoplasmic volume but a >60% reduction of the stimulation-associated initial cell shrinkage after cAMP-dependent and cholinergic stimulation, indicating that KCNQ1 function is necessary for the observed volume changes. The inhibitory effect of 293b was somewhat weaker during cholinergic than during cAMP-dependent stimulation, suggesting a higher relevance of additional mechanisms in the case of carbachol.

Charybdoxin at a concentration of 100 nM has been shown to block Ca²⁺-activated K⁺ channels in a gastric epithelial cell line (15). In rat distal colon, this compound blocked the early component of the K⁺ current after Ca²⁺-dependent stimulation (30). Evidence from Calu-3 airway epithelial cells suggests that charybdoxin-sensitive K⁺ channels mediate cell shrinkage with subsequent activation of Na⁺/K⁺/2Cl⁻ cotransport (17). Our data show that, in addition to a 293b-sensitive component, a charybdoxin-sensitive K⁺ conductance is equally involved in the cell shrinkage occurring after cholinergic stimulation of acid secretion in the parietal cell. Cl⁻ channels are also essential for HCl formation, and their molecular nature is discussed controversially. Malinowska et al. (21) have cloned the CLC-2 Cl⁻ channel from rabbit stomach and have speculated about its involvement in acid secretion. However, Hori et al. (16) did not find CLC-2 expression in gastric parietal cells by either PCR or immunohistochemistry, and the CLC-2 knockout mouse does not have reduced acid secretion (6). More recently, Nishizawa et al. have reported high enrichment of the chloride intracellular channel (CLIC)-related protein parichin in parietal cells, making this another very good candidate for apical Cl⁻ exit (24). Since no pharmacological inhibitors with discriminative value for these Cl⁻ channels are available, we used the unspecific blocker NPPB, which has been shown to block intracellular CLIC channels (1). NPPB, in a fully acid inhibitory concentration, completely prevented the stimulation-associated cell shrinkage. In addition, we had previously observed that NPPB has similar inhibitory effects on [¹⁴C]aminopyrine accumulation as diphenylamine-2-carboxylate (DPC), another nonspecific anion channel blocker (20). However, since NPPB has also been shown to act on K⁺ channels (11), we cannot rule out additional effects.

SCH28080 binds to the K⁺ transport site of the H⁺/K⁺ ATPase and inhibits K⁺ recycling via the proton pump. SCH28080, in a concentration that completely prevents any acid formation, also caused a partial inhibition of stimulation-associated shrinkage. This demonstrates that parietal cell shrinkage occurs even in the complete absence of proton accumulation in the canalicular space but that K⁺ cycling is necessary for the full stimulation-associated shrinkage to occur.

Next, we followed up on our previous results regarding the RVI after stimulation, which is dependent on Na⁺/H⁺ exchange, but not Na⁺/K⁺/Cl⁻ cotransporter (31). We had reported that the NHE1, NHE2, and NHE4 isoforms are expressed by the rabbit parietal cell (27). The involvement of different NHE isoforms in pH₇ and volume regulation can be pharmacologically dissected by using the compound HOE642, which at a concentration of 1 μM is specific for the NHE1 isoform (2). Earlier work from our group had demonstrated that the NHE1 and 2 isoforms are activated by intracellular acidification, whereas the NHE1 and NHE4 isoforms are activated by hyperosmolality (27). Furthermore, we had found that carbachol predominantly activates NHE1, while an increase in intracellular cAMP causes stimulation of all NHE isoforms expressed in the rabbit parietal cell (2). Targeted disruption of NHE4 and NHE2 leads to hypo- and achlorhydria, respectively (13, 29). The NHE1 knockout mice displays a milder gastric phenotype but may not live long enough to develop a stronger phenotype (4). Surprisingly, we here show a major contribution of the NHE1 isoform to the RVI after secretagogue-induced shrinkage, indicating that NHE1 plays a key role in rabbit parietal cell volume homeostasis during stimulated secretion. High concentrations of DMA have been reported to inhibit
heterologically expressed NHE4 (5) and were able to further reduce volume increase to very low levels. We thus conclude that, in the rabbit parietal cell, NHE1 and NHE4 contribute most to counteract the stimulation-associated volume loss. This fits with the relatively high expression levels for NHE1 and NHE4 in the rabbit parietal cells, compared with other gastric cell types (27). It fails to explain the gastric phenotype of the NHE2 knockout mouse. Possibly the situation is different in rabbit and mouse, or NHE2 plays an unknown role in parietal cell physiology.

In conclusion, we further investigated parietal cell volume regulation, a crucial homeostatic mechanism in this cell type with a high secretory activity. We are able to show that the cell shrinkage occurring after stimulation of acid secretion is dependent on K+ and Cl− channels. Our results demonstrate that a 293b-sensitive conductance, most likely KCNQ1, is an important player in this setting and that cholinergic stimulation additionally involves charybdo toxin-sensitive K+ channels. Furthermore, Cl− channel activity is necessary for the stimulation-associated cell volume decrease to occur. Blocking K+ cycling with the H+K+-ATPase inhibitor SCH28080 partially reverses parietal cell shrinkage in response to secretagogues. The NHE1 isoform of Na+/H+ exchangers is the main isoform involved in the subsequent regulatory volume increase.

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