Real Time Measurements of Water Flow in Amphibian Gastric Glands

MODULATION VIA THE EXTRACELLULAR Ca\(^{2+}\)-SENSING RECEPTOR

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The mechanisms for the formation of the osmotic gradient driving water movements in the gastric glands and its modulation via the extracellular Ca\(^{2+}\)-sensing receptor (CaR) were investigated. Real time measurements of net water flux in the lumen of single gastric glands of the intact amphibian stomach were performed using ion-selective double-barreled microelectrodes. Water movement was measured by recording changes in the concentration of impermeant TEA\(^{+}\) ions ([TEA\(^{+}\)]\(_{gl}\)) with TEA\(^{+}\)-sensitive microelectrodes inserted in the lumen of individual gastric glands. Glandular K\(^{+}\) ([K\(^{+}\)]\(_{gl}\)) and H\(^{+}\) (pH\(_{gl}\)) were also measured by using K\(^{+}\)- and H\(^{+}\)-sensitive microelectrodes, respectively. Stimulation with histamine significantly decreased [TEA\(^{+}\)]\(_{gl}\), indicating net water flow toward the gland lumen. This response was inhibited by the H\(^{+}/K^{+}\)-ATPase inhibitor, SCH 28080. Histamine also elicited a significant and reversible increase in [K\(^{+}\)]\(_{ot}\) that was blocked by chromanol 293B, a blocker of KCQNi1 \(K^{+}\) channels. Histamine failed to induce net water flow in the presence of chromanol 293B. In the “resting state,” stimulation of CaR with diverse agonists resulted in significant increase in [TEA\(^{+}\)]\(_{ot}\). CaR activation also significantly reduced histamine-induced water secretion and apical K\(^{+}\) transport. Our data validate the strong link between histamine-stimulated acid secretion and water transport. We also show that cAMP-dependent [K\(^{+}\)]\(_{ot}\) elevation prior to the onset of acid secretion generates the osmotic gradient initially driving water into the gastric glands and that CaR activation inhibits this process, probably through reduction of intracellular cAMP levels.

Water transport is a process of vital importance for the physiology of the digestive system. Significant amounts of water are secreted/absorbed every day in the GI tract, and the molecular mechanisms involved are well characterized for most segments (1). In the case of the stomach, however, although much interest has been centered on the ion transport mechanisms linked to acid or alkaline secretion, the molecular mechanisms of water transport are not fully understood. Most of the information in this field derives from rather early reports where water transport was measured either in amphibian or mammalian stomach by monitoring change in weight (2) or volume (3–5), by gravimetric (6) or radioactive methods (7, 8), or with fluorescent indicators in membrane vesicles (9).

In general, it is believed that in the stomach, as in other organ systems, water movement occurs secondarily to osmotic driving forces created by active ion transport (6, 10) and/or to hydrostatic pressure differences (2, 11). Since gastric water flow (which can amount to up to 2 liters/day in humans (1, 12)) is proportional to acid secretion, it was previously assumed that secreted H\(^{+}\) and Cl\(^{-}\) might create the osmotic driving force for water movement (6). Later, Berglindh et al. (13) proposed the “osmotic swelling” model in which the efflux of K\(^{+}\) from the acid-secreting cells into the canaliculi and into the gland lumen would create a gradient for the osmotic flow of water. To date, however, a clear correlation between the transport of specific ions and gastric water movement has yet to be established. More importantly, the temporal dynamics of gastric water flow have never been defined.

Here we studied how the osmotic gradient driving water into the lumen of the glands in the initial phases of acid secretion is generated using an approach that allows real time monitoring of fluid movement in single gastric glands. Water movement was measured by recording changes in the concentration of impermeant ions with ion-sensitive microelectrodes inserted in the lumen of gastric glands in the intact perfused amphibian stomach. Our data show a direct correlation between time course of transport of specific ions, namely K\(^{+}\), and water movement in the secretory state.

In this study, we also investigated how the activation of an unusual G-protein-coupled receptor, the extracellular Ca\(^{2+}\)-sensing receptor (CaR), influences water secretion in the stomach. CaR was first described in the parathyroid gland (14), and water movement in the gastric glands in the intact perfused amphibian stomach. CaR was first described in the parathyroid gland (14), and the molecular mechanisms involved are well characterized for most segments (1). In the case of the stomach, however, although much interest has been centered on the ion transport mechanisms linked to acid or alkaline secretion, the molecular mechanisms of water transport are not fully understood. Most of the information in this field derives from rather early reports where water transport was measured either in amphibian or mammalian stomach by monitoring change in weight (2) or volume (3–5), by gravimetric (6) or radioactive methods (7, 8), or with fluorescent indicators in membrane vesicles (9).
Water Transport in the Gastric Gland Lumen

Amino acids, and other polyvalent cations. The receptor has since been identified in many other cell types, including epithelial cells of the alimentary tract (for reviews, see Refs. 15 and 16).

The formation and secretion of gastric juice, which is composed mostly of water, hydrochloric acid (145 mmol/liter in humans), and pepsinogen, is under the control of hormonal and neural pathways and is essentially mediated by gastrin, histamine, and acetylcholine (17–21). In addition, we previously showed that changes in the concentration of external free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{ext}}\)], working via CaR, can influence the secretory function of gastric mucosa (22). We found that stimulation with carbachol, which mobilizes intracellular Ca\(^{2+}\) in the oxynto-peptic cells (OCs), resulted in a substantial local increase in the extracellular [Ca\(^{2+}\)] at the luminal face and a comparable depletion at the serosal aspect of amphibian acid-secreting cells (23). The increase in [Ca\(^{2+}\)] in the gastric gland lumen is due to activation of the plasma membrane Ca\(^{2+}\)-ATPase, which is highly expressed at the apical membrane of these cells, where it co-localizes with CaR.

In the amphibian gastric mucosa, carbachol is a well known agonist of bicarbonate (24–26) and pepsinogen secretion (27). We previously found that changes in [Ca\(^{2+}\)\(_{\text{ext}}\)] secondary to carbachol-induced increases in intracellular [Ca\(^{2+}\)] were sufficient and necessary to elicit both alkaline and pepsinogen secretion, independently of intracellular [Ca\(^{2+}\)] changes (22).

Recent reports show that CaR may also be involved in the modulation of fluid secretion in different tissues. Lactating mammary gland can “sense” extracellular Ca\(^{2+}\) and adjusts milk secretion as well as the secretion of parathyroid hormone-related protein and water in response to changes in [Ca\(^{2+}\)\(_{\text{ext}}\)] (29, 30). Finally, CaR, which is expressed along the entire gastrointestinal tract, seems to be involved in the modulation of fluid transport in the colon (31).

We found here that stimulation of CaR results in considerable attenuation of gastric water secretion under resting conditions. This modulation becomes less important, however, during maximal rates of acid secretion.

MATERIALS AND METHODS

Tissue and Solutions—The experiments were performed on gastric fundus mucosa of *Rana esculenta* in accordance with the Italian guidelines for animal experiments. Frogs were sacrificed by decapitation followed by destruction of the spinal cord and brain. The isolated mucosa was mounted horizontally between two halves of a top-open Lucite chamber (aperture 0.2 cm\(^2\)) with the serosal side facing up. The connective tissue layer was further removed with sharpened watchmaker forceps under direct microscopic observation in order to expose a number of glands for impalement with microelectrodes. Both the serosal and mucosal surfaces were constantly superfused with oxygenated Ringer’s solution at room temperature. Fast fluid exchange in the chamber was achieved within seconds from a shock-free, electronically controlled eight-way manifold.

The control Ringer’s solution had the following composition: 102.4 mmol/liter Na\(^{+}\), 4.0 mmol/liter K\(^{+}\), 1.4 mmol/liter Ca\(^{2+}\), 0.8 mmol/liter Mg\(^{2+}\), 91.4 mmol/liter Cl\(^{-}\), 17.8 mmol/liter HCO\(_3\)\(^{-}\) and 11 mmol/liter glucose. It was gassed with 5% CO\(_2\) in O\(_2\) and had a pH of 7.36.

Tissues were maintained in resting state by serosal addition of cimetidine (a histamine \(H_2\) receptor blocker used to prevent acid secretion) or stimulated by serosal perfusion with histamine. All chemicals were of reagent grade and purchased from Farmitalia Carlo Erba (Milan, Italy), Sigma, Fluka Chemie AG (Buchs, Switzerland), Alexis Biochemicals (Lausen, Switzerland), or SmithKline Beecham (Baranzate, Italy).

Transepithelial and Extracellular Ion Measurements—The transepithelial potential difference (V\(_t\)) was measured with a model 610C high impedance differential electrometer (Keithley, Cleveland, OH) using two flowing boundary calomel half-cells filled with 2.7 mol/liter KCl solution and connected to each bath solution downstream of the tissue. The serosal bath was connected to ground. The lumen of the gastric gland was punctured by lowering the ion-sensitive (TEA\(^{+}\), K\(^{+}\), H\(^+\), or Cl\(^{-}\)) double-barreled microelectrode, mounted on a Leitz micromanipulator, perpendicular to the surface of an exposed gland under oblique (45°) observation through a stereomicroscope at ×50 magnification (Wild, Heerbrugg, Switzerland). The correct positioning of the microelectrode tip in the gland lumen was established by the following criteria: (i) the near identity of the glandular luminal potential (V\(_{gl}\)) with the transepithelial potential (V\(_t\)); (ii) the near identity of the electrical resistance recorded between the microelectrode reference channel and serosal bath macroelectrode with the transepithelial resistance. All measurements were performed with a model FD 223 dual channel electrometer (World Precision Instruments, New Haven, CT) and recorded on a strip chart recorder (Kipp & Zonen, Delft, The Netherlands).

TEA\(^{+}\)-sensitive Microelectrodes—Double-barreled TEA\(^{+}\)-sensitive microelectrodes were constructed as described previously for pH-sensitive microelectrodes (24, 32). Briefly, two pieces of filament-containing aluminum silicate glass tubing of different diameter (1.5-mm outer diameter and 1.0-mm inner diameter and 1.1-mm outer diameter and 0.75-mm inner diameter) (Hilgenberg, Malsfeld, Germany) were fixed in parallel and melted together by first twisting and then untwisting them at melting point before they were pulled in a PE2 vertical puller (Narishige, Tokyo, Japan). Then the back of the thin channel was closed, and the thick channel was silanized for 180 s in dimethyldichlorosilane vapor (Fluka Chemie AG) and baked in the oven. The shank of the thick channel was back-filled with a small amount of the ligand mixture (World Precision Instruments), and its shaft was later filled with a solution containing 160 mmol/liter KCl and 1 mmol/liter TEA\(^{+}\).

For all microelectrodes, the reference channel contained 500 mmol/liter KCl, and an Ag/AgCl wire was inserted. The average slope and resistance of the electrodes were 57.5 ± 0.4 mV per decade change in [TEA\(^{+}\)] (n = 26) and 180 ± 4 gigaohms (selective channel) and 125 ± 3 megaohms (reference channel). All microelectrodes were calibrated in the upper half-chamber before each puncture and, if the micropuncture was successful, also after the puncture by flushing the chamber with a HEPES-
buffered Ringer’s solution containing different TEA⁺ concentrations (0.8, 1.0, and 1.5 mmol/liter). Although ion-selective microelectrodes respond to ionic activities and not to concentrations, calibrating solutions and results are reported as concentrations with the assumption that the activity coefficient does not change significantly during measurements (see Ref. 33). The slope of microelectrodes was not affected by pH (ranging between 4.0 and 8.0) or by spermine and Ca²⁺.

**pH-sensitive Microelectrode**—The tip of the selective channel was back-filled with H⁺ ligand (Hydrogen Ionophore II, Mixture A/Mixture B; Fluka, Buchs, Switzerland) and the shaft with a HEPES-buffered Ringer’s solution with pH 7.0. Average slope and resistance were 55.6 ± 0.4 mV/pH unit (n = 23), 292 ± 29 gigaohms (selective channel), and 187 ± 19 megaohms (reference channel). All microelectrodes were calibrated using HEPES-buffered Ringer’s solutions with pH values between 6.8 and 7.8.

**K⁺-sensitive Microelectrode**—The K⁺ ligand (K⁺-selective liquid ion exchanger, Mixture A; Fluka) was inserted in the tip of the selective channel, and the shaft was filled with a HEPES-buffered Ringer’s solution containing 4.0 mmol/liter K⁺. K⁺ microelectrodes were calibrated in HEPES-buffered Ringer’s solutions containing 0.8, 4.0, and 10.0 mmol/liter K⁺. The sensitivity was 52.0 ± 0.6 mV (n = 13) for a 10-fold change in K⁺ concentration.

**Cl⁻-sensitive Microelectrodes**—The Cl⁻ ligand (Cl⁻-selective ion exchanger, Mixture A; Fluka) was inserted in the tip of the selective channel while the shaft was filled with HEPES-buffered Ringer’s solution containing 91.4 mmol/liter Cl⁻. Cl⁻ microelectrodes were calibrated in Ringer’s solutions containing 50, 91.4, or 130 mmol/liter Cl⁻. Average slope was 56.7 ± 0.9 mV (n = 4).

**Acid Secretion Measurements**—Tissues were mounted vertically between two halves of a Lucite chamber having an exposed area of 0.64 cm². Each half-chamber consisted of a circular fluid canal of 2.5-ml total volume filled with modified Ringer’s solution that was constantly recirculated by means of a bubble lift. The control Ringer’s solution on the serosal side was gassed continually with 5% CO₂ in O₂ (pH 7.36).

The luminal solution was unbuffered and had the following composition: 102.4 mmol/liter Na⁺, 4.0 mmol/liter K⁺, 91.4 mmol/liter Cl⁻, 15 mmol/liter isethionate, 7 mmol/liter mannitol, and 11 mmol/liter D-glucose. To prevent accumulation of CO₂, this solution was gassed with 100% O₂ that was passed through a bottle containing Ba(OH)₂ solution (50 mmol/liter). Acid secretion was measured with the pH-stat method (Radiometer, Copenhagen, Denmark). The titration procedure was activated every 10 min using 5 mmol/liter NaOH as the titrant, respectively. The transepithelial potential (Vₜ) was monitored with a voltmeter using two calomel half-cells connected to each bath solution. HCl secretion was stimulated, adding 500 μmol/liter histamine to the serosal solution (no cimetidine).

**Data Analysis and Statistics**—Mean values are expressed ± S.E. of n individual micropuncture recordings. The significance of the observations was evaluated by Student’s t test for paired or unpaired data as appropriate, and p < 0.05 denoted a statistical difference.

**RESULTS**

**TEA⁺ as a Tool to Measure Water Transport in Single Gastric Glands**

The technique employed to measure water transport relies on an electrophysiological approach developed in our laboratory. The method, based on the use of ion-selective microelectrodes inserted in the lumen of gastric glands, allows continuous monitoring, for extended periods of time (up to 3 h), of ion concentrations in the lumen of single glands of the intact amphibian stomach (22–24, 32, 34, 35). Water movement was measured by continuously superfusing the mucosal side of the tissue with Ringer’s solution containing an impermeant ion (TEA⁺) and measuring fluctuations in its concentration. The use of impermeant ions (TEA⁺ or TMA⁺) to evaluate water transport with microelectrodes has already been successfully applied in other laboratories in different tissues (33, 36–39). The ligand used to construct our microelectrodes is sensitive to K⁺ and TEA⁺ but in the presence of high TEA⁺ concentrations (1 mmol/liter) is selective only to TEA⁺. Fig. 1A shows a typical trace of a microelectrode calibrated with different K⁺ concentrations in the absence (upper trace) and in the presence of 1.0 mmol/liter TEA⁺ (lower trace). In the presence of TEA⁺ the average slope for K⁺ dropped to near zero values, and the ion exchanger became sensitive only to [TEA⁺] changes. Therefore, changes in TEA⁺ concentration in the gland lumen ([TEA⁺]ₘ) can be correlated with dilution or concentration of the intraglandular solution (i.e., with water movement across the gastric epithelium).

After calibration in the perfusion chamber, the microelectrodes were advanced toward the basolateral membrane of OCs and positioned in the restricted space of the gland lumen. If, as shown in Fig. 1B (lower trace), microelectrodes were properly...
positioned in the gland lumen (for details, see "Materials and Methods") the time course of the response to changes in luminal [TEA\(^+\)] was much slower compared with when the tip was in the luminal bath (time to peak was 141.0 ± 11.8 s versus 20.3 ± 0.9 s; n = 4, respectively). Average slopes of bath/gastric gland lumen were not significantly different. The slope of microelectrodes was not affected by pH (ranging between 4.0 and 8.0) or by spermine and Ca\(^{2+}\). Fig. 1C shows that when the microelectrode tip was inserted in the lumen of a gastric gland, it was possible to record an immediate and reversible decrease in [TEA\(^+\)] in response to increased osmolarity of the mucosal perfusate (300 mmol/liter mannitol), indicating entry of water in the gland lumen.

**Water Transport at Rest and after Stimulation: Correlation with Acid Secretion**

The H\(^+/K^+/\)ATPase located on the apical membrane of the acid-secreting cells exchanges H\(^+\) for K\(^+\), and K\(^+\) is recycled from the lumen into the cytoplasm via apical K\(^+\) channels (40). These channels are necessary for the H\(^-/K^+\)-ATPase to function, and in fact their inhibition results in extremely effective reduction of acid secretion (41–45).

TEA\(^+\) is a known blocker of different types of K\(^+\) channels with varying degrees of efficacy (46). Therefore, we were concerned that the presence of TEA\(^+\) in the luminal bath could inhibit K\(^+\) movement across the apical membrane and block acid secretion. However, as shown in Fig. 2, the rate of histamine-stimulated acid secretion did not change when luminal TEA\(^+\) was added before (Fig. 2B) or after the addition of histamine (Fig. 2A). Another indication that TEA\(^+\) did not influence ion conductances (including K\(^+\) channels) was that mucosal membrane potential (\(V_{mm}\)), measured with intracellular microelectrodes was not altered by TEA\(^+\) (\(V_{mm} = -30.53 \pm 8.25\) mV in control and \(-30.00 \pm 8.41\) mV in the presence of TEA\(^+\), during histamine stimulation; \(n = 3\)). Taken together, these data indirectly exclude the inhibition of K\(^+\) movement through the apical membrane of OCs by TEA\(^+\) and indicate that the mucosa is still functional following exposure to luminal TEA\(^+\).

It is well established that stimulation of the frog stomach with histamine results in water flux from the serosal to mucosal surface (6, 10, 47) and is logically assumed to be the consequence of an osmotic mechanism originating from ionic secretion (2, 5, 48, 49). We applied TEA\(^+\)-sensitive microelectrodes to monitor changes in [TEA\(^+\)] in response to stimulation with histamine. As shown in Fig. 3, transepithelial potential (top trace) and glandular potential (middle trace) responded to stimulation with a typical, slow hyperpolarization due to ion transport mechanisms activated in stimulated cells (34, 50–52), whereas [TEA\(^+\)] decreased significantly (bottom trace), indicating net water flow toward the gland lumen. The response usually started after a lag period of 6–10 min (6.23 ± 0.43 min (S.E.), \(n = 4\)) and was fully reversible after the removal of histamine and perfusion with cimetidine. Fig. 4A summarizes the time course of changes in [TEA\(^+\)] in experiments with different concentrations of histamine, showing that the dilution of [TEA\(^+\)] increased significantly in parallel with concentrations of histamine.

These findings indicate direct correlation between [TEA\(^+\)] changes and acid secretion. The experiments summarized in Fig. 4B, where gland lumen pH (\(pH_{gl}\)) was measured with double-barreled pH-sensitive microelectrodes, show that pH\(_{gl}\) started to decrease about 10 min (9.63 ± 0.77 min; \(n = 4\)) after the addition of histamine. Although final pH\(_{gl}\) values varied...
It is well established that both K\(^+\) and Cl\(^-\) apical pathways are essential for the regulation of gastric secretion (53–56).

The Cl\(^-\)-conductive pathway identified, cloned, and characterized by Cuppoletti and co-workers (57, 58) is a protein kinase A-activated Cl\(^-\) channel that is voltage-sensitive and triggered by low pH. This Cl\(^-\) conductance plays a key role in mammalian parietal cells in the regulation of HCl secretion (53). We therefore examined whether Cl\(^-\) could be involved in the generation of the initial driving force for water by measuring changes in [Cl\(^-\)]\(_{lg}\) in the gland lumens with Cl\(^-\)-selective microelectrodes. As expected, resting [Cl\(^-\)]\(_{lg}\) was higher than [Cl\(^-\)]\(_{gl}\) in the Ringer’s solutions that superfused the mucosal side of the epithelium (99 ± 3.2 mmol/liter, n = 4 versus 91.4 mmol/liter; Fig. 5). This difference is probably explained by the “nonacidic” Cl\(^-\) secretion, usually observed in the resting state (59–61).

[Cl\(^-\)]\(_{lg}\) increased significantly in response to histamine (Fig. 5), which confirms previous observations that a Cl\(^-\) conductive pathway is activated by histamine (53, 57, 58). This increase was, however, significantly delayed with respect to the dilution of TEA\(^+\) (i.e. the water movement into the lumen) (11.2 ± 0.5 n = 4 min versus 6.23, p < 0.001), whereas its time course was not significantly different from that of gland lumen acidification. Therefore, although there is no doubt that Cl\(^-\) transport is essential for maintaining acid and fluid secretion, this anion does not appear to participate in the initial events that drive water into the gland lumen.

Since, as already discussed above, also K\(^+\) ions play a primary role in the activation of the catalytic cycle of the gastric pump, we evaluated the possibility that these ions might be involved in the formation of the osmotic gradient. Recently, Grahammer et al. (43) showed that the luminal K\(^+\)-channel KCQ1 is essential for acid secretion. These authors demonstrated that chromanol 293B (trans-6-cyano-4-[[N-ethylsulfonyl-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane), a putative specific inhibitor of apical KCQ1 conductance (62), strongly inhibits acid secretion in rat, dog, and rabbit stomach. As shown in Fig. 6, luminal chromanol 293B was able to inhibit histamine-stimulated H\(^+\) secretion in frog gastric mucosa, suggesting that also in frog stomach KCQ1, K\(^+\) channels may play a role in the recycling of K\(^+\) ions to be processed by H\(^+\)/K\(^+\) ATPase.

Next, histamine-induced [K\(^+\)]\(_{lg}\) changes were measured using double-barreled K\(^+\)-sensitive microelectrodes inserted in the gastric gland lumen. Fig. 6B shows that stimulation with histamine elicited a significant and reversible increase in [K\(^+\)]\(_{lg}\). This response was much faster (started 3.93 ± 0.22 min after the addition of histamine, n = 4) than the dilution of TEA\(^+\) (≈6 min; Figs. 3 and 4; p < 0.01) and was significantly reduced by pretreatment with chromanol 293B. These data support the idea that K\(^+\) ions are responsible for the osmotic gradient that

considerably (ranging between 6.5 and 3.0) depending on the concentration of histamine, time courses were always very consistent.

Furthermore, when the H\(^+\)-K\(^+\)-ATPase was inhibited by SCH 28080 (42), stimulation with histamine did not result in a significant dilution of gland luminal contents, whereas the typical histamine-induced dilution was observed in the same gland following removal of the inhibitor (Fig. 4C). Similar results were obtained also with omeprazole (not shown, n = 2). These findings indicate that apical ion transport mechanisms activated by histamine are probably responsible for the generation of the driving force for water movement.

**Ions Involved in the Generation of the Osmotic Gradient at the Onset of Acid Secretion**

We tried to identify the mechanisms involved in the formation of the osmotic gradient that drives water in the gastric glands in the initial moments after stimulation with histamine. This response was much faster (started 3.93 ± 0.22 min after the addition of histamine, n = 4) than the dilution of TEA\(^+\) (≈6 min; Figs. 3 and 4; p < 0.01) and was significantly reduced by pretreatment with chromanol 293B. These data support the idea that K\(^+\) ions are responsible for the osmotic gradient that...
drives water in the gastric gland lumen at the onset of gastric secretion. Control experiments showed that pretreatment with chromanol 293B effectively inhibited histamine-induced dilution of [TEA⁺]⁹_network in the gland lumen (Fig. 6C).

Finally, we also tested if, by analogy with the mammalian stomach (63), the Na⁺-K⁺-2Cl⁻ cotransporter-1 might be involved in the regulation of fluid secretion. Experiments performed with the Na⁺-K⁺-2Cl⁻ cotransporter-1 inhibitor bumetanide (100 μmol/liter) indicate that Na⁺-K⁺-2Cl⁻ cotransporter-1 does not appear to be involved in the regulation of fluid, K⁺, or acid secretion in our model (data not shown).

**Modulation of Water Transport by CaR**

At Rest and after Stimulation—The extracellular CaR, which is expressed along the entire gastrointestinal tract (31, 64), belongs to group C of the G protein-coupled receptor superfamily and has been shown to modulate many of the known effects of extracellular Ca²⁺ in a variety of tissues (15). We recently found that CaR is located in the apical membrane of OCs (23) and is involved in the modulation of alkali and pepsinogen secretion (22). Since it has been shown that this receptor can influence fluid transport in different tissues (28, 30, 31), we examined its role in the modulation of water movement in the gastric epithelium. Stimulation of CaR with either spermine (a CaR agonist present endogenously in the gastrointestinal tract (65)) or elevation of luminal [Ca²⁺] from 1.4 to 2 mmol/liter (a “physiological” concentration that is able to activate the receptor (22, 66)) resulted in a significant and reversible increase in [TEA⁺]⁹_network (Fig. 7A). Fig. 7B summarizes the data obtained under various conditions. Besides spermine and high luminal [Ca²⁺], we also stimulated CaR with carbachol, which is able to indirectly stimulate CaR due to its ability to induce asymmetrical changes in external [Ca²⁺] (i.e. elevating luminal [Ca²⁺] from 1.4 to 2.0 mmol/liter while decreasing serosal [Ca²⁺] from 1.4 to 1.0 mmol/liter) (23). Fig. 7B shows that carbachol elicited an increase in [TEA⁺]⁹_network and that mimicking carbachol-induced asymmetrical fluctuations in extracellular [Ca²⁺] also resulted in an increase in [TEA⁺]⁹_network similar to that observed in response to other agonists. The latter response was entirely due to the action of high luminal [Ca²⁺], since lowering basolateral [Ca²⁺] alone did not cause any significant change in [TEA⁺]⁹_network (Fig. 7B). Thus, in nonstimulated frog gastric mucosa, activation of CaR resulted in a significant reduction of water in the gland lumen.

Since water transport and gastric acid secretion are closely correlated in frog stomach (Figs. 3 and 4), we investigated the action of CaR on histamine-induced water transport (Fig. 8A). Stimulation with low histamine concentrations resulted in a dilution of glandular solution that was not affected by the addition of spermine in the luminal perfusate, whereas spermine still elicited an increase in [TEA⁺]⁹_network before and after histamine in the same gland. However, pretreatment with spermine elicited the typical increase in [TEA⁺]⁹_network and significantly reduced histamine-induced water secretion (Fig. 8B).

Since we have previously demonstrated that in frog gastric mucosa CaR is coupled with the classical pertussis toxin-sensitive Gαi (22), it is likely that the effect of CaR on water movement is the result of decreased intracellular cAMP levels. Since gastric acid secretion is a cAMP-dependent process (18, 67, 68), one could predict that activation of CaR might result in inhibition of gastric acid secretion. To test this possibility, we per-
formed experiments with double-barreled pH-sensitive microelectrodes and measured directly pH variations in the gland lumen. Stimulation of CaR with spermine did not revert the histamine-induced decrease in pH gl both at high (Fig. 9A; two types of responses are shown overlapped) and low concentration of histamine (Fig. 9B). On the other hand, preactivation of CaR with spermine first alkalinized the gland lumen, a typical response observed previously (22), and subsequently caused a significant reduction in the rate of histamine-induced gland acidification (Fig. 9C). When the same protocol was applied using higher concentrations (100 μmol/liter) of histamine, a typical response observed previously (22), and subsequently caused a significant reduction in the rate of histamine-induced gland acidification (Fig. 9C). When the same protocol was applied using higher concentrations (100 μmol/liter) of histamine, activation of CaR did not result in a detectable modulation of the acid secretion rate (not shown). This indicates that CaR may have a significant regulatory action on acid secretion only when the epithelium is not fully stimulated to secrete acid.

**Involvement of cAMP**—Our data suggest that the significant K+ accumulation in the gastric gland lumen observed at the onset of acid secretion may represent the osmotic driving force for water from nutrient to luminal side (Fig. 4). Since it is known that the histamine-activated apical K+ channels are cAMP-dependent (45, 69), we expect that stimulation of CaR should affect K+ secretion and consequently also water movement. Fig. 10 illustrates that increasing cAMP, either histamine or isobutylmethylxanthine led to significant elevation of [K+] gl, whereas treatment with SQ22,536, which depresses cAMP production by inhibiting adenyl cyclase, resulted in a reduction of [K+] gl. Interestingly, stimulation of CaR by either spermine or elevation of luminal [Ca2+] or carbachol also resulted in significant reduction of [K+] gl. These data show that K+ secretion is strongly dependent on cAMP production and that CaR activation results in marked inhibition of apical K+ movement.

**DISCUSSION**

Experimental evidence from a number of early studies converge on the hypothesis that the osmotic gradient driving water transport in the lumen of the stomach originates from the ionic secretory machinery activated by histamine (2, 5, 48, 49). Using an electrophysiological approach that allows real time monitoring of fluid and ion movements, we measured for the first time net
Water transport in the gastric gland lumen

Water flux within the lumen of gastric glands of the intact amphibian stomach, providing direct evidence for the strong link between histamine-induced net water flow and acid secretion.

We showed that histamine-induced water flux precedes, to a great extent (~3 min), the onset of acid secretion. This time lag cannot be explained by different response times of the two different types of microelectrodes/resins employed (i.e., TEA⁺ and pH microelectrodes) to measure water flow and acid secretion. The response time of the two microelectrodes was in fact virtually the same: 20.34 ± 0.35 s (n = 35) for TEA⁺ and 21.07 ± 0.38 s (n = 20) for pH microelectrodes. These findings indicate that the osmotic gradient driving water in the gland lumen has to originate from ion movements taking place just before the initiation of acid secretion, which rules out H⁺. Other possible candidates are Cl⁻ and K⁺. Stimulation of the oxyntopeptic cells to secrete acid results in an extensive rearrangement of the apical membrane consisting in the fusion of the cytoplasmatic tubulovesicular compartment with the apical membrane and consequent translocation of the H⁺/K⁺ ATPase, in parallel with K⁺- and Cl⁻-conductive pathways (53, 55, 57, 70–72). Both ions are essential for maintenance of acid secretion (13, 54, 70, 72).

Chloride ions are involved in the formation of the osmotic gradient in a large number of tissues, from salivary glands (73) to choroid plexus epithelium (74) to cholangiocytes (75). Recently, parchorin, a novel protein with significant homology to the family of chloride intracellular channels, has been cloned and characterized in the apical membrane of rabbit parietal cells (76). Parchorin is known to be involved in the formation of tubulovesicular compartment with the apical membrane and consequent translocation of the H⁺/K⁺ ATPase, in parallel with K⁺- and Cl⁻-conductive pathways (53, 55, 57, 70–72). Both ions are essential for maintenance of acid secretion (13, 54, 70, 72).

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Logsdon and Machen (78) found that Cl⁻ is not required either for the initiation of H⁺ secretion or for the maintenance of stimulated morphology of frog stomach. Villegas and Sananes (79) have shown that replacement of chloride by sulfate in the solutions bathing frog stomach does not affect water transport, whereas it inhibits histamine-induced acid secretion.

The fact that the histamine-induced increase in [Cl⁻]gl is significantly delayed with respect to water secretion favors the idea that Cl⁻ is not the initiator of the osmotic driving force. It is likely, however, that Cl⁻ is indispensable for the potentiation of the gradient during later stages of the secretory response, when basolateral and apical influx and efflux mechanisms are fully activated.

In contrast, a number of observations in this study show that movement of K⁺ from oxyntopeptic cells to the gland lumen is necessary for initiation of water secretion in frog stomach. We found that the first event recorded following stimulation with histamine is the increase in [K⁺]gl (~4 min after stimulation; Fig. 5). Our data are consistent with the observation by Logsdon and Machen (78) that removal of K⁺ from solutions results in an occluded morphology of the gland lumen in stimulated gastric glands, indicating a drastic reduction in intraglandular hydrostatic pressure. The fact that chromanol 293B, a blocker of KCQN1 channels, strongly inhibits the histamine-induced increase in [K⁺]gl (Fig. 6) and the dilution of the gland lumen further supports the idea that K⁺ is the promoter of the osmotic gradient driving water in the gland lumen, at least at the onset of acid secretion. To date, three different K⁺ channels (KCNO1, Kir2.1, and Kir4.1), identified in the apical membrane of parietal cells (43–45, 80, 81) are potential candidates for apical recycling of K⁺.

These data lead to the identification of a specific sequence of ion transport events activated in response to histamine. First, elevation in intracellular cAMP results in opening of apical K⁺ channels (45) and consequent exit of K⁺ into the gland lumen. As to the counterion involved in this very first phase, we suggest that H⁺, which rules out H⁺ into the gland lumen. As to the counterion involved in this very first phase, we suggest that a possible candidate might be HCO₃⁻. This idea derives from our observations both in this (Fig. 9A, gray plot) and in previous studies (24) that in the 6–10 min before pH₂ starts to decline, a transient and small but significant alkalinization of the gland lumen was measured in 50% of the gland lumina. Second, as a result of K⁺ and HCO₃⁻ efflux, an osmotic gradient sufficient to drive water into the gland lumen is created. Third, the presence of K⁺ in the gland lumen activates the H⁺/K⁺ ATPase, and H⁺ moves into the lumen accompanied by Cl⁻ ions. The resulting decrease in pH₂ activates pH-sensitive apical Cl⁻ channels (57), which further increases [Cl⁻]gl and results in potentiation of the osmotic gradient. We can speculate that at this stage, water transport becomes fully activated, and the hydrostatic pressure rises to push the content of the gland lumen into the greater lumen of the stomach. Water secretion may then account for the observed formation of channels of fluid at the opening of gastric pits (82), ultimately facilitating the flow of acid and pepsin through the mucus layer coating the luminal surface of the stomach.

We have recently demonstrated that activation of the extracellular CaR in the “resting” stomach (in the presence of H₂ receptor inhibitors) elicits alkaline and pepsinogen secretion in frog stomach from OCs (22). We show here that in the resting state, CaR activation leads to reduction in gland lumen water content. A reduction of water flux would be functionally appropriate for the weak alkaline secretion elicited by CaR to be effective. An alkalinization of the gland lumen might be important to prevent premature activation of pepsinogen to pepsin (known to be favored by acidic pH (83)), which may damage the oxyntopeptic cells. Since CaR is functionally coupled to a pertussis toxin-sensitive Gα type in the OCs (22), activation of the receptor should decrease the availability of cellular cAMP. Our data show that activation of CaR in the resting state gives rise to a transient reduction of [K⁺]gl similar to that elicited by SQ22,536, a well known inhibitor of cAMP production, indicating that the K⁺ conductance involved is cAMP-dependent (see also Fig. 10). These findings imply that there is a basal rate of cAMP-dependent K⁺ extrusion toward the gland lumen, which in turn is responsible for basal water movement under resting conditions.

The scenario is different when mucosae are stimulated with histamine. After the acid-secretory mechanism is fully operation, activation of the pertussis toxin-sensitive Gα coupled pathway by CaR is probably insufficient to affect the physiolog-
ical progression of water and acid secretion (Figs. 8A and 9, A and B). On the other hand, when intracellular cAMP levels are not too high (i.e. at low histamine concentrations or during basal turnover of adenyl cyclase), preincubation with CaR agonists and the consequent engagement of the G<sub>A</sub> pathway may be sufficient to counteract the stimulatory action of cAMP on water and acid secretion (Figs. 8B and 9C). Our data reveal a subtle equilibrium between the stimulatory activity of G<sub>A</sub> and antagonism by G<sub>B</sub> both serve to influence intracellular cAMP levels and thereby fine tune the secretion of water and acid under resting conditions.

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Water Transport in the Gastric Gland Lumen

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