ΔF508 Mutation Results in Impaired Gastric Acid Secretion*

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The cystic fibrosis transmembrane conductance regulator (CFTR) is recognized as a multifunctional protein that is involved in Cl− secretion, as well as acting as a regulatory protein. In order for acid secretion to take place a complex interaction of transport proteins and channels must occur at the apical pole of the parietal cell. Included in this process is at least one K+ and Cl− channel, allowing for both recycling of K+ for the H,K-ATPase, and Cl− secretion, necessary for the generation of concentrated HCl in the gastric gland lumen. We have previously shown that an ATP-sensitive potassium channel (KATP) is expressed in parietal cells. In the present study we measured secretagogue-induced acid secretion from wild-type and ΔF508-deficient mice in isolated gastric glands and whole stomach preparations. Secretagogue-induced acid secretion in wild-type mouse gastric glands could be significantly reduced with either glibenclamide or the specific inhibitor CFTR-inh172. In ΔF508-deficient mice, however, histamine-induced acid secretion was significantly less than in wild-type mice. Furthermore, immunofluorescent localization of sulfonylurea 1 and 2 failed to show expression of a sulfonylurea receptor in the parietal cell. Included in this process is at least one K+ and Cl− channel, allowing for both recycling of K+ for the H,K-ATPase, and Cl− secretion, necessary for the generation of concentrated HCl in the gastric gland lumen. We have previously shown that an ATP-sensitive potassium channel (KATP) is expressed in parietal cells. In the present study we measured secretagogue-induced acid secretion from wild-type and ΔF508-deficient mice in isolated gastric glands and whole stomach preparations. Secretagogue-induced acid secretion in wild-type mouse gastric glands could be significantly reduced with either glibenclamide or the specific inhibitor CFTR-inh172. In ΔF508-deficient mice, however, histamine-induced acid secretion was significantly less than in wild-type mice. Furthermore, immunofluorescent localization of sulfonylurea 1 and 2 failed to show expression of a sulfonylurea receptor in the parietal cell, thus further implicating CFTR as the ATP-binding cassette transporter associated with the KATP channels. These results demonstrate a regulatory role for the CFTR protein in normal gastric acid secretion.

A defect in or absence of the cystic fibrosis transmembrane conductance regulator (CFTR) protein is responsible for the autosomal recessive, multiorgan disease cystic fibrosis. Defective or deficient copies of the protein on the cell surface result in abnormalities in viscosity and electrolyte content of various exocrine secretions. To date more than 1000 different mutations in the CFTR gene have been identified and associated with cystic fibrosis, the most common being the ΔF508 mutation. 70% of defective alleles are affected by this mutation causing a deletion of a phenylalanine residue at position 508. The defective protein is synthesized but recognized as abnormal and targeted for degradation. When defective ΔF508 protein is placed in a cell-free lipid bilayer, it retains a substantial part of its function (1–5).

The CFTR protein is a cAMP-regulated chloride channel that belongs to the superfAMILY of ATP-binding cassette transporters. It contains two membrane-spanning domains, each consisting of six helices, as well as two nuclear binding domains responsible for ATP hydrolysis, and a regulatory domain with phosphorylation sites for various protein kinases. Both amino (N) and carboxyl (C) tails of the protein are oriented cytoplasmically and mediate CFTR interaction with other ion channels, receptors, and the cytoskeleton (6–10). Initially thought to be merely a chloride conductance channel, the cystic fibrosis transmembrane conductance regulator, as the name implies, has been shown to regulate several other cellular processes. CFTR interacts and plays a regulatory role in the activity of the outwardly rectifying chloride channel (11–14), epithelial sodium channel (15, 16), as well as the renal outer medullary K+ channel type 2 (ROMK2 or Kir1.1b) (17–21).

The acid secretory cell of the gastric gland is the parietal cell. Stimulation by either hormonal (histamine and gastrin) or neuronal (acetylcholine) secretagogues via their corresponding receptors on the basolateral plasma membrane results in translocation and insertion of the H,K-ATPase from its inactive state in the cytoplasmic tubulovesicle elements to the apical membrane of the secretory canaliculari. Proton secretion then occurs by exchanging an H+ ion for a K+ ion at the expense of 1 ATP molecule via the H,K-ATPase. This proton secretion is coupled with the extrusion of a Cl− ion via an apical Cl− channel. To maintain the exchange of H+ for K+ it is necessary to have an apical K+ channel that secretes K+ into the lumen of the gland to provide a continuing K+ gradient for the H,K-ATPase. This cyclic K+ movement and the concurrent H+ and Cl− secretion leads to the generation of 0.1 N HCl in the lumen of the gland, which is then secreted into the interior of the stomach (22–28). The molecular identity of both the K+ channel and the Cl− channel on the apical surface of the parietal cell is still in question; however, recently a variety of K+ channel proteins have been identified in the parietal cell so that the secretion of K+ may either be associated with multiple channel proteins, or complexes of these different K+ channels that have been identified (29–33). Based on the putative role of CFTR in modulat-
CFTR and Gastric Acid Secretion

Isolation of Gastric Glands and Digital Imaging for pH

Wild-type and transgenic mice were sacrificed by overdose of isoflurane anesthesia. Following laparotomy a total gastrectomy was performed, and the stomach was immediately rinsed with ice-cold HEPES-buffered Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 32.2 mM HEPES, and 5 mM glucose, pH 7.4) (Table 1) to eliminate any residual food matter, and kept in HEPES solution on ice until use. 0.5-cm square sections were cut from the corpus and transferred to the stage of a dissecting microscope where individual gastric glands were hand-dissected using a previously described technique (66). After isolation, the glands were transferred to coverslips pre-coated with adhesive Cell-Tak (BD Cell-Tak Cell and Tissue Adhesion, BD Biosciences) and mounted in a thermostatically controlled chamber maintained at 37 °C on an inverted microscope (Olympus IX50) attached to a digital imaging system (Universal Imaging, Downingtown, PA) 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 acetomethyl ester (BCECF-AM, Molecular Probes, Eugene, OR) for 10 min in HEPES at 37 °C. After the glands were loaded, the chamber was flushed with HEPES-buffered Ringer solution to remove non-de-esterified dye. BCECF was excited at 490 ± 10 and 440 ± 10 nm, and the emitted fluorescence light was measured at 535 ± 10 nm using an intensified charge-coupled device camera. Data were collected every 12 s, and the ratio at 490/440 nm was initially recorded as arbitrary pH units, which were then converted to absolute intracellular pH (pHᵢ) using a high K⁺/nigericin calibration technique (67). Data are expressed as changes in pHᵢ (ΔpHᵢ) per minute. Acid extrusion was monitored in the absence of NaHCO₃ as intracellular alkalinization after the removal of Na⁺ from the bath and using the NH₄Cl prepulse technique, which caused reproducible and sustained intracellular acidification. Alkalinization rate (ΔpHᵢ/min) for the calculation of Na⁺-independent pHi recovery (H,K-ATPase activity) was measured in the pH range of 6.50–7.00. Na⁺-free solution eliminated the possibility of contribution to alkalinization rates by the Na/H-exchanger.

Secretagogues—100 μM histamine, 100 μM carbachol, or 100 μM pentagastrin were used to induce acid secretion by wild-type mouse gastric glands. 100 μM histamine was used to assess acid secretory capability of isolated gastric glands from ΔF508-defective mice.

Pharmacological Inhibitors of CFTR—CFTR inhibition was carried out using 100 μM glibenclamide. All drugs were present...
in combination with BCECF during incubation of the glands for 10 min before the experiment, as well as in all perfusate solutions excluding the K⁺/nigericin calibration solution. In addition to glibenclamide, the recently developed more CFTR-specific inhibitor CFTR-inh172 was also used at a dose of 10 μM in SLO-permeabilized mouse gastric glands as described by Ammar et al. (63). During the 10-min incubation period, 1 μg/ml SLO was used to permeabilize the cell in the presence of histamine and CFTR-inh172. CFTR-inh172 and histamine were then included in all of the above-mentioned perfusate solutions. Experiments with similar doses of histamine, SLO, and vehicle (Me₂SO) in the absence of CFTR-inh172 were also performed as controls.

**Isolated Perfused Whole Stomach pH Measurements**—A total gastrectomy was performed on wild-type and ΔF508 (−/−) mice, following anesthesia with isoflurane and ligation of both the gastroesophageal and gastroduodenal junctions. Once excised, 0.2 cc of non-buffered saline was injected into the isolated whole stomach. The entire stomach was placed in a bath of oxygenated HEPES-buffered Ringer solution at 37 °C, pH 7.4, for 60 min in the absence of secretagogue (control) or in the presence of 200 μM histamine. Following incubation, the injected non-perfused saline solution was aspirated from the stomach, and its pH was measured.

**Immunohistochemistry**—Male C57BL/6 mice (20–25 g) were anesthetized with pentobarbital intraperitoneal and perfused through the left ventricle with PBS buffer, pH 7.4, followed by paraformaldehyde-sucrose periodate fixative (68). Following perfusion for 5 min with fixative, the stomachs were removed, cleaned from food residues, and fixed overnight at 4 °C by immersion in paraformaldehyde-sucrose periodate. Stomachs were then washed three times with PBS, and the sections were cut at a thickness of 5 μm after cryoprotection with 2.3 M sucrose and 50% polyvinylpyrrolidone in PBS for at least 12 h. Immunostaining was carried out as described previously (53). Briefly, sections were incubated with 1% SDS for 5 min, washed three times with PBS, and incubated with PBS containing 1% bovine serum albumin for 15 min prior to the primary antibody. The primary antibodies mouse monoclonal anti-human β gastric H⁺/K⁺-ATPase (Affinity Bioreagents, Golden, CO) and goat anti-SUR-1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), were diluted at 1:1000 and 1:100, respectively, in PBS and applied overnight at 4 °C. Sections were then washed twice for 5 min with high NaCl PBS (PBS plus 2.7% NaCl), once with PBS, and incubated with the secondary antibodies (donkey-anti-mouse Alexa 546, donkey-anti-goat Alexa 488, or donkey-anti-mouse Alexa 488, Molecular Probes, Eugene, OR) at a dilution of 1:200 for 1 h at room temperature. Sections were then washed twice with high NaCl PBS and once with PBS before mounting with Vectashield (Vector Laboratories, Burlingame, CA). The specimens were viewed with a Nikon E-800 microscope or a Zeiss LSM-410 confocal microscope.

**Statistics**—All data are summarized as means ± S.E. Significance was determined using an unpaired Student’s t test with p < 0.05 considered to be statistically significant.

**RESULTS**

Secretagogue-induced H,K-ATPase Activity in Isolated Gastric Glands from Wild-type Mice in the Presence and Absence of 100 μM Glibenclamide—The first series of experiments in this study assessed CFTR involvement in parietal cell secretagogue-induced acid secretion by H,K-ATPase using the CFTR inhibitor glibenclamide at a dose of 100 μM. To confirm that stimulation of the Na⁺-independent pHᵢ recovery represented H,K-ATPase activity, glands were preincubated with 100 μM of the specific inhibitor of gastric H,K-ATPase omeprazole for 10 min in the presence of the specific secretagogue used. Omeprazole prevented the stimulatory effect of all secretagogues on the Na⁺-independent pHᵢ recovery rate.

Histamine-induced, Na⁺-independent intracellular recovery (0.088 ± 0.006 pH unit/min, n = 65 parietal cells from 11 glands from 11 mice) was abolished in the presence of 100 μM glibenclamide (0.013 ± 0.001 pH unit/min, n = 79 parietal cells from 8 glands from 6 mice) (Fig. 1). In addition to 100 μM histamine, the other classic secretagogues were used to stimu-
late H,K-ATPase activity and examine glibenclamide (100 μM)-dependent inhibition of acid secretion. 100 μM carbachol-induced intracellular alkalinization rates (0.086 ± 0.005 pH unit/min, n = 29 parietal cells from 5 glands from 4 mice) were significantly inhibited in the presence of 100 μM glibenclamide (0.020 ± 0.003 pH unit/min, n = 44 parietal cells from 4 glands from 4 mice). Intracellular recovery rates induced by 100 μM pentagastrin (0.102 ± 0.006 pH unit/min, n = 54 parietal cells from 7 mice; pentagastrin plus glibenclamide: n = 60 from 5 glands from 4 mice). *p value < 0.0001.

Secretagogue-induced H,K-ATPase Activity in Isolated SLO-permeabilized Gastric Glands from Wild-type Mice in the Presence and Absence of 10 μM CFTR-inh172—To specifically target the CFTR protein, the inhibitor CFTR-inh172 was used in SLO-permeabilized mouse gastric glands. Histamine-induced, Na⁺-independent intracellular recovery (0.135 ± 0.015 pH unit/min, n = 11 parietal cells from 2 glands from 2 mice) was significantly reduced in the presence of 10 μM CFTR-inh172 (0.038 ± 0.005 pH unit/min, n = 38 parietal cells from 4 glands from 2 mice) (Fig. 3).

Immunohistochemistry—Immunohistochemistry confirmed the presence of the β subunit of H,K-ATPase inside the parietal cells of mouse gastric glands from the corpus (Fig. 4A). However, no signal for SUR 1 and 2 could be detected inside the parietal cells of the same section (see Fig. 4A).

Histamine-induced H,K-ATPase Activity in Isolated Gastric Glands from Gene-targeted Mice Homozygous for the ΔF508 Mutation—To confirm a role for CFTR in H,K-ATPase-mediated acid secretion by the parietal cell, 100 μM histamine was used to stimulate Na⁺-independent H⁺ extrusion in isolated gastric glands from cystic fibrosis mice homozygous for the ΔF508 mutation. In comparison to alkalinization rates of histamine-induced isolated gastric glands from wild-type mice (0.088 ± 0.006 pH unit/min, n = 65 parietal cells from 11 glands from 11 mice), glands isolated from transgenic ΔF508 cystic fibrosis mice (Fig. 5) had a substantially diminished response to
CFTR and Gastric Acid Secretion

Recent studies have focused on the role of CFTR in gastric acid secretion. In patients with cystic fibrosis, there is a known correlation between the severity of the disease and the presence of mutations in the CFTR gene. It has been hypothesized that CFTR may play a role in regulating gastric acid secretion, which is consistent with the known physiological function of CFTR in other epithelial cells, such as the airway epithelial cells.

In a study using mice homozygous for the F508 mutation, it was observed that these mice demonstrated a significantly blunted acid secretion response to histamine compared with wild-type mice. This was evident both at the whole organ level in stomach preparations and at the tissue level in gastric glands isolated from these mice. The intragastric pH of histamine-induced luminal acid secretion in ΔF508 CF stomach was significantly higher (4.3 ± 0.3 (n = 3)) (Fig. 7).

DISCUSSION

Since being cloned in 1989 (7), CFTR has been shown to be present extensively along the gastrointestinal tract. Despite evidence for its presence in the parietal cell in the stomach (69–71), it has not been assigned a role in parietal cell physiology as yet, nor have there been convincing reports of gastric pathology in patients with cystic fibrosis. In this study, we present direct evidence for a functional role for the CFTR protein in acid secretion by the parietal cell. Furthermore, mice with cystic fibrosis (ΔF508) were found to have significantly blunted acid secretion in response to histamine in both isolated gastric glands as well as whole stomach preparations.

Recently a study conducted on CFTR (−/−) mouse antral gland base cells using pH-Stat titration and the pylorus ligation technique failed to identify a role for CFTR in gastric acid secretion (44). In contrast in the present study, individual gastric glands were dissected from the corpus of wild-type and Δ508 CFTR-deficient mice that were loaded with a pH-sensitive dye, and measured with a digital real-time imaging device to allow direct measurements of intracellular pH and acid secretion as calculated from proton efflux from the cell. Mice with cystic fibrosis, homozygous for the most common allele causing the disease in humans, the ΔF508 mutation, demonstrated defective gastric acid secretion in this study. These results compliment our measurements of impaired proton efflux (acid secretion) following application of the CFTR-specific inhibitor CFTR-inh172 or glibenclamide. One possible explanation for the divergent results may be due to the complete ablation of the CFTR-inh172 or glibenclamide. One possible explanation for the divergent results may be due to the complete ablation of the CFTR gene potentially causing activation of compensatory mechanisms (i.e. up-regulation of non-CFTR regulated K+ channels) thereby preventing reductions in acid secretion and giving an apparently normal profile. We chose to use mice with the ΔF508 mutation, because it is the most common mutation in humans.

Our data demonstrate a convincing role for CFTR in parietal cell acid secretion, raising the issue as to what may be the cellular and molecular mechanisms behind its involvement. The multifunctional nature of the CFTR protein is well established as are its interactions with several other apical channels and transporters (9). As a chloride conductance channel, however,
CFTR, having a conductance of 8ps (72), is unlikely to be a candidate for the 28pS (73) chloride conductance reported as that responsible for chloride secretion by the parietal cell into the gastric lumen. This current has characteristics similar to ClC-2, and this has been postulated as the Cl− current (74, 75). Considering its pleiotropic nature, CFTR most likely participates in gastric acid secretion via interaction and modulation of other ion channels or transporters. CFTR may interact with H,K-ATPase in either trafficking from the tubulovesicles to the secretory canaliculi, or as a cAMP provider for protein kinase A-dependent phosphorylation and activation. However, an interaction between the two proteins has not been described despite their common expression in several other organs, including the kidney and colon. An association with parietal cell apical KATP channels seems to be a likely role for CFTR-modulated H,K-ATPase-mediated secretagogue-induced acid secretion. The presence of KATP channels, including Kir2.1, Kir4.1, and Kir7.1, has been shown in parietal cells (30–32), and our laboratory has demonstrated a significant role for KATP channels in the recycling of K+ for acid secretion. Our laboratories have also previously shown by coexpression studies in oocytes that CFTR interacts with and modulates ROMK2 and could likely be the ATP-binding cassette transporter associated with ROMK2 in the kidney, taking into account the absence of a sulfonylurea (SUR) receptor in renal epithelia and the relative abundance of CFTR (18). Similarly, we demonstrate the absence of SUR expression in the parietal cell. Therefore the presence of CFTR as the ATP-binding cassette transporter regulating KATP activity may characterize its role in acid secretion. In addition, Malinowska et al. (32) observed a discrepancy in gating kinetics between gastric vesicles and Kir2.1-expressed in oocytes. Gribble et al. (77) demonstrated that Kir2.1 is unaffected by SUR1 when coinjected in Xenopus oocytes. Taken together with our results from the present study, it is likely that CFTR may be the ATP-binding cassette transporter associated with Kir2.1 in the parietal cell, thus explaining the discrepancy observed by Malinowska et al.

Despite significant inhibition of acid secretion by glibenclamide and CFTR-inh172 and substantially reduced secretagogue-induced acid secretion in gastric glands isolated from cystic fibrosis mice as compared with wild-type in our experiments, acid secretion is not entirely eliminated. Residual acid secretion may be due to cAMP-independent Ca2+-dependent intracellular pathways that activate H,K-ATPase in a CFTR-independent fashion. This residual acid secretion would be expected to be sensitive to conventional inhibitors and may explain the data with H2-receptor antagonists and proton pump inhibitors that were shown clinically to decrease acid secretion and raise duodenal pH and improved fat absorption in CF patients (4, 35). Ca2+-dependent compensatory pathways could be up-regulated in these two instances illustrating the diverse complex intracellular mechanisms that converge at H,K-ATPase-mediated acid secretion in the parietal cell. In fact, Roch et al. (78) demonstrated the regulation of KATP channels by intracellular Ca2+ in addition to cAMP in cystic fibrosis epithelial cells.

Our data support a role for a CFTR-modulated cAMP-dependent pathway for acid secretion. In this model apical CFTR acts by regulating apical K+ channels and cAMP-dependent phosphorylation of H,K-ATPase thereby activating H+ exchange for K+ in addition to cAMP-dependent phosphorylation of ClC-2 and CFTR. The former will mediate Cl− conductance, whereas the latter, when present, activates K+ channels to recycle K+ for H,K-ATPase. In contrast to other organs affected in cystic fibrosis, we describe a role for CFTR in acidification rather than alkalinization. In the duodenum (79–81) and airways (76, 82, 83), the absence of CFTR-mediated HCO3− secretion in cystic fibrosis accounts for the reduced pH observed in the intestinal lumen and airway surface liquid, respectively. It would seem counterintuitive for gastric CFTR, on the other hand, to mediate HCO3− secretion into the gastric lumen in conjunction with cAMP-activated secretagogue-induced acid secretion. Raising intracellular pH in the face of activated acid secretion is circumvented through anion exchangers present basolaterally in the parietal cell.

In conclusion, we have provided evidence supporting a role for CFTR in gastric acid secretion. The elucidation of a functional role for CFTR in the stomach may have important implications as an additional target for the suppression of acid secretion in patients with problems of non-controlled hyperacidity.

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REFERENCES

1. Egan, M. E., Glocker-Pagel, J., Ambrose, C., Cahill, P. A., Pappoe, L., Balamuth, N., Cho, E., Canny, S., Wagner, C. A., Geibel, J., and Caplan, M. J. (2002) Nat. Med. 8, 485–492
2. Haardt, M., Benharouga, M., Lechardeur, D., Kartner, N., and Lukacs, G. L. (1999) J. Biol. Chem. 274, 21873–21877
3. Mickle, J. E., and Cutting, G. R. (1998) Annu. Rev. Physiol. 57, 499–528
4. Rowe, S. M., Miller, S., and Sorscher, E. J. (2005) N. Engl. J. Med. 352, 2001–2007
5. Zeitchik, P. L. (2000) Kidney Int. 57, 832–837
6. Li, C., and Naren, A. P. (2005) Pharmacol. Ther. 108, 208–223
7. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzela, Z., Zielenksi, J., Lok, S., Plavsic, N., and Chou, J. L. (1989) Science 245, 1066–1073
8. Riordan, J. R. (2005) Annu. Rev. Physiol. 67, 701–718
9. Schwiebert, E. M., Benos, D. J., Egan, M. E., Stutts, M. J., and Guggino, W. B. (1999) Physiol. Rev. 79, 5145–5166
10. Vanekerberghen, A., Cuppens, H., and Cassiman, J. J. (2002) J. Cyst. Fibros. 1, 13–29
11. Egan, M., Flotte, T., Afione, S., Solow, R., Zeitchik, P. L., Carter, B. J., and Guggino, W. B. (1992) Nature 358, 581–584
12. Gabriel, S. E., Clarke, I. L., Boucher, R. C., and Stutts, M. J. (1993) Nature 363, 263–268
13. Jovov, B., Ismailov, I. I., Berdiev, C. M., Sorscher, E. J., Dedman, J. R., Kaetzel, M. A., and Benos, D. J. (1995) J. Biol. Chem. 270, 29194–29200
14. Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) Cell 81, 1063–1073
15. Boucher, R. C., Stutts, M. J., Knol, M. R., Cantley, L. I., and Gatto, J. T. (1986) J. Clin. Invest. 78, 1245–1252
16. Knowles, M. R., Stutts, M. J., Spock, A., Fischer, N., and Gatto, J. T., and

3 S. M. Sidani, P. Kirchhoff, T. Socrates, L. Stelter, E. Ferreira, C. Caputo, K. E. Roberts, R. L. Bell, M. E. Egan, and J. P. Geibel, unpublished data.
