Biochemical, histological, and inhibitor studies of membrane carbonic anhydrase in frog gastric acid secretion

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Gastric acid secretion is dependent on carbonic anhydrase (CA). To define the role of membrane-bound CA, we used biochemical, histological, and pharmacological approaches in the frog (Rana pipiens). CA activity and inhibition by membrane-permeant and -impermeant agents were studied in stomach homogenates and microsomal fractions. H+ secretion in the histamine-stimulated isolated mucosa was measured before and after mucosal addition of a permeant CA inhibitor (methylenezolamide) and before and after mucosal or serosal addition of two impermeant CA inhibitors of differing molecular mass: a 3,500-kDa polymer linked to aminobenzolamide and p-fluorobenzylaminobenzolamide (molecular mass, 454 kDa). Total CA activity of frog gastric mucosa is 2.280 U/g, of which 10% is due to membrane-bound CA. Membrane-bound CA retains detectable activity below pH 4. Histochemically, there is membrane-associated CA in surface epithelial, oxynticopeptic, and capillary endothelial cells. Methanolamide reduced H+ secretion by 100%, whereas the two impermeant inhibitors equally blocked secretion by 40% when applied to the mucosal side and by 55% when applied to the serosal side. The presence of membrane-bound CA in frog oxynticopeptic cells and its relative resistance to acid inactivation and inhibition by impermeant inhibitors demonstrate that it subserves acid secretion at both the apical and basolateral sides.

CARBONIC ANHYDRASE IV (CA IV) is present in the plasma membranes of endothelial cells and numerous secretory cells (3, 25, 39). Unlike cytosolic CA II, which catalyzes the interconversion of CO2 and HCO3− within the cell, CA IV is anchored externally to the cell membrane by a glycosyolphosphatidylinositol tail (7). Positioned in this fashion, the catalytic activity of CA IV is available to the adjacent extracellular space. Histological and cell fractionation studies (7, 13, 15, 16, 20, 21, 32, 39) have revealed considerable membrane-bound CA activity in both surface epithelial and parietal cells of mammalian stomach, constituting 5–35% of total CA activity. Studies (3, 7) using isozyme-specific CA IV antibodies have revealed that CA IV represents part of this activity.

Inhibition of gastric CA with diffusible permeant sulfonamide inhibitors markedly suppresses acid secretion (see Ref. 32 for review). However, the role of CA IV or membrane-bound CA has not been established. Its function can be investigated using impermeant sulfonamides that remain extracellular by either marked hydrophilicity imparted by an ionized state at physiological pH (benzolamide (37) and quaternary ammonium sulfonamide (9)) or high molecular mass. However, benzolamide is not absolutely impermeant, and the toxicity and weak inhibiting activity of quaternary ammonium sulfonamide limits its use to isolated cell and organ studies. Earlier large molecular mass inhibitors such as the dextran-linked sulfonamides of Tinker et al. (36) and Karlmark et al. (11) had certain problems, including dissociation of the drug from the polymer (8) and anaphylactic reactions to dextrans.

The problems of earlier sulfonamide-linked polymers stimulated the synthesis of a 3,500-kDa polymer linked to aminobenzolamide (F3500); aminobenzolamide irreversibly bound to a nontoxic polymer of the dicarboxy derivative of polyoxyethylene (6). In vivo studies (17) using this polymer have demonstrated successful tight sulfonamide binding and lack of toxicity. F3500 has revealed the significant contribution of membrane-bound CA in renal bicarbonate reabsorption. Recently, we (34) synthesized an analog of benzolamide, p-fluorobenzylaminobenzolamide (pFBAB), which is very hydrophilic and only slightly larger than its parent compound. It retains high affinity for membrane-bound CA (33) and is considerably easier to synthesize than F3500.
In the present study, we examined the role of membrane-bound CA and cytoplasmic CA in H⁺ secretion in the frog stomach using the impermeant inhibitors F3500 and pFBAB and methazolamide, a highly diffusible and permeant inhibitor. To better understand and correlate the function of membrane-bound CA in frog gastric acid secretion, we performed histochemical studies in the frog stomach. In addition, given the highly acidic milieu in which an apical membrane-bound CA operates, we tested the pH resistance of CA IV in vitro.

MATERIALS AND METHODS

CA isozymes and inhibitors. Purified CA II was obtained from human blood by an affinity chromatography technique (12). Membrane-bound CA IV was prepared as a microsomal suspension from bovine kidneys (39).

Methazolamide was obtained from Lederle Laboratories (Pearl River, NY). F3500 was synthesized according to a previously published method (6). The synthesis of pFBAB was achieved by reductive alkylation of aminobenzalamide with p-fluorobenzaldehyde (34).

Enzyme activity and sensitivity to pH and inhibitors. The inhibitory potency of the CA inhibitors toward cytosolic and membrane-bound CA fractions of the frog gastric mucosa (prepared as described below) was determined at 0°C by monitoring the catalysis of CO₂ hydration by canine red cell CA (5). Briefly, the method involves the incubation of two enzyme units (EU) of canine red cell CA with known amounts of inhibitor in distilled water containing 25 mg/l bromthymol blue indicator at 0°C for a minimum of 2 min. The solution is then saturated with 100% CO₂, and the hydration reaction is initiated by the addition of 2 ml of 50 mM barbital buffer at pH 7.9. The catalyzed time (tₚ) is recorded to obtain a color change of the indicator, approximately pH 6. The reaction is also run in the absence of inhibitor to obtain the uncatalyzed time (t₀). The number of enzyme units in the system is given by EU = (t₀-tₚ)M. The IC₅₀ for a drug is the molar amount that reduces the number of enzyme units by 50%.

Gastric mucosa and red cells from Rana pipiens were obtained from anesthetized frogs after a 5-min partial immersion in a 1.5% solution of the methanesulfonate salt of 3-aminobenzoic acid ethyl ester. The abdomen was opened, and the stomach was perfused free of blood with an infusion of isotonic saline via the aorta. When the mesenteric effluent was clear, the stomach was excised and opened laterally, and the mucosa was weighed, cut into small pieces, and finally rinsed twice with distilled water. Before mounting, this tissue was rinsed with cold 6 mM phosphate buffer at pH 7.2 to remove the gluteraldehyde.

The histochemical technique used in this study was the resin version of the cobalt-precipitation technique described previously by Ridderstrale (23, 24). The samples were dehydrated by increasing concentrations of ethanol and embedded in a water-soluble glycol methacrylate (Historesin, Kulzer, Heidelberg, Germany). The tissue samples were infiltrated with a mixture of resin monomer and ethanol for 3 h, followed by resin monomer for 3 h, before embedding and polymerization at room temperature. Sections (2-μm thick) were cut with a microtome (RM 2165, Leica Instruments) using glass knives. Sections were incubated, floating in the incubation medium, for 3 and 6 min. The incubation medium contained (in mM) 3.5 CoSO₄, 53 H₂SO₄, 11.7 KH₂PO₄, and 157 NaHCO₃. After incubation, sections were rinsed in 0.67 mM phosphate buffer (pH = 5.9), transferred to 0.5% (NH₄)₂S, and finally rinsed twice with distilled water. Before mounting, some sections were counterstained with azure blue. Reaction specificity was checked by adding 10⁻⁵ M acetazolamide to the incubation medium and incubating sections as described above. The procedure resulted in a black precipitate at sites of CA activity. Sections incubated with acetazolamide showed no significant staining, except for weak shading of the nuclei.

Gastric acid secretion measurements. Isolated frog gastric mucosa was obtained as described above and then mounted in an Ussing-type chamber. The details of the basic chamber...
construction and the solution compositions used on the apical and basolateral sides were as given previously by Rehm et al. (22). The solution composition used on the basolateral (nutrient) side was (in mM) 102 Na\(^{+}\), 4 K\(^{+}\), 0.8 Mg\(^{2+}\), 81 Cl\(^{-}\), 0.8 SO\(_4^{2-}\), 25 HCO\(_3^{-}\), 1 phosphate, and 10 glucose. The apical solution was made NaCl free by replacement of choline for sodium and sulfate for chloride with sucrose added to make up any osmotic difference (2). In all studies, apical and basolateral solutions (temperature, 25°C) were gassed continuously with 95% O\(_2\)-5% CO\(_2\). Histamine (0.1 mM) was added to the basolateral solution to stimulate secretion. Apical H\(^{+}\) secretory rates were measured over times ranging up to 10 min. The apical (secretory) solution was titrated with 2.5 mM NaOH to maintain a pH of 5, utilizing a voltage of 100 mV with the basolateral side positive. Three to four measurements were made. A plot of NaOH (in \(\mu\)eq) added vs. time yielded a straight line, the slope of which was the secretory rate. Figure 1 shows a representative example of a control histamine-stimulated stomach. For inhibition with F3500 and pFBAB, a weighed quantity of either drug was dissolved in the apical or basolateral solution before addition to the chamber, and this was allowed to equilibrate with the tissue for 5 min before initiation of stimulated secretion.

**RESULTS**

*Inhibitor and enzyme activity measurements.* The structures, molecular masses, and inhibitory activities of the three compounds against purified mammalian CA II and IV isoenzymes are given in Fig. 2. The inhibition against both isozymes in vitro roughly follows a molecular size relationship with methazolamide showing the greatest potency, followed by pFBAB, and then F3500. All drugs show a 20- to 25-fold greater activity against CA II than CA IV. When the data in Fig. 2 are compared with the inhibition constants for

<table>
<thead>
<tr>
<th>K(_i) (nM) 25°C</th>
<th>CA-II</th>
<th>CA-IV</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methazolamide</td>
<td>23±3</td>
<td>530±47</td>
<td>204</td>
</tr>
<tr>
<td>(n=4)</td>
<td>(n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3500</td>
<td>80±6</td>
<td>2200±189</td>
<td>3500</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFBAB</td>
<td>50±4</td>
<td>950±12</td>
<td>454</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td></td>
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</tr>
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</table>
the frog tissues in Table 1, they are roughly four- to fivefold higher. This largely reflects the temperature differences at which the assays were run (25°C vs. 0°C) and the expected temperature-related decrease in inhibitor affinity.

To demonstrate the effective exclusion of pFBAB from intracellular (cytosolic) CA, we tested pFBAB penetration into human red cells. Erythrocytes are a simple cell system that permits quantitative determination of intracellular drug accumulation without the complication of membrane binding because red cells have no membrane-bound CA. In five experiments at 37°C, with blood of 50% hematocrit and 500 μM external concentration, red cell pFBAB concentration was only 5 ± 2 μM (SD) at 1 h. This compares with 35 ± 18 μM for benzolamide (a relatively impermeant inhibitor), 225 ± 31 μM for methazolamide (a permeant inhibitor), and a value below the detection limit (<2 μM) for F3500, the 10-fold larger impermeant polymer.

Table 1 shows the CA activity in frog red cells and mucosal homogenate and in a preparation of mucosal microsomes along with the inhibitory potencies of methazolamide, F3500, and pFBAB. Analysis of residual hemoglobin as a marker of contaminating red cells showed that only 30 U of activity could be attributed to red cell CA. The CA activity in mucosal microsomes (180 EU/g) was tested for residual cytosolic enzyme by treatment with 1% SDS, a concentration known to inactivate CA II but not CA IV (26). There was no change in the CA activity (data not shown). Thus ~10% of the overall CA activity in frog gastric mucosa resides in the membrane fraction with a resistance to SDS denaturation similar to mammalian CA IV.

The pH dependence of log (kcat/Km) under acid conditions is shown in Fig. 3. The solid line shows that the data are consistent with a pKₐ near 7 for the zinc-bound water at the active site of the enzyme. The slope of unity at pH < 6 remains consistent with the single ionization of the zinc-bound water to a pH as low as 3.5.

Gastric CA histochemistry. Figure 4 shows views of the frog gastric mucosa comprising the outer surface epithelium and the underlying glandular zone, consisting of oxynticopeptic cells. Staining for CA activity is evident in nuclei and cytoplasm of some surface epithelial cells. All capillaries are heavily stained, as are occasional red cells trapped within. Staining in the oxynticopeptic cells is seen along cell borders, with obvious staining apparent along the basolateral membranes. The small surface area of the apical membrane in these narrow unstimulated gland lumens is unstained. Weak cytoplasmic staining is present together with heavily stained nuclei.

Acid secretion measurements. Table 2 shows the H⁺ secretory rates for the histamine-stimulated frog gastric mucosa along with dose-response data for the permeant inhibitor methazolamide, as well as the secretory rates after inhibition by the two membrane-impermeant inhibitors when applied to either the apical or basolateral side. Both impermeant inhibitors are capable of decreasing acid secretion, but compared with the control secretory rate of 0.051 μeq·min⁻¹·cm⁻², F3500 and pFBAB gave only ~40% reductions in H⁺ secretion when applied to the apical side and a slightly greater reduction (55%) when applied to the basolateral side. These results should be contrasted with the total abolition of H⁺ secretion that occurred when intracellular and membrane-bound CA was inhibited by 100 μM methazolamide, in agreement with Carrasquer and Schwartz (2) who used acetazolamide, another permeant inhibitor.

DISCUSSION

This is the first study to demonstrate a membrane-bound CA function in gastric acid secretion. Our main findings are that the frog gastric mucosa contains a cell membrane-associated mammalian-like CA IV activity, as shown by histochemical and biochemical techniques. This membrane-bound CA represents almost 10% of total mucosal CA activity, and it retains significant activity in an acid milieu in contrast to other CA isozymes. Using two different size membrane-impermeant drugs with high activity against CA IV applied to the apical or basolateral surface, we find that this CA activity subserves almost 40% and 55%, respectively, of stimulated H⁺ secretion.

Table 1. CA activity and sensitivity to CA inhibitors of frog gastric mucosal homogenate and microsomes and red cells

<table>
<thead>
<tr>
<th></th>
<th>Total CA Activity, EU/g</th>
<th>Methazolamide</th>
<th>F3500</th>
<th>pFBAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>5</td>
<td>2,280 ± 209</td>
<td>5 ± 1</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>Microsomes</td>
<td>5</td>
<td>180 ± 25</td>
<td>110 ± 4</td>
<td>690 ± 49</td>
</tr>
<tr>
<td>Red cells</td>
<td>7</td>
<td>6,600 ± 780</td>
<td>4 ± 1</td>
<td>28 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of experiments. The inhibitor constant (Kᵢ) was found at 0°C. CA, carbonic anhydrase; F3500, 3,500-kDa polymer linked to aminobenzolamide; pFBAB, p-fluoro-benzyl-aminobenzolamide; EU, enzyme units.
Presence and characteristics of membrane-bound CA.

Membrane-bound CA activity in the gastric mucosa of many vertebrate species has been amply documented by histochemical and biochemical techniques in the surface epithelial cells and gastric glands (13, 15, 16, 20, 21, 32, 38). In mammalian parietal cells, apical and basolateral membranes stain positively with the histochemical technique (7, 13, 20, 30). The staining in the frog (Fig. 4) is more prominently found at the basolateral (antiluminal) border of the oxynticopeptic cells. The lack of evident staining in the apical membranes is not entirely explicable because the nondiffusible inhibitors were active when applied to the luminal side. Because these histochemical results were obtained in unstimulated stomachs, it may be possible that CA is moved into the apical membrane with the onset of stimulated acid secretion. In mammals, this activity has been ascribed to CA IV (3, 7) using isozyme-specific antibodies. However, a recent report (19) of a second membrane-bound CA isoenzyme in renal proximal tubules, designated CA XIV, suggests that not all membrane-bound CA activity is necessarily CA IV. This may explain the curious finding (1) that in pig parietal cell tubulovesical membranes, which are heavily enriched in H^+-K^+-ATPase and have high CA activity, the ~30-kDa protein suspected to be a CA did not stain with an anti-CA IV antibody.

Our histochemical findings confirm CA activity in cell membranes of oxynticopeptic cells of the gastric mucosa of *Rana pipiens*, similar to staining observed in other frog species (21, 31). In contrast to histochemical results in mammals, in which cytoplasmic staining is heavy (21, 31), cytoplasmic staining is weaker in amphibian stomach, and membrane staining appears accentuated against this weaker background. The isozymes present in nonmammalian species have not been rigorously delineated by gene or protein analysis, but it appears that in frog stomach, membrane-bound CA activity is necessarily CA IV. This may explain the curious finding (1) that in pig parietal cell tubulovesical membranes, which are heavily enriched in H^+-K^+-ATPase and have high CA activity, the ~30-kDa protein suspected to be a CA did not stain with an anti-CA IV antibody.

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Table 2. Inhibition of gastric acid secretion in isolated frog stomach by CA inhibitors of differing membrane permeability

<table>
<thead>
<tr>
<th>Drug Concentration, µM</th>
<th>Secretory Rate, µeq·min^{-1}·cm^{-2}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0</td>
<td>0.051 ± 0.003</td>
<td>7</td>
</tr>
<tr>
<td>Methazolamide 1</td>
<td>0.045 ± 0.005</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>0.023 ± 0.003*</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>0.0076 ± 0.002†</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>&lt;0.002†</td>
<td>3</td>
</tr>
<tr>
<td><strong>Apical application</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3500 300</td>
<td>0.029 ± 0.002*</td>
<td>3</td>
</tr>
<tr>
<td>600</td>
<td>0.035 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td>1,200</td>
<td>0.033 ± 0.003*</td>
<td>3</td>
</tr>
<tr>
<td>pFBAB 100</td>
<td>0.035 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td>250</td>
<td>0.031 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td><strong>Basolateral application</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3500 600</td>
<td>0.024 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td>1,200</td>
<td>0.023 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td>pFBAB 100</td>
<td>0.025 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td>250</td>
<td>0.024 ± 0.003*</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05, †P < 0.01 vs. control (paired Student's t-test).
sulfonamides gave nearly similar maximal levels of inhibition when applied luminally or antiluminally. Our data establish that apical and basolateral membrane-bound CA are responsible for almost 40% and 55% of gastric acid secretion, respectively, with the remainder dependent on intracellular CA II, which could only be accessed by methazolamide.

Functions of gastric CA in acid secretion. Figure 5 provides an overview of cell and membrane events relevant to the acid secretion and possible roles of CA in parietal or oxynticopeptic cells of the stomach. How apical membrane-bound CA in the stomach subserves H⁺ secretion is not totally answered by our studies. In the presence of HCO₃⁻ (swallowed in saliva, contained in food, and secreted by surface epithelial cells), membrane-bound CA permits rapid dehydration of H₂CO₃ to CO₂ as luminal HCO₃⁻ is titrated by H⁺ secretion. The newly generated CO₂ then readily diffuses across the apical membrane (see Fig. 5, reaction 1). This would be operative at the start of secretion until the pH fell below 6.

We have shown that CA IV is functional in an acidic milieu in which bicarbonate is no longer quantitatively present (pH < 6). Although we could not extend our measurements below a pH of 3.5 for technical reasons, this may be a moot point. Studies utilizing confocal microscopy and pH-sensitive fluorescent dyes (4) or small pH microelectrodes (28) capable of resolving pH down to the epithelial cell layer and gastric crypts have shown pHs no lower than 3.5 despite bulk luminal values <1 under stimulated conditions. However, it must be realized that it is not yet established how low the extracellular pH is in the immediate vicinity of apical membrane-bound CA. Schreiber and Scheid (28) have advanced the novel but controversial concept that special buffering and H⁺ release properties of gastric mucus form a vehicle for proton transport toward the gastric lumen. In this model (28), newly secreted protons are highly buffered by cosecreted mucus. As the mucus migrates outward, protons are released into the bulk luminal fluid as pepsin acts on and reduces the buffering capacity of mucus. Mucus production and its H⁺ buffering capacity may not accommodate all stimulated gastric acid secretion, but to the extent that it can facilitate acid secretion, perhaps apical membrane-bound CA also permits rapid buffering of secreted H⁺ by gastric mucus (Fig. 5, reaction 2).

We also studied the function of basolateral membrane-bound CA, which we and others (21, 31) observed histochemically, by application of membrane-impermeant inhibitors to the serosal side. Our data showing a 55% reduction of gastric acid secretion is in accord with Loveridge et al. (14) who used an antibody with CA-inhibiting properties from the serum of patients with pernicious anemia. When the antibodies were applied to the basolateral side they suppressed acid secretion by nearly 80% in the stimulated frog stomach. Basolateral membrane-bound CA may subserve acid secretion in two ways. The extrusion of H⁺ across the apical membrane by H⁺-K⁺-ATPase generates OH⁻, which in the presence of CO₂ and cytosolic CA II rapidly reacts to form bicarbonate. The HCO₃⁻ is then extruded across the basolateral membrane by either a Cl⁻/HCO₃⁻ anion exchanger (35) or an electrogenic Na⁺-HCO₃⁻ cotransporter (27). In the extracellular space (Fig. 5, reaction 3), the extruded HCO₃⁻ and available H⁺ are catalyti-
cally reacted to regenerate CO$_2$. Thus a potential rate-limiting buildup of extracellular HCO$_3^-$ in the vicinity of the anion transporters is minimized by the activity of a basolateral membrane CA.

The function of cytosolic CA is crucial to H$^+$ secretion. This is evident in our data with methazolamide in the frog stomach and many other studies using permeant inhibitors (see Ref. 32 for review). Cytosolic CA supports the high turnover of H$^+$-K$^+$-ATPase by permitting rapid conversion to HCO$_3^-$ of OH$^-$ produced in the hydrolysis of ATP and translocation of H$^+$ across the apical membrane (Fig. 5, reaction 4). The equimolar intracellular generation of the hydroxyl ion as a H$^+$ is translocated to the lumen must be dissipated quickly to forestall a rate-limiting alkalinity on the ATPase reaction.

In conclusion, high rates of gastric H$^+$ secretion are dependent on both membrane-bound and cytosolic CA. The relatively acid-resistant apical membrane CA operates in the acidic environment of the gastric gland lumen in HCO$_3^-$ reabsorption at the onset of stimulated secretion and then possibly to facilitate rapid H$^+$ binding to gastric mucus. Cytosolic CA subserves high H$^+$-K$^+$-ATPase turnover by catalyzing OH$^-$ conversion to HCO$_3^-$. Basolateral membrane and capillary endothelial CA subserve rapid transfer of cytosolic HCO$_3^-$ to the blood by membrane anion exchangers and Na$^+$-coupled extrusion.

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