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

ERIK R. SWENSON,1 TIMOTHY W. TEWSON,2 PER J. WISTRAND,3 YVONNE RIDDERSTRALE,4 AND CHINGKUANG TU5
1Department of Medicine, Department of Veterans Affairs Medical Center, and 2Department of Radiology, University of Washington, Seattle, Washington 98108; 3Department of Neuroscience, Uppsala University, SE-751 24 Uppsala; 4Department of Animal Physiology, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden; and 5Department of Pharmacology, College of Medicine, University of Florida, Gainesville, Florida 32610

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Swenson, Erik R., Timothy W. Tewson, Per J. Wistrand, Yvonne Ridderstrale, and Chingkuang Tu. Biochemical, histological, and inhibitor studies of membrane carbonic anhydrase in frog gastric acid secretion. Am J Physiol Gastrointest Liver Physiol 281: G61–G68, 2001.— Gastric acid secretion is dependent on carbonic anhydrase (CA). To define the role of membrane-bound CA, we used biochemical, histochemical, 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 (methazolamide) 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-fluorobenzyl-aminobenzolamide (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. Methazolamide 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.

Address for reprint requests and other correspondence: E. R. Swenson, Pulmonary Section, S-111-Pulm, Dept. of Veterans Affairs Medical Center, 1660 South Columbian Way, Seattle, WA 98108 (E-mail: eswenson@u.washington.edu).

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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 aminobenzamidate 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 (tₐ) 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 (tₑ). The number of enzyme units in the system is given by EU = (tₑ - tₐ)/Mₑ. 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-amino benzoic acid ethyl ester. To better understand the catalyzed exchange and allow more precise measurements of rates at lower pH values, the catalyzed exchange is very rapid (17). Testing below pH 3.5 was not possible because of the very fast uncatalyzed rate, even at 10°C, which depletes the isotope before measurements can be taken.

Gastric CA histochemistry. The gastric mucosa from *Rana pipiens* was obtained from anesthetized frogs as described above except that they were not perfused free of blood. The isolated mucosa was then placed in a buffered physiological solution (2) at 25°C and continuously gassed with 95% O₂-5% CO₂. After a 30-min incubation period, the mucosa was then rinsed three times with the physiological solution. Fixation was accomplished by transferring the mucosa to a 2.5% glutaraldehyde solution at pH 7.2, containing 19 mM NaCl and 4 mM Na₂HPO₄, and incubating with 100% CO₂ for 6 h at 3–4°C. Afterward, this tissue was rinsed with cold 6 mM phosphate buffer at pH 7.2 to remove the glutaraldehyde.

The histochemical technique used in this study was the resin version of the cobalt-precipitation technique described previously by Ribberstrale (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 7.2) and then rinsed with distilled water. Before mounting, some sections were counterstained with nuclear dye solutions.

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 Mg²⁺, 81 Cl⁻, 0.8 SO₄²⁻, 25 HCO₃⁻, 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% O₂-5% CO₂. 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 μeq) 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 1. CA activity and sensitivity to CA inhibitors of frog gastric mucosal homogenate and microsomes and red cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total CA Activity, EU/g</th>
<th>Methazolamide K_i, nM</th>
<th>F3500 K_i, nM</th>
<th>pFBAB K_i, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>5 2,280 ± 209</td>
<td>5 ± 2</td>
<td>30 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Microsomes</td>
<td>5 180 ± 25</td>
<td>110 ± 14</td>
<td>690 ± 49</td>
<td>450 ± 34</td>
</tr>
<tr>
<td>Red cells</td>
<td>7 6,600 ± 780</td>
<td>4 ± 1</td>
<td>28 ± 4</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

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

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 (k_cat/K_m) under acid conditions is shown in Fig. 3. The solid line shows that the data are consistent with a pK_a 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.
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 has similarities to CA IV. The data (Fig. 2 and Table 1) show that membrane-bound activity is 20 times less sensitive to CA inhibitors than the cytosolic enzyme and is not denatured by SDS, characteristics in mammals that distinguish cytosolic CA II from CA IV (5). In distinct contrast to other CA isozymes, which lose all activity by denaturation pH <5, CA IV retains significant activity in the acid pH range (Fig. 2) over which it must function at the extracellular apical surface (see below).

The results of CA inhibition in the isolated frog gastric mucosa provide further evidence of membrane-bound and extracellularly oriented CA. Two different, but otherwise very impermanent, potent CA-inhibiting

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

<table>
<thead>
<tr>
<th></th>
<th>Drug Concentration, μM</th>
<th>Secretory Rate, μeq min(^{-1})cm(^{-2})</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.051 ± 0.003</td>
<td>7</td>
</tr>
<tr>
<td>Methazolamide</td>
<td>1</td>
<td>0.045 ± 0.005</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.023 ± 0.003*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0076 ± 0.002†</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>&lt;0.002†</td>
<td>3</td>
</tr>
<tr>
<td>Apical application</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3500</td>
<td>300</td>
<td>0.029 ± 0.002*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.035 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1,200</td>
<td>0.033 ± 0.003*</td>
<td>3</td>
</tr>
<tr>
<td>pFBAB</td>
<td>100</td>
<td>0.035 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.031 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td>Basolateral application</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3500</td>
<td>600</td>
<td>0.024 ± 0.003*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1,200</td>
<td>0.023 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td>pFBAB</td>
<td>100</td>
<td>0.025 ± 0.004*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<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 CO₂. Thus a potential rate-limiting buildup of extracellular HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻. Basolateral membrane and capillary endothelial CA subserve rapid transfer of cytosolic HCO₃⁻ to the blood by membrane anion exchangers and Na⁺-coupled extrusion.

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REFERENCES


