Effect of Acetazolamide and Melittin on Polarization of the Frog Gastric Mucosa Proton Pump (44057)

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Abstract. **We have shown that polarization of an electrogenic H^+/K^+ATPase pump located in the secretory (luminal) membrane of the frog gastric mucosa is the major factor contributing to the change in open circuit potential difference (OCPD) induced by voltage clamping. This transmucosal polarization was markedly reduced by H_2 blockers famotidine and cimetidine, and by the H^+/K^+-ATPase inhibitors omeprazole and SCH 28080. SCN^-, a nonspecific H^+ secretion inhibitor, did not affect the polarization. In the present experiments, the effects of two other inhibitors of H^+ secretion were examined, namely, acetazolamide (AA), a carbonic anhydrase inhibitor, and melittin (MEL), an inhibitor of the H^+/K^+-ATPase enzyme. When AA 10^{-3} *M* or MEL 10^{-5} *M* was added to the nutrient solution, H^+ secretion was completely inhibited. While MEL markedly reduced the polarization induced by voltage clamp, AA did not affect the polarization. These data support the concept that MEL directly affects the electrogenic H^+/K^+-ATPase pump while the inhibition of H^+ secretion by AA is by an indirect mechanism. The data further support the electrogenicity of the H^+/K^+-ATPase.** [P.S.E.B.M. 1996, Vol 213]

When current is sent across the frog gastric mucosa, there is a polarization of the open circuit voltage which has been shown to be independent of the capacitance of the tissue (1, 2). Moreover, a nonlinear resistance was ruled out as the cause of the polarization (1, 2). The observed polarization could be due to ion redistribution with change in diffusion electromotive forces (EMFs) and/or a change in the EMF of the active pumps during current sending. We have shown that polarization is still present despite Na^+-free, Cl^--free, and high K^+ (79 m*M*) bathing solutions (3–5). These data suggested that a mechanism other than the K^+ and Cl^- diffusion EMFs or the electrogenic Na^+/K^+-ATPase must be responsible, at least in part, for the polarization effect. We also found that the polarization was inhibited by inhibitors of H^+ secretion, the H_2 blockers famotidine (4) and cimetidine (5) and by inhibitors of the H^+/K^+-ATPase enzyme omeprazole (4) and SCH 28080 (5). SCN^-, a nonspecific H^+ secretion inhibitor, did not affect the polarization (5). Furthermore, voltage clamp polarization did not occur in K^+-free solutions (4). These data showed that the proton pump, in which K^+ is involved, is mostly responsible for the voltage clamp polarization and thus support the concept that the proton pump is electrogenic.

In present experiments the effects on the voltage clamp polarization of two other inhibitors of H^+ secretion were examined, namely, acetazolamide (6), a carbonic anhydrase inhibitor, and melittin (the honey bee venom), an inhibitor of the H^+/K^+-ATPase enzyme (7–9). We will show that the data support the concept that melittin inhibits the electrogenic H^+/K^+-ATPase in the gastric mucosa and acetazolamide does not.

Materials and Methods

Experiments were performed on fundi of stomachs of Rana pipiens by an *in vitro* method in which the stomachs were mounted between a pair of cylindrical chambers (10). All experiments began with standard Cl^- solutions on both sides of the mucosa, to check the viability of the mucosa. The Cl^- nutrient (serosal) solution contained (in m*M*): Na^+, 102; K^+,

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4; Ca^{2+}, 1; Mg^{2+}, 0.8; Cl^-, 81; SO_4^{2-}, 0.8; HCO_3^-, 25; phosphate, 1; and glucose, 10; and the Cl^- secretory (mucosal) solution, which is hypertonic (11), contained Na^+, 156; K^+, 4; and Cl^-, 160. Then these solutions were modified to NaCl-free solutions. Cl^- was replaced with SO_4^{2-}, and sucrose was added to make up any osmotic deficit. Na^+ was replaced with choline. Acetazolamide or melittin were added to the nutrient (serosal) solution to a concentration of 10^{-3} *M* and 10^{-5} *M*, respectively.

The H^+ secretory rate before addition of the inhibitors, the transmucosal resistance, and the transmucosal potential difference (PD) were measured. Two pairs of electrodes were used, one for sending current across the mucosa and the other for measuring the PD. The PD is considered positive when the nutrient side is positive relative to the secretory side of the stomach. The resistance was determined as the change in PD per unit of applied current. Current (20 μA/1.3 cm^2 of tissue area) was applied for 1 or 2 sec, first in one direction and 2 or 3 sec later in the other direction. For voltage clamping, the voltage was clamped in steps of 20 mV up to 80 mV above the open circuit PD (OCPD) by sending current from secretory (mucosa) to nutrient (serosa). During the voltage clamp period, the current was interrupted periodically for about 2 sec in order to obtain the open circuit voltage. The H^+ secretory rate was determined by the pH stat method of Heinz and Durbin (12). The pH of the secretory solution was generally maintained between 4.7 and 5.0, and the pH of the nutrient solution was about 7.2–7.3. Both sides of the gastric mucosa were gassed with 95% O_2/5% CO_2 throughout these experiments, and 0.1 m*M* histamine in the nutrient solution was used to stimulate secretion. Histamine was not present when acetazolamide or melittin were added. Linear regression analysis (Method I: Comparing two straight lines using separate regression fits—Testing for parallelism) (13) was used for statistical analysis of the regression lines. For other purposes, the Student's *t* test with paired observations was used.

Results

Data will be presented such that Figure 1 will show results from one experiment in control, with addition of acetazolamide and with addition of melittin in the nutrient solution. Although we have shown that polarization of the frog gastric mucosa was mostly due to polarization of the proton pump and not to ion redistribution of Na^+ or Cl^-, all experiments presented in this paper were in NaCl-free solutions to exclude ion redistribution of Na^+ or Cl^-, or the electrogenic Na^+/K^+-ATPase (14) as possible contributors to the observed polarization (3–5). Figure 2 and Table I will present the summary of all the data (three experimental groups).

Effect of Voltage Clamping (VC) on OCPD in Na^+-Free, Cl^--Free, and 4 m*M* K^+ Solutions, in Control, with 10^{-3} Acetazolamide or with 10^{-5} *M* Melittin in the Nutrient Solution. Figure 1 shows data from a representative experiment in each of the three conditions. The voltage was clamped in steps of 20 mV from 20 to 100 mV, nutrient side positive. The figure presents the voltage clamping (VC) and the OCPD versus time. The OCPD was recorded continuously before voltage clamping. Although the lines representing OCPD are plotted as a continuous line, they were recorded periodically, every 1–2 min. During voltage clamping, the OCPD was recorded by releasing the clamp for 1–2 sec. The OCPD values were practically constant during the 10-min periods. No attempt was made to record the OCPD for the first 30–60 sec, since the early effects were well studied and documented previously (1, 2).

Figure 1A shows data obtained during the control period, in the absence of inhibitors. The OCPD increased from about 5 mV pre-clamping to a maximum of about 35 mV when the voltage was clamped at 100 mV. A VC of about 95 mV above the pre-clamp level increased the OCPD by about 30 mV.

The mean value of H^+ secretion for 15 experiments during the control period was 3.4 (SEM ± 0.2) μA hr^{-1} cm^{-2}.

Figure 1B presents data from one experiment with acetazolamide in the nutrient solution. In the presence of acetazolamide, the OCPD increased from about −2 mV pre-clamping to a maximum of about 23 mV when the voltage was clamped at 80 mV. A VC of about 82

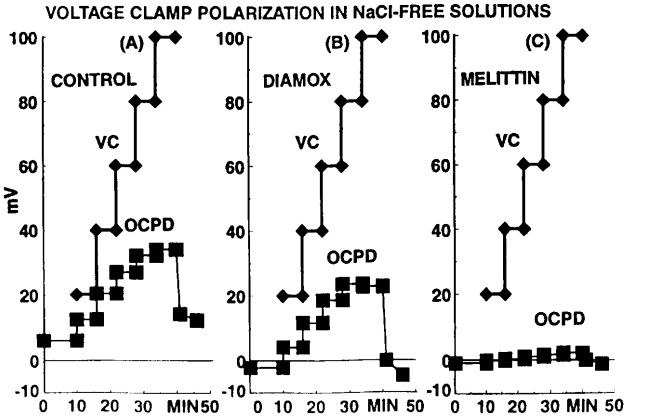

Figure 1. Voltage clamp potential (VC) and open circuit PD (OCPD) are plotted versus time with NaCl-free/4 m*M* K^+ solutions: control without inhibitors (A); 10^{-3} *M* acetazolamide in nutrient solutions (B); and with 10^{-5} *M* melittin in nutrient solution (C). Voltage clamp was obtained manually, and its plot is factual. OCPD was continuously plotted during the pre-clamp period. During VC, OCPD was obtained by opening the circuit for about 2 sec, every 1–2 min. Values for OCPD are average values for the 10-min periods (<5% deviation from mean). Values of OCPD were not recorded during the first 30–60 sec of VC (see text).

mV above the pre-clamp level increased the OCPD by about 25 mV.

There was no H^+ secretion in the presence of acetazolamide.

Figure 1C presents data from one experiment with melittin in the nutrient solution. In the presence of melittin, the OCPD barely increased from about −1 mV to a maximum of 3 mV when the voltage was clamped at 100 mV. A VC of 101 mV above the pre-clamp level increased the OCPD by about 4 mV—that is, the venom markedly decreased the voltage clamp polarization.

There was no H^+ secretion in the presence of melittin.

A graphic representation of the experiments summarized in Table I is shown in Figure 2. The increase in OCPD (polarization) induced by voltage clamping (OCPD [vs] − OCPD) is plotted versus the difference between the voltage clamp PD and the control OCPD (VCPD − OCPD). The regression parameters of the increase in OCPD (polarization) versus the increase in transepithelial PD by voltage clamping (OCPD [vc] − OCPD) are presented in Table I. The slopes of the lines represent the polarization of the PD, as an increase in the open circuit potential per 100 mV increase in PD, by voltage clamping. The polarization was 27.4 mV percent in the absence of inhibitors. With 10^{-3} *M* acetazolamide in the nutrient solution, the polarization was 30.4 mV percent, not different from control. With 10^{-5} *M* melittin in the nutrient solution, the polarization was 9.1 mV percent, which was significantly lower than control and acetazolamide experiments ($P < 0.01$).

Discussion

Although from work in vesicles there is evidence for the existence of a neutral H^+/K^+-ATPase pump in the gastric mucosa secretory membrane (15–18), from work on intact tissue there is ample evidence that H^+ secretion is electrogenic (12, 19–21). In recent publications (3–5), we have provided further evidence of the electrogenicity of the proton pump in the intact stomach and presented a phenomenological construct originally proposed by Forte *et al.* (18), in which the two mechanisms were put together. Furthermore, we showed (4, 5) that the polarization induced by producing a voltage clamp across the gastric mucosa was markedly reduced by H_2 blockers (22, 23), famotidine (4) and cimetidine (5), and by omeprazole (4), which in the active form is a sulfonamide that inhibits the H^+/K^+-ATPase by reacting with its sulfhydryl groups (24, 25) and by SCH 28080 (5), a reversible competitive inhibitor of the K^+-induced hydrolysis of the H^+/K^+-ATPase (26).

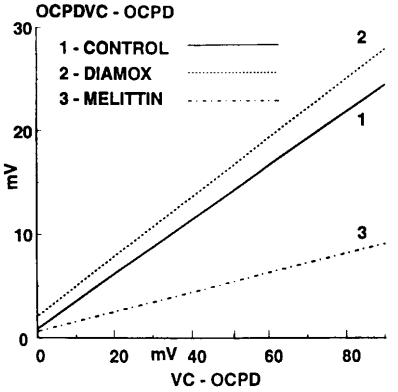

Figure 2. Effect of voltage clamp on open circuit PD in NaCl-free/4 m*M* K^+ solutions. Increase in OCPD during voltage clamp ($OCPD_{VC}$ − OCPD) plotted versus increase in voltage by voltage clamp over the pre-clamp PD (VCPD − OCPD). The slope represents the increment in OCPD (polarization) induced by voltage clamp. Values of regression parameters presented in Table I.

Table I. Effect of Voltage Clamping on Open Circuit Potential in NaCl-Free Solutions (Regression Line Parameters)

	n^a	Slope	Intercept	*R*
Control	14	27.4	1.1	0.72
+ acetazolamide	6	30.4	2.1	0.94
+ melittin	9	9.1[b,c]	0.5	0.47

[a] *n*, number of experiments.
[b] Significantly different from control solutions (without inhibitors) ($P < 0.01$)
[c] Significant difference from acetazolamide ($P < 0.01$).

While the inhibitors mentioned above interfered with the voltage clamp polarization, SCN^-, a known inhibitor of H^+ secretion (27–33), which does not affect the pump but induces back diffusion of HSCN, did not interfere with the polarization (5). These findings support the concept that, in order to inhibit the voltage clamp polarization, one must inhibit the proton pump *per se*. This notion was given additional support by the finding that there was no voltage clamp polarization when K^+ was removed from the bathing solutions (4).

In present experiments, new information has been provided on the effect of acetazolamide and melittin on the H^+ pump. Acetazolamide was known to inhibit H^+ secretion (6), presumably by inhibiting carbonic anhydrase in the oxyntic cells of the gastric mucosa. Melittin is known to inhibit the gastric mucosa H^+/K^+-ATPase (7–9), but the effect on H^+ secretion in the intact mucosa was not known. In present experiments, both 10^{-5} *M* melittin in the nutrient solution or 10^{-3} *M* acetazolamide abolished the H^+ secretion in the intact gastric mucosa.

Data obtained with the voltage clamp experiments show that melittin inhibits the voltage clamp polarization as other inhibitors of the H^+/K^+-ATPase pump (H_2 blockers, omeprazole, and SCH 28080)—that is, melittin inhibits H^+ secretion by a direct effect on the pump. Thus, we have confirmed that melittin has a

direct inhibitory effect on the pump in the intact gastric mucosa as previously observed by other techniques. Since melittin binds to the ATPase pump on the cytosolic side, the question is, How does it get into the cell? We do not know the answer at this time.

Although it is well known that acetazolamide inhibits H^+ secretion, this is the first experimental evidence that the inhibitor does not affect the H^+/K^+-ATPase pump directly. That is, apparently, acetazolamide inhibits H^+ secretion in some indirect way (like SCN^-—see above) by inhibition of carbonic anhydrase. The voltage clamp polarization with acetazolamide was about the same as in control experiments without acetazolamide.

In summary, acetazolamide and melittin are inhibitors of gastric H^+ secretion: acetazolamide does it by an indirect mechanism and the honey bee venom, melittin, by a direct effect on the pump. The results, moreover, give further support to the concept of an electrogenic gastric H^+/K^+-ATPase pump.

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