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ON THE MECHANISM OF ACID SECRETORY INHIBITION BY ACETAZOLAMIDE

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SUMMARY

1. The relationship between electrical potential difference and acid secretion by the bullfrog gastric mucosa was measured in the presence and absence of acetazolamide. At every value of potential the acid secretory rate was less with the drug present.

2. Intracellular pH changes were monitored in the intact tissue using bromcresol purple with a dual wavelength spectrophotometer. Addition of acetazolamide resulted in an alkaline shift in the intracellular pH.

3. Measurement of respiratory chain components showed an increased level of reduction of cytochrome a_3 in response to the addition of acetazolamide.

4. The results are interpreted to support the hypothesis that acetazolamide acts primarily on intracellular acid-base regulation as opposed to a direct effect on the hydrogen secretory mechanism. This action is presumably due to the inhibition of carbonic anhydrase.

INTRODUCTION

Acetazolamide (Diamox) is a known inhibitor of carbonic anhydrase, and this action has been implicated in its inhibitory effect on gastric acid secretion[1,2,13] presumably indicating an important role for carbonic anhydrase in the formation of acid. Recently, considerable doubt has been raised about the action of Diamox on gastric secretion. DURBIN AND HEINZ[3] found that Diamox inhibited gastric chloride secretion by *in vitro* bullfrog gastric mucosa with only a small inhibition of hydrogen ion secretion. HOGBEN[4] suggested that the decrease in electrical potential which accompanies inhibition of chloride transport might be responsible for the inhibition of secretion and failed to obtain an inhibition when the potential was held at zero volts.

The major function of carbonic anhydrase in the gastric mucosa is thought to be as a catalyst for the hydration of CO_2 to form H^+ and HCO_3^-. The H^+ may serve to neutralize excess base formed during secretion or as a source of H^+ for secretion while the HCO_3^- is transported out of the cell, presumably in a passive manner, possibly exchanging for Cl^-. Inhibition of carbonic anhydrase by Diamox might then inhibit acid secretion by decreasing the availability of H^+ either directly, if it is a source of H^+, or indirectly by allowing the intracellular pH to become alkaline.

The purpose of this study is to reexamine the effect of Diamox on acid secretion and to provide a crucial test for the various mechanisms by which it might exert an inhibitory effect.

METHODS

Bullfrogs (*Rana catesbieana*) were decapitated and pithed and the stomach quickly removed. The mucosa was separated from the muscular layer by blunt dissection and mounted between two lucite half chambers having an opening of 1.1 cm^2 in area. When the tissue was to be used with the spectrophotometric methods, the stomach was perfused *via* the descending aorta with oxygenated Ringer solution prior to removing the stomach. This procedure is required to remove hemoglobin which interferes with the spectral measurements[5]. The chamber used with the spectrophotometer was similar in design to the one above but the front and rear surfaces were optically clear to permit transillumination of the tissue and the area of the opening was 2.4 cm^2.

The nutrient surface of the tissues was bathed with a solution containing in mM: Na^+, 101; K^+, 4.0; Ca^{2+}, 1.0; Mg^{2+}, 0.8; SO_4^{2-}, 0.8; Cl^-, 81.0; HCO_3^-, 25.0; phosphate, 1.0; glucose, 5.0; and the secretory surface with a solution containing the same cation content but with Cl^- as the only anion and no glucose. The nutrient solution was gassed with 95 % O_2, 5 % CO_2 and the secretory side with 100 % O_2. All experiments were carried out at 18–20°.

Acid secretion was measured by the pH-stat method[3] using an automatic titration system (Radiometer). Electrical potential differences were monitored with calomel half cells connected to the chamber by agar–ringer bridges and current was passed using Ag, AgCl electrodes *via* agar–ringer bridges. Potentials and current were recorded on a Polygraph (Grass Instr., Model 5). In all experiments interactions between the glass electrode and the potential and current-passing electrodes were compensated for.

The optical measurement of cytochromes and the pH indicator bromcresol purple were performed with a dual wavelength spectrophotometer[6]. For measurement of intracellular pH, bromcresol purple (Fisher Sci.) was incorporated into the tissue by exposing the tissue on the nutrient side to the dye ($1 \cdot 10^{-4}$ M) for 1–2 h. The chamber was then rinsed several times over a period of 15–30 min to remove excess dye and the experiment begun. No modification of the tissue is required for monitoring the endogenous cytochromes. Wavelength pairs used for monitoring cytochrome a_3 and bromcresol purple were 445–465 nm and 588–630 nm[8], respectively.

RESULTS

It has been demonstrated that changing the electrical potential across the gastric mucosa can alter the rate of acid secretion[9] and it is possible that the inhibitory effect of Diamox is due to a change in the potential so as to make the secretory side more positive relative to the nutrient side and thus less favorable to H^+ secretion. To test this hypothesis the effect of applied potential on acid secretory rate was measured (Fig. 1). In each experiment the tissue was stimulated to secrete with $1 \cdot 10^{-4}$ M histamine and the potential was forced to a chosen value by applying

current from an external source. The potential was held at the new value for 5 min and then allowed to return to its initial value. The recovery period usually required 3–10 min. Any tissue which did not return to within 10 % of its initial potential and acid secretory rate between potential steps was discarded. After an appropriate series of potential steps Diamox ($3 \cdot 10^{-2}$ M) was added to the nutrient bathing medium and the changes in secretion and potential were followed for 45–60 min (Fig. 2). The series of potential steps was then repeated, again allowing for recovery between each value.

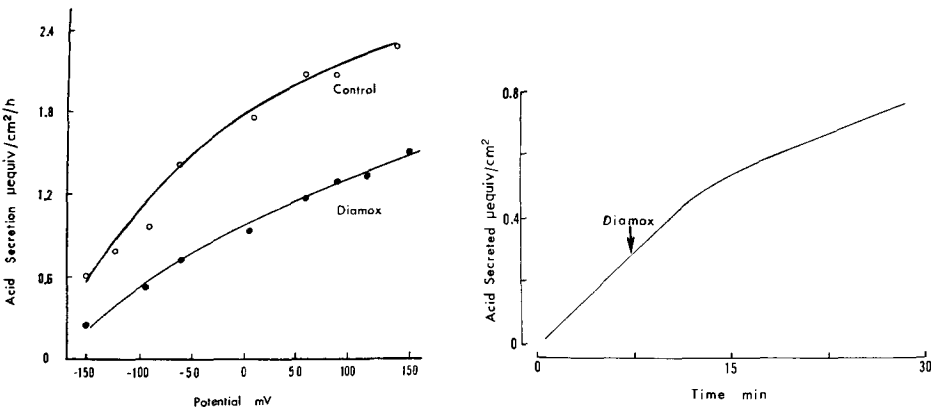

Fig. 1. Effect of potential difference on acid secretory rate in the presence and absence of Diamox. Each point represents the mean of six experiments. Polarity refers to nutrient *versus* secretory surfaces. (See text for details.)

Fig. 2. Time course of Diamox effect on acid secretory rate. A new steady secretory rate is established in approx. 10 min after the addition of Diamox ($3 \cdot 10^{-2}$ M). Trace is redrawn from the original pH-stat recording.

The results of these experiments clearly show that the potential change associated with Diamox inhibition cannot account for the decrease in acid secretory rate. The control potentials averaged 11.2 mV, a value which is lower than that reported in previous studies of Diamox[3,4] but more consistent with actively secreting tissues[9]. Diamox reduced the potential by an average of 4.2 mV, a change which is far too small to account for the large decrease in acid secretion. The acid secretory rate was markedly inhibited by Diamox from a mean value of 1.98 μequiv/cm^{-2} per h to 0.90 μequiv/cm^{-2} per h. These values correspond to a mean slope of 0.27 μequiv/cm^{-2} per h per mV change while the slopes of the experimental curves in the vicinity of zero potential are respectively 0.006 and 0.004 μequiv/cm^{-2} per h per mV change for control and Diamox-treated tissues.

The hypothesis that Diamox inhibits secretion by virtue of its effect on carbonic anhydrase leads to the prediction of an alkaline shift in the intracellular space. To test this hypothesis intracellular pH changes were monitored with the dualwavelength spectrophotometer using the indicator dye bromcresol purple. A typical experiment is shown in Fig. 3. The addition of Diamox ($1 \cdot 10^{-2}$ M) to the nutrient medium results in a rapid and substantial increase in absorption indicating an alkaline pH change. The pH change reaches an apparently stable value in 3–5 min and remains relatively constant for at least 1 h (the longest time that the trace was monitored).

In previous studies it was shown that changes in acid secretion produced by

histamine and thiocyanate were accompanied by changes in the oxidation–reduction level of the respiratory chain components[5]. It was therefore of interest to see if Diamox inhibition of secretion results in redox changes similar to those associated with thiocyanate inhibition. The effect of Diamox addition on cytochrome a_3 is shown in Fig. 4. It may be seen from this experiment that Diamox does indeed produce a redox change in cytochrome a_3. However, in this case acid secretory inhibition is associated with a reduction of the cytochrome, while thiocyanate inhibition produces an oxidation[5].

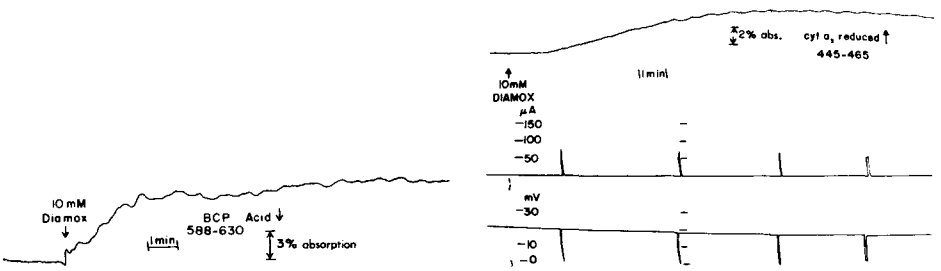

Fig. 3. Effect of Diamox on intracellular pH. The intracellular pH indicator bromcresol purple (BCP) was incorporated into the tissue and changes were monitored with the dual wavelength spectrophotometer using the sample wavelength of 588 nm. Scale bars give time and percent absorption change. Upward change indicates increasing pH. The magnitude of the pH change corresponding to a 3 % change in absorption of bromcresol purple cannot be calculated due to uncertainties in its intracellular pK and distribution (see DISCUSSION). Diamox added to nutrient solution at arrow.

Fig. 4. Effect of Diamox on cytochrome a_3 and electrical parameters. Upper trace, cytochrome a_3; middle trace, short-circuit current; lower trace, transepithelial potential difference. Diamox added to nutrient solution at arrow, upward deflection indicates cytochrome reduction. A 2 % increase in absorption corresponds to an increase in the concentration of reduced cytochrome a_3 of approx. 10 μM. Open-circuit potential was brought to zero by applying a brief current (spikes) to measure short-circuit current. Potential values refer to nutrient surface positive. Note small decrease in potential and short-circuit current accompanying cytochrome change.

DISCUSSION

The present results confirm that Diamox inhibits acid secretion by the frog gastric mucosa. Elucidation of the site and mechanism of action of this agent would represent a significant step toward the understanding of the molecular mechanisms involved in gastric acid secretion.

Two hypotheses have received rather wide support over the past several years and this work attempts to distinguish between them. HOGBEN has suggested that Diamox acts primarily to inhibit chloride transport thereby pharmacologically short-circuiting the tissue[4]. qH^+ is thought to decrease due to a less favorable electrochemical gradient. Decreases in transmucosal potential and chloride transport in response to Diamox have been reported[3,4] (also see Fig. 4). This hypothesis would predict that qH^+* could be restored by returning the potential to the pre-Diamox level by passing current from an external source. Our results indicate that at every potential qH^+ is significantly less after Diamox, and that potentials as high as 150 mV secretory side negative could not restore qH^+ to the spontaneous rate before the drug. This is an agreement with the findings of REHM[10] for *in vivo* dog stomach.

* $\overline{qH^+}$, the rate of acid secretion.

The second hypothesis suggests that Diamox acts to decrease the availability of H^+ for the transport mechanism by inhibiting carbonic anhydrase. This is consistent with its action as an inhibitor of the isolated enzyme. Related compounds have been shown to inhibit acid secretion in proportion to their potency as carbonic anhydrase inhibitors[1]. Consistent with the findings of other investigators, however, concentrations of Diamox required to inhibit qH^+ in the present work far exceeded those required to inhibit the isolated enzyme[11]. This discrepancy remains unexplained.

Inhibition of carbonic anhydrase might decrease the availability of H^+ by one of two mechanisms, depending on the source of H^+ for the transport mechanism. There are three probable sources; H^+ liberated from metabolized substrate; H^+ produced by hydrolysis of H_2O; and H^+ from the hydration of CO_2 to form H_2CO_3 which subsequently dissociates into H^+ and HCO_3^-. It is not presently known which of these sources specifically provide H^+ for the pump or whether they all contribute to a common pool. If H^+ from the hydration of CO_2 is available to the pump, inhibition of carbonic anhydrase would act directly to reduce pump substrate. If, on the other hand, only H^+ from the first two sources were available, carbonic anhydrase inhibition would act to decrease the cells capacity to buffer the excess OH^- produced. Regardless of which of these mechanisms for carbonic anhydrase involvement is operative, an inhibition of the enzyme in the secreting mucosa would be expected to result in an intracellular alkalinization.

The direct observation of an alkaline shift in the intracellular pH is consistent with the hypothesis that Diamox inhibition of acid secretion is due to the effect of the drug on carbonic anhydrase. Unfortunately neither the exact magnitude of the pH shift nor the final pH level can be determined using the indicator dye method. This is because of the uncertainties involved in pK shifts due to protein binding of the dye and in determining the exact intracellular localization. Thus a small fraction of dye might be detecting a large pH shift or a large fraction of dye might be detecting a small pH shift.

Although a detailed comparison of kinetics is not possible due to uncertainties involved in the measurements, the time course of the pH shift is consistent with the idea that pH changes precede the inhibition of acid secretion. The time to reach a stable pH was 3–5 min (Fig. 3) in all experiments while acid secretion required 5–15 min (Fig. 2) to reach a new steady level. This is consistent with a primary effect of Diamox on carbonic anhydrase as opposed to a direct effect on the transport mechanism. The cytochrome changes appear to lie somewhere intermediate between the pH changes and the change in acid secretion.

The change in the redox level of cytochrome a_3 associated with Diamox inhibition of acid secretion is interesting in that redox changes have been observed previously with other agents which alter qH^+, including histamine and thiocyanate[5]. Diamox and thiocyanate both decrease qH^+ but appear to act *via* different mechanisms since the former produces a reduction in cytochrome a_3 while the latter has been reported to produce an oxidation. Moreover, the observation of different redox changes with similar acid secretory charges indicates that the redox level of the cytochromes is not changing in direct response to the rate of secretion *per se*.

CHANCE *et al.*[12] have demonstrated that a decrease in pH produces an oxidation of pyridine nucleotide and cytochrome b in isolated mitochondria. If similar mechanisms operate in the gastric mucosa, the increase in intracellular pH could account

for the observed cytochrome reduction. This idea is supported by the relative time course of the two parameters. This suggests an important role of intracellular pH in the control of the redox level of the cytochrome chain. Confirmation of this idea and its implications for the coupling between oxidative metabolism and acid secretion must await further experiments.

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