Biochimica et Biophysica Acta, 640 (1981) 512—520

BBA 79077

EFFECTS OF OUABAIN ON FROG GASTRIC MUCOSA IN VITRO

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(Received July 9th, 1980)

Key words: Ouabain; Potential difference; H^+ secretion; Gastric mucosa; $(Na^+ + K^+)$-ATPase

Summary

The effect of the addition of ouabain to the nutrient solution was determined on resistance, potential difference (p.d.) and H^+ secretion rate. In NaCl media, 10^{-3} M ouabain decreased significantly the p.d. from 25.6 mV to 16.1 mV in 30 min and to 11.0 mV in 60 min. No significant changes occurred in resistance and H^+ secretion rate. In Na_2SO_4 (Cl^--free) media, ouabain produced a biphasic effect on p.d. The p.d. changed from —28.0 mV (nutrient-negative) to a nadir of —37.4 mV in 7 min and then increased to —16.4 mV in 60 min. At the nadir there was no significant change in resistance or H^+ secretion rate but at 60 min, unlike Cl^- media, resistance increased by 36% and H^+ secretion rate decreased by 43%. To decide whether the ouabain-caused decrease in H^+ rate in Na_2SO_4 media was due to an effect on the H^+ pump or on resistance of the return pathways, the voltage was clamped at 0 and 40 mV. Clamping the voltage showed that in the case of a marked decrease in the H^+ secretion rate, the H^+ transport mechanism itself was inhibited (and not the parallel pathway). The decrease in p.d. due to ouabain in Cl^- and SO_4^{2-} media indicates that the $(Na^+ + K^+)$-ATPase mechanism may be electrogenic.

Introduction

Both H^+ and Cl^- are actively secreted by mammalian and frog stomachs. It has been proposed recently [1] that Cl^- secretion may be a 'secondary' type of active transport located in the nutrient membrane of the oxyntic cell. The primary transport would be an ATPase-dependent Na-K exchange pump located in the nutrient membrane which transports Na^+ from the cell and K^+ into the cell. It is further postulated that there is a neutral mechanism in the

nutrient membrane which transports NaCl into the cell — the chemical potential of NaCl is maintained at a lower level in the cell than in the nutrient fluid due to the $(Na^+ + K^+)$-ATPase pump. Thus Cl^- is transported uphill.

Transport across the secretory membrane may take place as follows: (a) Cl^- could move from the secreting cell into the lumen down its electrochemical gradient which results from H^+ transport via an electrogenic H^+ pump; or (b) both Cl^- and H^+ could be actively transported across the secretory membrane either by independent electrogenic pumps or by a neutral HCl mechanism.

In order to investigate to what extent the Na-K pump on the nutrient membrane controls the secretion of ions, experiments were designed using ouabain. It is well accepted that ouabain is an inhibitor of the $(Na^+ + K^+)$-ATPase pump [2]. Previous studies with ouabain in the frog gastric mucosa showed an inhibitory effect on H^+ secretion [3,4].

In the work reported herein, we found that ouabain inhibited the H^+ secretion rate in Cl^--free media. In order to investigate whether ouabain affected the H^+ pump per se or the return pathway, voltage-clamping experiments were performed in the absence and presence of ouabain. It has been shown [5] that increasing the p.d. by voltage clamping, that is, making the nutrient side more positive relative to the secretory side, increases the H^+ secretion rate. Voltage clamping does not re-establish the H^+ secretion rate in the presence of inhibitors of the H^+ pump such as dinitrophenol [6].

Methods

Experiments were performed on gastric mucusae of *Rana pipiens* by an in vitro method in which the mucosae are mounted between a pair of cylindrical chambers [7]. All experiments were started with physiological solutions on both sides of the mucosa, the compositions of which were as follows. The standard Cl^- nutrient (or serosal) solution contained (in mM): Na^+, 102; K^+, 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 standard Cl^- secretory (or mucosal) solution: Na^+, 102; K^+, 4; and Cl^-, 106. In Cl^--free (SO_4^{2-}) experiments, the standard SO_4^{2-} nutrient solution contained (in mM): Na^+, 101; K^+, 4; Ca^{2+}, 1; Mg^{2+}, 0.8; SO_4^{2-}, 41.3; HCO_3^-, 25; phosphate, 1; glucose, 10; and sucrose, 40; and the standard SO_4^{2-} secretory solution contained (in mM): Na^+, 100; K^+, 4; SO_4^{2-}, 52; and sucrose, 64. The transmembrane resistance, the transmembrane potential difference (p.d.) and the H^+ secretion rate were recorded. Two pairs of electrodes were used, one for sending current across the mucosa and the other for measuring the p.d. The p.d. was taken as positive when the nutrient side was positive relative to the secretory side of the frog gastric mucosa. The resistance was determined as the change in p.d. per unit of applied current. Current (10—20 $\mu A \cdot cm^{-2}$) was applied for 1 or 2 s, in one direction and, 2 or 3 s later, in the other direction. No significant rectification was observed. The H^+ secretion rate was measured by the pH stat method introduced by Durbin and Heinz [8]. The pH of the secretory solution was maintained generally between 4.6 and 5.0. Both sides of the mucosa were gassed with a mixture of 95% O_2/5% CO_2. Histamine was added to the nutrient solution to a concentration of 10^{-4} M.

Experiments were performed with 10^{-3} M ouabain in the Cl^- and Cl^--free nutrient solutions for maximal effects. As mentioned in the introduction, the possible effect of ouabain on the H^+ pump was tested by the technique of voltage clamping.

Effect of ouabain in the presence of Na and Cl. Fig. 1 shows the effect of 10^{-3} M ouabain in the nutrient solution in an experiment with standard Cl^- solutions on both sides of the frog gastric mucosa. The resistance did not change appreciably after addition of ouabain. The p.d. fell from about 25 mV to about 15 mV in 10 min and to about 12 mV in 40 min. The H^+ secretion rate increased slightly following ouabain addition. Table I shows data from nine experiments performed as that presented in Fig. 1. Data were obtained up to 60 min after addition of ouabain in seven out of the nine experiments. The p.d. decreased significantly ($P < 0.01$), by 9.5 mV in 30 min and by 14.6 mV in 60 min, from the control level. The H^+ secretion rate and the resistance did not change significantly during a 1 h period.

Effect of ouabain in SO_4^{2-} (Cl^--free) media. Fig. 2 shows the effect of 10^{-3} M ouabain in an experiment in Cl^--free (SO_4^{2-}) solutions. Ouabain did not affect the H^+ secretion rate in this experiment but it induced a biphasic effect on the p.d. Immediately following ouabain addition, the p.d. changed from about −28 to a nadir of about −38 mV, at which point the p.d. began to change towards zero, reaching a value of −23 mV in about 70 min.

Table II gives data from seven experiments similar to that shown in Fig. 2. The mean change in p.d. to the nadir after the addition of ouabain to the nutrient solution was 9.4 mV from −28.0 to −37.4 mV ($P < 0.01$). No significant change occurred in resistance or H^+ secretion rate during this period.

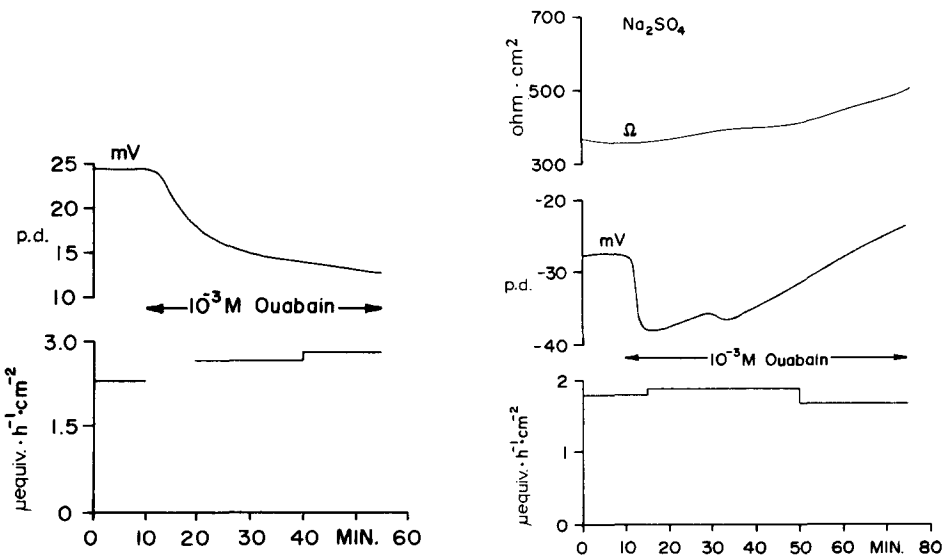

Fig. 1. Effect of ouabain in Cl^- solutions. Ouabain added at time 10 min. Top line, transmucosal potential in mV. Bottom line, H^+ secretion rate.

Fig. 2. Effect of ouabain in SO_4^{2-} solutions. Ouabain added at time 10 min. Top line, transmucosal resistance in ohm · cm^2. Middle and bottom lines are the p.d. and H^+ secretion rate as in Fig. 1.

TABLE I

EFFECT OF OUABAIN IN Cl^- SOLUTIONS

The transmucosal resistance, transmucosal potential difference, and H^+ secretion rate were measured. Values are means ± standard deviations. *P* values are obtained from paired data by the Student's *t*-test. Columns labeled *R*, p.d. and $\dot{H}$ refer, respectively, to the control values of resistance, transmembrane potential difference and H^+ secretion rate. Columns labeled ΔR, Δp.d. and $\Delta\dot{H}$ refer to changes in these three parameters 30 min and 60 min after the addition of ouabain to the nutrient solution. The first set of values refers to results obtained in nine experiments 30 min after addition of ouabain, the second set to seven experiments 60 min after addition.

R (ohm · cm²)	Δ*R* (ohm · cm²)	p.d. (mV)	Δp.d. (mV)	$\dot{H}$ (μequiv. · h^{-1} · cm^{-2})	$\Delta\dot{H}$ (μequiv. · h^{-1} · cm^{-2})
133 ± 45	−12 ± 18 ($P > 0.05$)	25.6 ± 6.8	−9.5 ± 5.3 ($P < 0.01$)	3.98 ± 0.72	0.17 ± 0.40 ($P > 0.20$)
132 ± 52	−12 ± 22 ($P > 0.10$)	25.5 ± 7.6	−14.6 ± 6.6 ($P < 0.01$)	3.97 ± 0.82	0.12 ± 0.31 ($P > 0.20$)

TABLE II

EFFECT OF OUABAIN IN Cl^--FREE SOLUTIONS

The transmembrane resistance, transmembrane potential difference and H^+ secretion rate were measured. Details are as in Table I except that the first set of values refers to results obtained at the nadir, 7 min after ouabain addition, for seven experiments.

R (ohm · cm²)	Δ*R* (ohm · cm²)	p.d. (mV)	Δp.d. (mV)	$\dot{H}$ (μequiv. · h^{-1} · cm^{-2})	$\Delta\dot{H}$ (μequiv. · h^{-1} · cm^{-2})
376 ± 164	27 ± 42 ($P > 0.10$)	−28.0 ± 5.3	−9.4 ± 3.3 ($P < 0.01$)	1.62 ± 1.00	0.17 ± 0.29 ($P > 0.20$)
376 ± 164	124 ± 36 ($P < 0.01$)	−28.0 ± 5.3	11.6 ± 9.3 ($P < 0.02$)	1.62 ± 1.00	−0.73 ± 0.52 ($P < 0.01$)

The mean time for the nadir was 7 min, and the mean half-time was 3 min. The initial change in p.d. following ouabain addition occurred after about 1 min. 60 min after ouabain addition the resistance increased by 124 from 376 ohm · cm^2 ($P < 0.01$); the p.d. changed by 11.6 from −28 to −16.4 mV ($P < 0.02$); and the H^+ secretion rate decreased by 0.73 from 1.62 μequiv. · h^{-1} · cm^{-2} ($P < 0.01$).

Effect of ouabain in SO_4^{2-} media during voltage clamp. In order to see whether the decrease in H^+ secretion observed following ouabain addition was due to an effect on the H^+ transport mechanism, the effect of voltage clamping at 0 and 40 mV was determined before and after the addition of ouabain.

During the voltage clamp period, the external current necessary to maintain the voltage clamp was recorded. Periodically, the external circuit was opened briefly in order to record the open-circuit p.d. and the resistance * during the voltage clamping period was obtained from the change in p.d. divided by the

* $R_{vc} = (|p.d._{vc} - p.d._{oc}|/I_{vc}) - R_s$, where R_{vc} = the resistance during voltage clamp; $p.d._{vc}$ = voltage clamp p.d.; $p.d._{oc}$ = open circuit p.d.; I_{vc} = external current to maintain voltage clamp, and R_s = solution resistance.

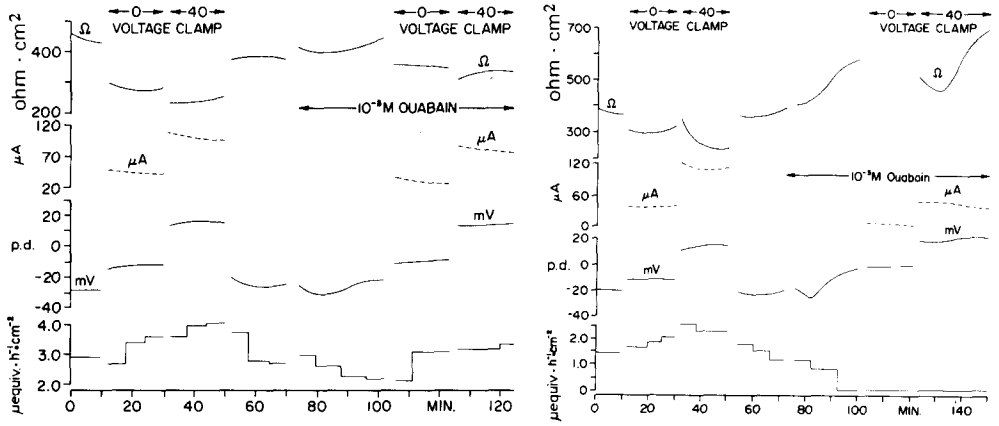

Fig. 3. Effect of voltage clamp and ouabain in SO_4^{2-} solutions with mild response to ouabain. Each half has three periods: open circuit, 0 mV voltage clamp and 40 mV voltage clamp. Top panel, transmucosal resistance in ohm/cm^2. Second panel, current necessary to keep at 0 and 40 mV, nutrient positive in μA. To obtain $\mu A \cdot cm^{-2}$, divide by 1.3. Third panel, transmucosal open circuit p.d. in mV. Bottom panel, H^+ secretion rate in μequiv. $\cdot h^{-1} \cdot cm^{-2}$. Left half, control; right half, plus ouabain.

Fig. 4. Effect of voltage clamp and ouabain in SO_4^{2-} solutions with strong response to ouabain. All parameters are as in Fig. 3.

current. For our purpose, clamping the voltage between the electrodes at 0 or 40 mV (nutrient-positive) was adequate, that is, we did not attempt to clamp the voltage across the tissue at these levels.

Fig. 3 presents data from an experiment with ouabain and voltage clamp. Before the addition of ouabain, 45 μA were necessary to clamp the voltage at 0 mV. During this period the H^+ secretion rate increased from 2.8 during the open-circuit period to about 3.5 μequiv. $\cdot h^{-1} \cdot cm^{-2}$. The open-circuit p.d. changed from —28 mV during the open-circuit period to about —10 mV during the zero voltage clamp period. The zero voltage clamp resistance was about 300 ohm $\cdot$ cm^2, compared to about 450 ohm $\cdot$ cm^2 during the preceding open-circuit period.

About 115 μA were needed to clamp the voltage at 40 mV. In this case, the H^+ secretion rate further increased and the open-circuit p.d. increased to about 18 mV. Upon return to the open-circuit condition, the H^+ secretion rate, the p.d. and the resistance returned toward the control (pre-voltage clamp) levels.

Addition of ouabain (10^{-3} M) to the nutrient solution resulted in the typical biphasic change in p.d., that is, a decrease (more negative) followed by an increase. (Note: The scale in Fig. 3 is much more compressed than that in Fig. 2.) The resistance increased and the H^+ secretion rate decreased by less than 20%. (Note: H^+ scale does not go to zero.) In this experiment, voltage clamp in the presence of ouabain resulted in similar changes on H^+ secretion rate, open-circuit p.d. and resistance as those observed in the absence of ouabain.

Fig. 4 shows data from one experiment performed similarly to the experiment presented in Fig. 3. The effects of voltage clamping during the control

period (before ouabain) were similar in the two experiments. The effects of ouabain and voltage clamping were quite different with respect to the H^+ secretion rate. Addition of ouabain completely abolished the H^+ secretion rate and it remained at zero during voltage clamping. The changes in open-circuit p.d. following ouabain and during voltage clamping were similar in the experiments of Figs. 3 and 4. The open-circuit resistance increased to a greater extent, following ouabain (to about 600 ohm · cm^2), in Fig. 4 than in Fig. 3. The resistance could not be calculated in the experiment of Fig. 4 during the 0 mV voltage clamp because in this particular experiment the current and change in p.d. were very small.

Figs. 3 and 4 are representative of the different types of responses that we observed after ouabain addition, that is, not all mucosae responded similarly to ouabain. Of nine experiments, three showed a marked inhibition, as depicted in Fig. 4, three others showed a relatively small inhibition, as depicted in Fig. 3, and the remaining three showed a moderate inhibition.

Effect of ouabain in SO_4^{2-} media in the presence of cimetidine. When H^+ secretion is inhibited in SO_4^{2-} media, the p.d. becomes positive [6,9]. A study of the factors which may contribute to this positive electromotive force in SO_4^{2-} solutions using cimetidine as an inhibitor of H^+ secretion has been the subject of a recent paper from our laboratory [10]. Although Na^+ transport

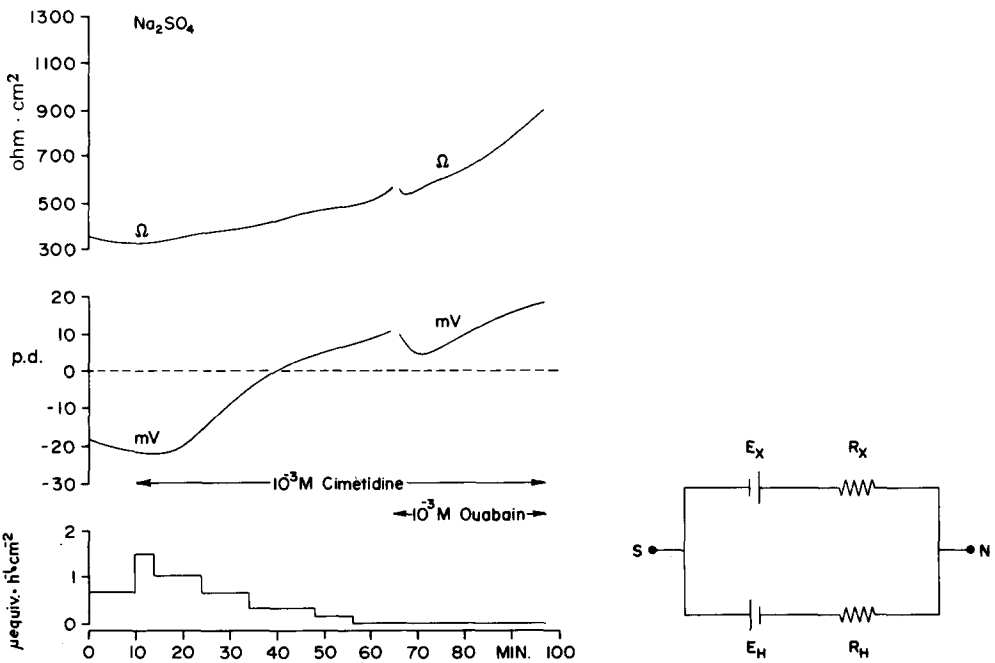

Fig. 5. Effect of cimetidine and ouabain in SO_4^{2-} solutions. Cimetidine added at 10 min and ouabain at 65 min to nutrient solution. The curves are as in Fig. 2.

Fig. 6. Electrogenic H^+ transport in SO_4^{2-} solutions. S, secretory solution. N, nutrient solution. E_H, H^+ electromotive force. R_H, H^+ pathway resistance. E_X, parallel pathway electromotive force. R_X, parallel pathway resistance.

from secretory to nutrient was found to be a possible factor contributing to the positive electromotive force, it was ruled out as the exclusive factor since removal of Na^+ (replaced with choline) did not abolish the positive p.d.

In the context of our present work with the H^+ pump inhibited by cimetidine, it became important to know to what extent the $(Na^+ + K^+)$-ATPase pump may contribute to the positive p.d. in Cl^--free media. Fig. 5 presents data from one such experiment. Addition of 10^{-3} M cimetidine to the nutrient solution resulted in an increase in p.d. from about −20 to 10 mV. The H^+ secretion rate decreased to zero and the resistance increased.

Addition of ouabain to the nutrient solution to a concentration of 10^{-3} M resulted in a decrease in p.d. from 10 to about 4 mV. As in the case of the secreting stomach in SO_4^{2-} media, the p.d. reached a nadir and then increased to values above the pre-ouabain levels. Seven experiments were performed similarly to that shown in Fig. 5 and the biphasic response was observed in all of them. The p.d. remained substantially positive until the end of the experiments.

Discussion

The fact that ouabain produces a rapid decrease in p.d. in the frog mucosa in both Cl^- and SO_4^{2-} media is evidence for the electrogenicity of the $(Na^+ + K^+)$-ATPase pump. Post and Jolly [11] found an $Na^+ : K^+$ ratio of 3 : 2 for the ATPase of the erythrocyte, which supports the concept of electrogenicity. The same ratio was observed by Thomas [12] in the snail neurone. Furthermore, Thomas found that injection of Na^+ but not K^+ or Li^+ into a resting nerve resulted in hyperpolarization of the cell. This hyperpolarization could be blocked by ouabain and by removal of K^+ from the exterior of the cell. The latter is strong evidence for the electrogenicity of the pump. If the $(Na^+ + K^+)$-ATPase pump of the gastric mucosa has an $Na^+ : K^+$ coupling ratio of 3 : 2 or any coupling ratio greater than 1, it would be electrogenic, tending to make the nutrient positive. Inhibition of the pump should result in a decrease in p.d.

As pointed out above, the initial rapid decrease in p.d. due to ouabain would be expected on the basis of an electrogenic pump. However, the subsequent slow decline in Cl^- solutions is undoubtedly due to an indirect effect of the pump on the ionic composition of the cell. The fact that the p.d. remained positive with a normal H^+ secretion rate 60 min after ouabain addition to Cl^- solutions suggests that aside from the ATPase pump there is at least one other mechanism giving rise to a positive p.d.

Previously, a positive p.d. was found in SO_4^{2-} solutions when H^+ secretion was inhibited with cimetidine [10]. This p.d. was partly attributed to active transport of Na^+. In the absence of Na^+, a residual positive p.d. persisted which could be due to active anion transport such as HCO_3^- and/or SO_4^{2-} from nutrient to secretory. As shown in Fig. 5, ouabain did not abolish the positive p.d. induced by cimetidine in SO_4^{2-} media. This finding is further evidence for a mechanism other than the $(Na^+ + K^+)$-ATPase pump to account for the positive p.d. in Cl^--free media.

The positive p.d. observed in Cl^- solutions 60 min after ouabain addition may be due to an active transport mechanism for Cl^- and/or to the persistence

of ion gradients between the cell and the media due to the pre-ouabain activity of the (Na^+, K^+) pump. In this situation, high H^+ rates were observed and, therefore, it is quite reasonable to assume that the Cl^- rate approximates the H^+ secretion rate. Consequently, Cl^- was actively transported since it was moving against its electrochemical gradient. Possibilities for active Cl^- transport include a neutral HCl pump, an electrogenic Cl^- pump or a Cl^--HCO_3^- exchange mechanism. This latter mechanism, which would be located in the nutrient membrane, would derive its energy from the HCO_3^- gradient between the cell and the nutrient fluid. Of these three mechanisms the latter two could account for the positive p.d., provided that the coupling ratio of the Cl^--HCO_3^- pump does not equal unity.

Effect of ouabain on H^+ secretion. Inhibition of H^+ secretion by ouabain in the frog stomach has been reported previously in Cl^- solutions [3,4]. Although both authors suggested that the effect is due to a change in the ionic composition of the cell, one of them [4] attributed the effect, in part, to a direct inhibition of Cl^- transport by ouabain. In the present studies, ouabain did not have a noticeable effect, in Cl^- solutions, on the H^+ secretion rate but gave a significant decrease in the H^+ rate in SO_4^{2-} (Cl^--free) solutions. The inhibition of H^+ secretion in SO_4^{2-} media obviously cannot result from an inhibition of Cl^- transport.

We cannot give a clear explanation of the difference between our results and those of Davenport [3] and Cooperstein [4] in Cl^- media. The difference may arise from differences in methods. For example, Davenport used a sac technique under hyperbaric conditions, presumably without exogenous CO_2. Moreover, under these conditions, ouabain failed to inhibit when the K^+ concentration in the bathing solution was markedly elevated. The difference between our results and Cooperstein's may simply be due to the fact that the latter results are obtained 3—5 h after the addition of ouabain. Of significance is the fact that the H^+ secretion rate was not measured directly but calculated from the short-circuit current and the net Cl^- flux.

There is good evidence in favor of H^+ secretion by an electrogenic pump in SO_4^{2-} solutions [6,9]. A scheme of this theory is presented in Fig. 6. The H^+ mechanism is represented by the H^+ limb, including an electromotive force, E_H, and a resistance, R_H. The H^+ current through this pathway is electrically coupled to the parallel pathway comprising a resistance R_x and an electromotive force E_x for all other ions. The H^+ secretion rate could be decreased either by a direct effect on the H^+ pathway or indirectly by inhibiting the movement of ions through the parallel pathway. If the inhibitory effect of ouabain took place on the parallel pathway, an alternate external parallel pathway could be provided by voltage clamping, in which case the H^+ secretion rate would be essentially the same in the absence and in the presence of ouabain. Voltage clamping would give a lower H^+ secretion rate in the presence of ouabain compared to the control if ouabain affected the H^+ pathway.

Although ouabain inhibited H^+ secretion in SO_4^{2-} solutions, the effect varied from a minimal change to a complete abolition of secretion. In Fig. 3 there was a relatively small decrease in the H^+ secretion rate and voltage clamping increased the rate approximately to the controls. In Fig. 4, there was a marked

decrease in H^+ secretion rate, and voltage clamping at 0 and 40 mV failed to increase the rate. In the latter experiments, comprising three experiments out of nine, it is clear that ouabain affected the H^+ transport mechanism per se.

Acknowledgements

This work was supported in part by National Institutes of Health Grant EY03161 and by National Science Foundation Grant PCM-7828018. Cimetidine was a gift from John G. Paul of Smith, Kline and French Laboratories. We wish to thank Jeanne E. Willhite, Goldie Miller and Chung-Yuan Chu for excellent technical assistance.

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