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Effect of Calcium on the H^+/K^+ ATPase of Hog Gastric Microsomes

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Summary. The K^+-stimulated, ouabain-insensitive ATPase activity present in vesicles of microsomal fractions from hog gastric mucosa can be demonstrated in fresh preparations by adding Ca^{2+} (μM range) to the incubation medium. Ca^{2+} effect is similar but not additive to the effect of gramicidin or freezing. High Ca^{2+} concentrations (1 mM) produce an inhibitory effect on the K^+-stimulated ATPase activity. This effect is not seen in the presence of gramicidin. Calcium increases the magnitude of ATP-driven H^+ uptake in vesicles exposed to K^+ for periods of time up to 60 min. At longer times of exposure (120 min) the response does not differ from controls. It is concluded that Ca^{2+} at low concentrations (μM range) enhances the K^+ permeability of the vesicular membrane. At higher concentrations (mM range), Ca^{2+} becomes inhibitory to the K^+ permeability. A role for Ca^{2+} as a second messenger in stimulus-secretion coupling in the parietal cell is discussed.

The role of ATP as the energy source for gastric acid secretion is still controversial. The presence of the well-defined Na^+-K^+ ATPase in tissues that carry out Na^+ and K^+ transport, is in itself evidence for the involvement of ATP in those tissues. In stomach, the primary observation of an anion stimulated, SCN^- inhibited ATPase suggested a possible involvement of a similar mechanism in the process of HCl secretion [18]. However, the contribution of this enzyme may be taken as doubtful since it might be from mitochondrial origin. A very similar ATPase has been found in mitochondrial fractions from the gastric mucosa [6, 36, 39] and other tissues [29].

Recently a new kind of ATPase has been described in microsomes of bullfrog gastric mucosa, which is a K^+-stimulated, ouabain-insensitive, Mg^{2+}-dependent ATPase [9, 10]. An enzyme with similar characteristics has also been described in gastric microsomes from some mammalian species [7, 20, 34]. Moreover, the ability of these mammalian preparations to perform vectorial ATP-driven H^+ transport under the proper condi-

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tions [20], strongly suggests the participation of this microsomal enzyme in the process of acid secretion.

The rates of ATP hydrolysis and H^+ uptake by mammalian gastric microsomal vesicles are in a way controlled by the permeability of the vesicular membrane to K^+. This ATPase system may be responsible for the translocation of H^+ across the tubulo-vesicular and/or apical membrane of the oxyntic or parietal cell. If this is the case, a mechanism should be provided by which an inactive ATPase could turn into an active one as a result of hormonal stimulation. In other words, there should be a coupling between stimulus and secretion. One way of coupling could be by controlling the permeability of the tubulo-vesicular and/or apical membrane to potassium. An increase in K^+ permeability brings about an increase in ATPase activity and H^+ uptake in microsomal vesicles [10, 35]. If the tubulo-vesicular system and the apical plasma membrane are the source of the microsomal fraction this could be a mechanism by which the rate of acid secretion could be controlled in the intact cell.

The permeability of microsomal vesicle membranes to potassium can be increased by different agents or procedures (ionophores and freezing-thawing, for example); however, one should be able to find a more physiological agent. Calcium ion has been shown to increase potassium permeability in other systems such as the red blood cell [21, 31], liver cell [41] and nerve membranes [1]. On the other hand, calcium has been shown to act as a second messenger in mediating stimulus-secretion coupling in a variety of systems [32], some of which involve solute and water transport [2, 30, 37]. In addition, Ca^{++} has been shown to be essential for acid secretion [8, 12, 14, 16, 17].

Trying to elucidate the molecular mechanism for the secretion of H^+, we report here the effects of calcium on the H^+/K^+ ATPase system of hog gastric microsomes. The results are interpreted on the basis of a role for calcium as a cellular messenger in mediating stimulus secretion coupling in the parietal cell.

Materials and Methods

Preparation of Microsomal Fractions

Empty hog stomachs from freshly killed animals, were obtained from a local slaughter-house. The antral and the cardiac regions were cut away and the mucosa removed from the outer muscle by scalpel dissection. The mucosal cells were scraped off the underlying connective tissue and each gram of tissue was immediately homogenized (5 strokes at

2000 rpm) at 0 °C in 5 ml of a solution of 0.25 M sucrose buffered with 10 mM Tris-HCl at pH 7.4, in a Teflon glass homogenizer. The homogenate was spun at $500 \times g$ for 10 min, the supernatant spun at $20{,}000 \times g$ for 20 min, and the last supernatant spun at $100{,}000 \times g$ for 120 min. The pellet from the last centrifugation was resuspended in enough quantity of the sucrose-Tris solution to give about 5–8 mg of protein/ml of final suspension. The material so prepared was kept in the refrigerator (fresh preparation) or in the freezer (frozen preparation).

Assay of the ATPase Activity

20 µl of the microsomal suspension were generally preincubated at 37 °C for 5 min in the presence of (final concentrations): 50 to 150 mM Tris-HCl buffer (pH 6.9); 5 mM $MgCl_2$ and, according to the experimental design, requisite amounts of NaCl, KCl, other salts, and 1 mM ouabain. The reaction was started by adding to the medium Na-free Tris-ATP (2 mM final concentration). The final reaction volume was 1 ml. After 10 min, the incubation was terminated by the addition of 1 ml of ice cold 5% $HClO_4$ to the incubation tubes. The samples were chilled and centrifuged. The liberated phosphate was determined in the deproteinized solution [19]. All samples were run in triplicate or quadruplicate, and each experiment carried out at least twice with different preparations. ATPase activity is expressed as nmoles of phosphate produced per mg of protein per min after substraction of a blank run in parallel without the 20 µl of microsomal suspension, which were added only after the addition of the $HClO_4$. Preliminary experiments demonstrated that, under our incubation conditions, a linear relationship existed between the liberation of phosphate and the time of incubation. The protein content of the original suspension was measured by means of Folin reagent [22].

ATP-Driven H^+ Uptake

ATP-hydrolysis driven H^+ uptake measured by the ATP pulse method of Mitchell and Moyle [25] in fresh microsomal fractions. A glass reaction cell with magnetic stirring, with a final volume of 4 ml, was used. The concentration of microsomes in the reaction cell was of 0.6–1.0 mg of microsomal protein per ml. The basic reaction medium contained 200 mM sucrose, 5 mM $MgCl_2$, and 5 mM glycylglycine. Depending on the experiment, varying concentrations of KCl, ranging from 0 to 100 mM were also present. ATP was added as aliquots of a solution containing 10 mM Na_2-ATP and 10 mM $MgCl_2$ (Mg-ATP solution). The pH of this solution was adjusted to produce no change in pH in the incubation medium without microsomes. H^+ uptake was measured as changes in outer pH. These measurements were made at pH 6.1–6.13 at 25 °C. In this range of pH, no net acid is produced in the hydrolysis of ATP [40]. A combined glass/calomel electrode (Radiometer GK2321C) and a sensitive pH meter (Radiometer PHM 64) coupled to a recorder were used for the measurements.

Ouabain (Strophanthin-G), gramicidin, olygomycin, Tris, ATP and glycilglycine were purchased from the Sigma Chemical Company, St. Louis, Mo. All other reagents were from E. Merck AG., Darmstadt, Germany.

Results

ATP-Hydrolyzing Activity

In preliminary experiments the effect of gramicidin and freezing-thawing on the K^+-ATPase activity reported by other authors [10, 20,

Table 1. Effect of 10 μM Ca^{2+} on the ATPase activity of fresh preparations

Medium	ATP hydrolyzed (nmoles/mg prot. min)	ΔK
Mg^{2+}	256	–
$Mg^{2+}+K^+$	269	+13
$Mg^{2+}+Ca^{2+}$	252	–
$Mg^{2+}+Ca^{2+}+K^+$	467	+215
Ca^{2+}	62	–
$Ca^{2+}+K^+$	70	+8

The following procedure was used: 20 μl of microsomal preparations (containing about 5-8 mg prot/ml) were added to 1 ml of the incubation medium containing as final concentration (in mM): Tris-HCl (pH 6.9) from 150 to 50 (according to the experiment) to maintain the ionic strength; $MgCl_2$, 5; ATP-Tris, 2; KCL, 5; Ouabain, 1; Ca^{2+}, when added, 10 μM. After 5 min preincubation at 37 °C, the reaction was started by adding the ATP to the medium, and it was stopped after 10 min by addition of 1 ml of ice cold 6% perchloric acid to the incubation. The samples were chilled, centrifuged, and assayed for the presence of P_i.

34] were confirmed in the present preparation. Gramicidin and freezing-thawing largely increased the K^+-stimulated component of the ATPase, presumably by virtue of their effect on the K^+-permeability of the vesicular membrane.

The effect of Ca^{2+} on the ATPase system is shown in Table 1: There is a basal, Mg^{2+}-dependent ATPase activity, which is slightly stimulated by the presence of K^+. The presence of 10 μM Ca^{2+} produces an important increment of the K^+-stimulated activity without any effect on the Mg^{2+}-dependent component. We can also see that the K^+-stimulated ATPase is Mg^{2+} but not Ca^{2+} dependent since K^+ was not able to activate it in the presence of Ca^{2+} alone. On the other hand, Ca^{2+} (in the tested concentration) showed to be a poor substitute for the basal Mg^{2+}-dependent ATPase.

This stimulatory effect of Ca^{2+} could be due either to a direct effect on the K^+-ATPase system or to an effect on the K^+ permeability of the vesicles in a manner similar to the already shown effect of gramicidin and freezing-thawing. The experiments to be described indicate that the effect is rather on the K^+ permeability.

Figure 1 shows the effect of increasing K^+ concentrations on the ATPase activity of microsomal preparations in different conditions. The fresh preparation shows a maximal activity at 100 mM external K^+

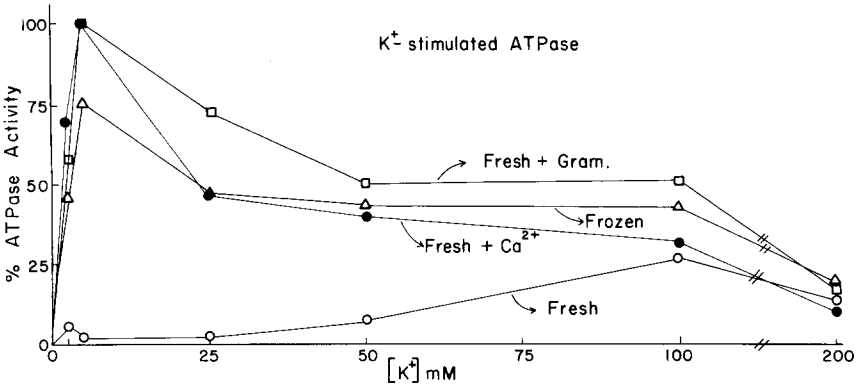

Fig. 1. Effect of increasing K^+ concentration (as KCl) on the Mg^{2+}-dependent ATPase activity of frozen and fresh microsomal preparations, with or without 10 μg/ml gramicidin or 10 μM Ca^{2+}. In the experiments summarized in this and in the following figures the reaction was carried out as indicated in *Methods*. Values are expressed as percentage of the maximal activity. Each point is based upon 4 replicates

in a diffusion kinetics pattern. Thus it appears that K^+ at this concentration permeates the vesicles pushed by the gradient force. However, this activity only represents about 30% of the maximal activity reached under the other conditions. The addition of Ca^{2+} or gramicidin to the fresh preparation or prefreezing-thawing of the vesicles induces a large increase of the K^+-ATPase activity and causes a shift in the optimal K^+ concentration in the stimulation of the enzyme. It can also be seen that maximal activity is of about the same magnitude, and it is reached at 5 mM K^+ in all the above mentioned conditions.

The kinetics of activation of the microsomal ATPase by K^+ in the low range concentrations in the presence of 10 μM Ca^{2+} or gramicidin in the fresh preparation is shown in Fig. 2. A saturation type of kinetics is observed with concentrations up to 5 mM. The values obtained for V_{max} and K_a for K^+ activation of the enzyme are identical in both conditions. It indicates that Ca^{2+} does not change the affinity of the ATPase for potassium.

The effect of different added Ca^{2+} concentrations on the K^+-ATPase activity is shown in Fig. 3. K^+-ATPase activity increases with increasing Ca^{2+} concentrations up to 10 μM in a saturation kinetics pattern. The apparent K_a for Ca^{2+} activation was calculated to be on the order of 0.4 μM. This effect of Ca^{2+} is not additive to the already shown effect of gramicidin, as we can see in this figure. Similarly, we cannot see any effect of Ca^{2+} in the already K^+ permeable frozen preparations either. On the other hand, it is interesting to point out that higher concentrations

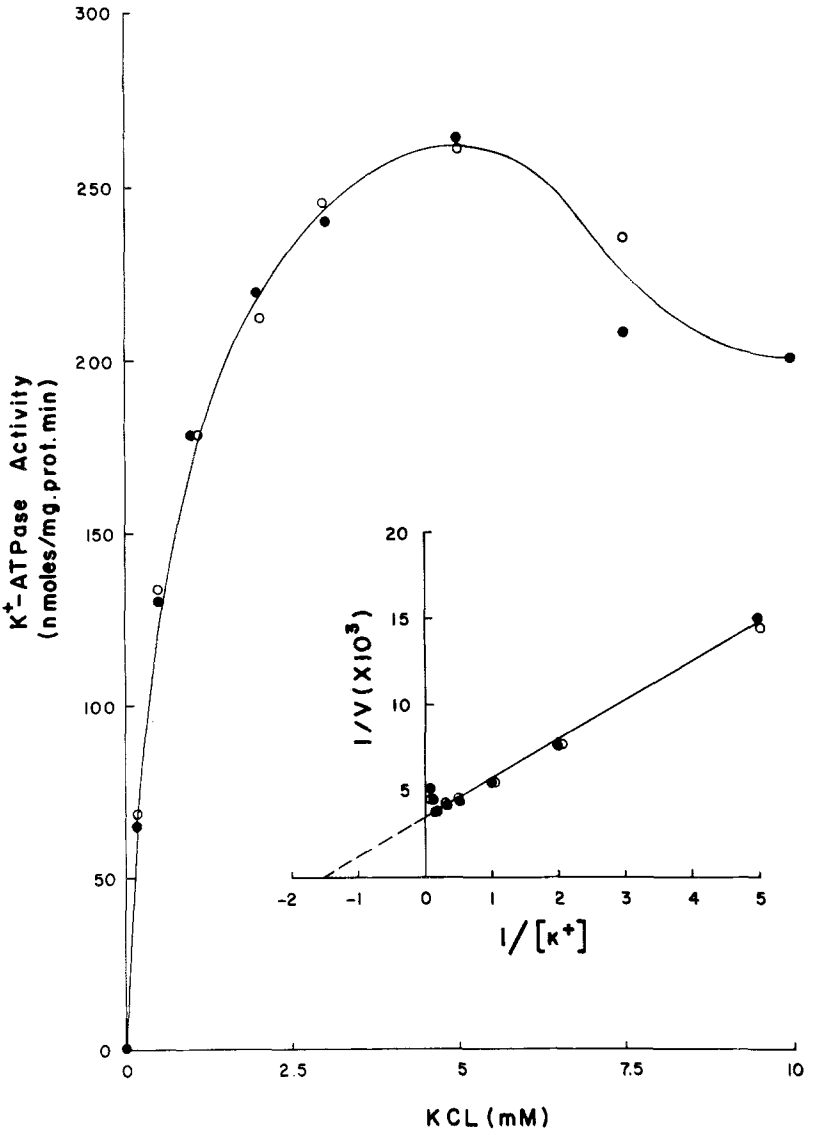

Fig. 2. Kinetics of activation of the Mg^{2+} dependent microsomal ATPase by K^+ in the fresh preparation in the presence of 10 μM Ca^{2+} (o—o) or 10 μg/ml gramicidin (●—●). The activities correspond to the difference between the total ATPase ($Mg^{2+}+K^+$) minus the basal ATPase (Mg^{2+} alone). Each point is based on four replicates. Points corresponding to 7.5 and 10 mM K^+ are not taken in account for calculations. Lineweaver-Burk plot for the calculation of K_a shown in the insert

of Ca^{2+} (above 100 μM) appear to inhibit the K^+-stimulated component of the ATPase. This inhibition may be due to an effect at these concentrations on the K^+ permeability, since it is very much reduced in the presence of the K^+ ionophore.

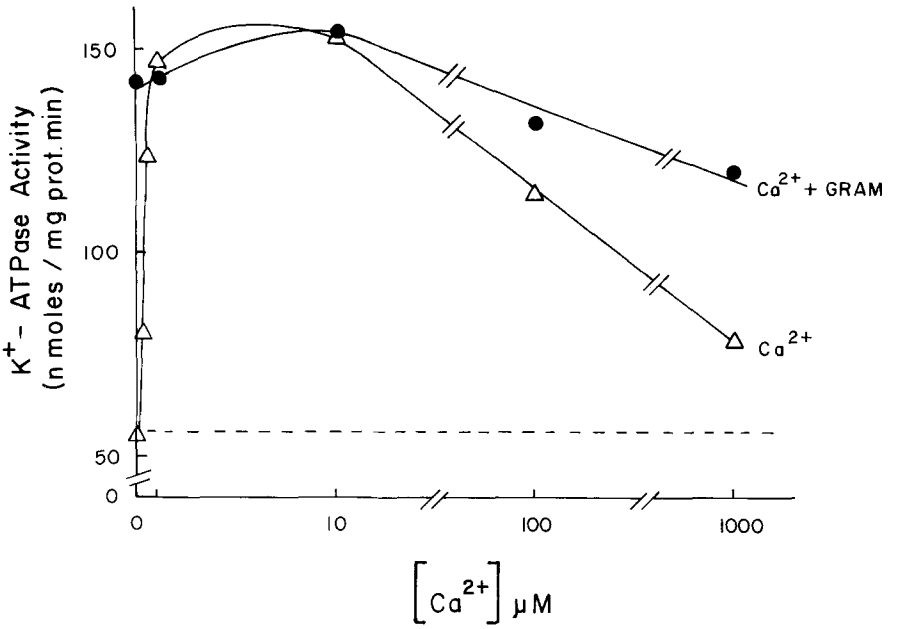

Fig. 3. Effect of increasing Ca^{2+} concentration on the K^+-ATPase activity of fresh preparations in the presence or absence of gramicidin. K^+ concentration, 5 mM. Each point is based upon four replicates

ATP-Driven H^+ Uptake

The effect of a 100 nmoles pulse of ATP on H^+ uptake (ΔpH_o) by fresh microsomal vesicles is shown in Fig. 4. Trace *a* shows the effect of ATP in a OK^+ medium; trace *b* shows the effect in control conditions when K^+ was present in the medium; trace *c* shows the effect when Ca^{2+} was present together with K^+. In the present experiment, Ca^{2+} was added 5 min prior to the addition of 100 mM KCl. Potassium was present for 15 min prior to the addition of ATP. In the absence of K^+, ATP did not have any effect on medium pH (trace *a*). Addition of ATP in the presence of K^+ causes a prompt change in pH_o (medium becomes alkaline) that reaches a maximum in about 20–30 sec with a slow decay thereafter. In the presence of Ca^{2+} a larger change in medium pH (H^+ uptake) is obtained with the same amount of ATP. Similar results were obtained when 5 μg/ml oligomycin were present in the above experiment.

The results obtained in ATPase assays described above indicated that the major effect of calcium was that of increasing potassium permeability of the vesicular membrane. To confirm these findings and

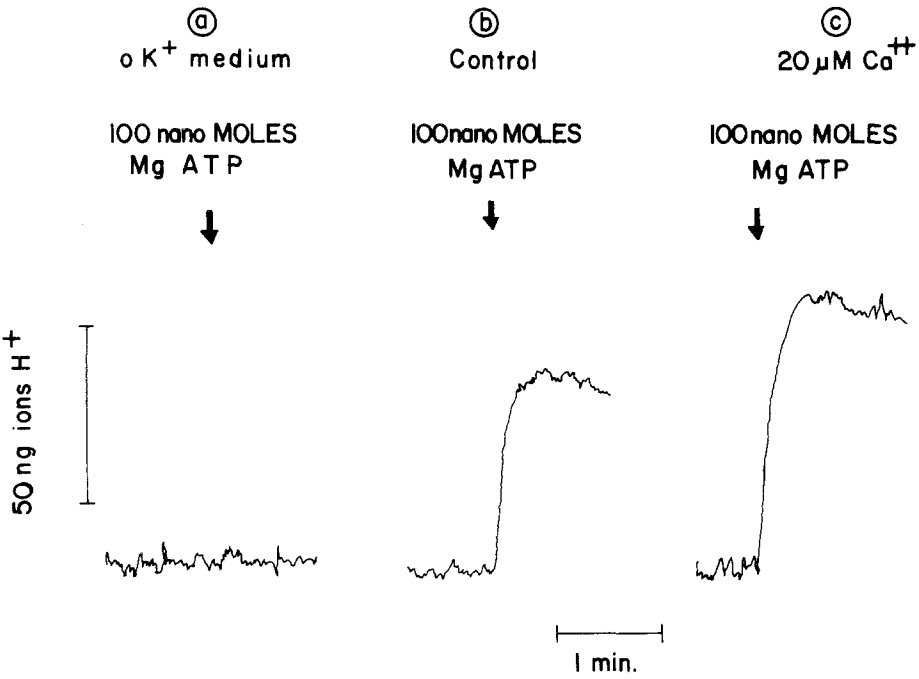

Fig. 4. Effect of a 100 nmoles pulse of ATP upon H^+ uptake by fresh microsomal vesicles (*a*) in the absence of K^+, (*b*) in the presence of 100 mM KCl, and (*c*) in the presence of 100 mM KCl and 20 μM Ca^{2+}. Calcium in experiment *c* was added 5 min prior to the addition of KCl. In *b* and *c*, KCl was added 15 min before the ATP pulse

to study the effects of calcium on H^+ transport itself, the effects of different concentrations of K^+ and the time of exposure to this ion on H^+ uptake by microsomal vesicles were studied in the presence and absence of calcium. These results are presented in Fig. 5. The magnitude of ATP-driven H^+ uptake is dependent on the concentration of potassium and the time the vesicles have been exposed to this ion prior to the induction of transport by ATP. Ca^{2+} reduced the time of exposure to K^+ required to obtain maximal H^+ uptake. The effect of calcium was better observed at higher concentrations of K^+ (100 mM) and at shorter times of exposure, before equilibration in the control aliquot takes place. The response at longer times of exposure (120 min) does not differ in the presence or absence of calcium, indicating that this ion has no effect on the transport mechanism *per se*, but rather on potassium permeability.

This reduction in the time of exposure to K^+ necessary for maximal

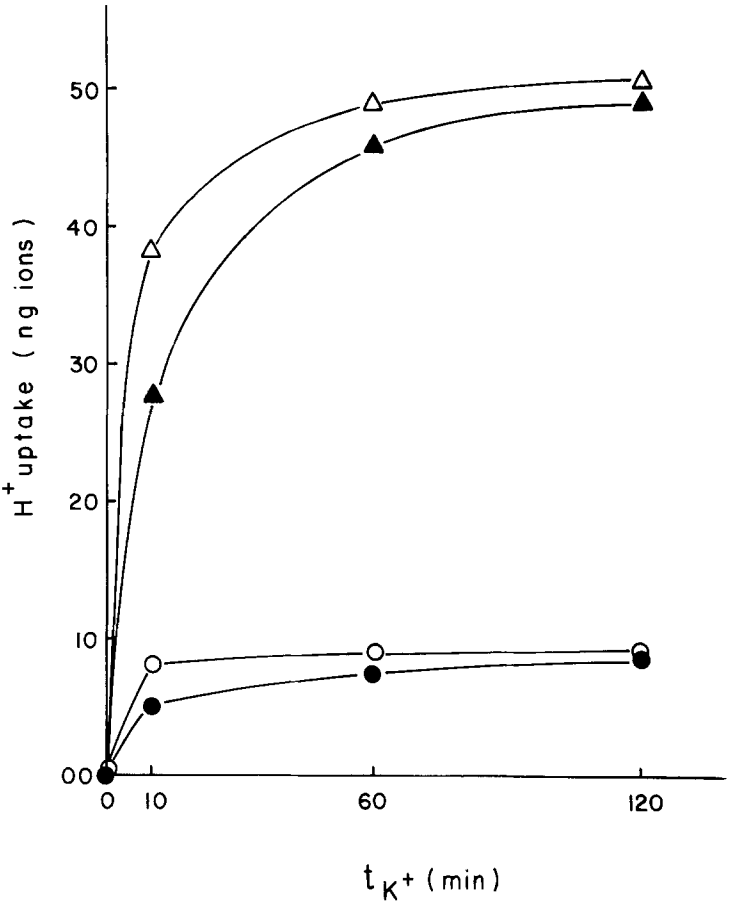

Fig. 5. Effect of time of preincubation with different concentrations of KCl on ATP-driven H^+ uptake by fresh microsomal vesicles with (△ ○) or without (▲ ●) the addition of 10 μM Ca^{2+}. Aliquots from the same preparations were preincubated with 5 (circles) or 100 mM KCl (triangles) and for different lengths of time prior to the addition of a-100 nmoles pulse of ATP

H^+ uptake is similarly observed when the experiment is done with frozen-thawed vesicles with no Ca^{2+} in the medium. This is shown in Fig. 6. In this case, the time required for equilibration is reduced from over 60 min (Fig. 5 control) to 10 min or less. In addition to the increase in K^+ permeability, an increment in H^+ permeability can be observed, as judged by the fast decay in medium pH after the ATP pulse (Fig. 6, insert). It can also be observed that the total ΔpH_o is much smaller than in the fresh preparation (*see* Fig. 4 for comparison).

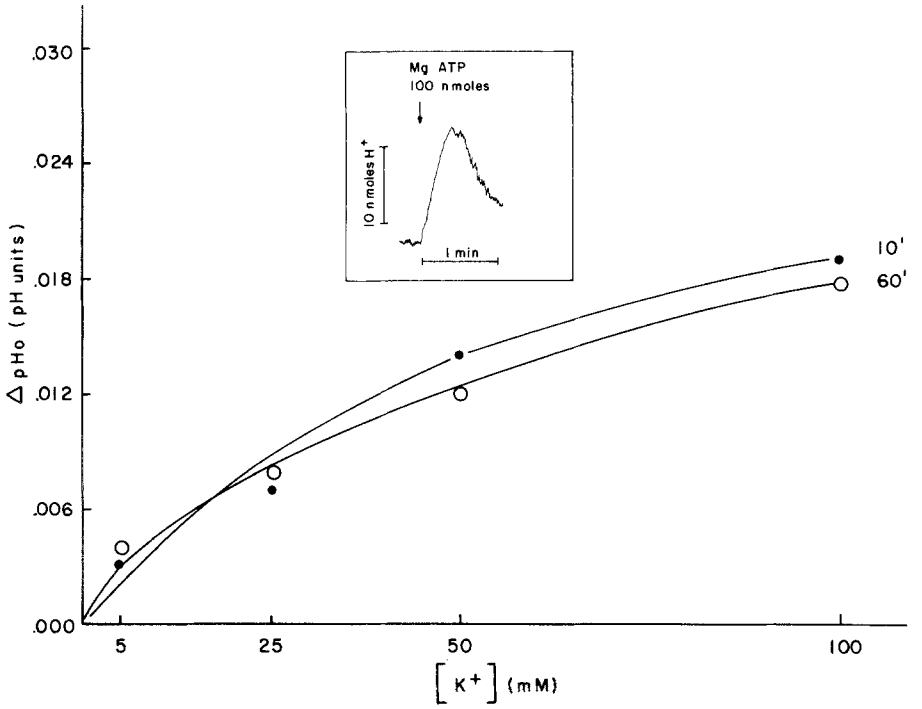

Fig. 6. Effect of time of preincubation and concentration of KCl upon ATP-driven H^+ uptake by frozen-thawed microsomal vesicles. Experimental procedure was as in Fig. 5. Insert shows the time course of H^+ uptake. Note the fast decay in outer pH after attaining maximal uptake

Discussion

Microsomes isolated from the fundic region of the hog stomach mucosa contain a K^+-stimulated, H^+ transporting ATPase [4, 30]. The rate of hydrolysis of ATP by this system is in a way controlled by the permeability of the microsomal vesicle membrane to potassium. Furthermore, the amount of H^+ uptake by the vesicles is dependent on the availability of potassium inside the vesicle to be exchanged by protons in the ATP driven process.

In this work a crude microsomal preparation was used in order to diminish the K^+ leakness of the vesicles induced by further purification [4, 34, 35].

The *Results* show that Ca^{2+} added in micromolar concentrations is able to induce a large increment in ATPase activity in the presence of K^+. This stimulation of ATPase activity is not due to a direct stimulation

of the enzyme by Ca^{2+}, but rather to an increase in the permeability of the vesicular membrane to potassium. It has been shown [10, 20], and we have also confirmed in this paper, that increasing the permeability of the vesicle to K^+ enhances the rate of hydrolysis of ATP. Our interpretation of an increase in K^+ permeability of the vesicle brought about by calcium is based on the following facts: (i) Calcium requires K^+ for stimulating the enzyme; (ii) The characteristics of the stimulation of the ATPase by potassium in the presence of calcium are identical to those in the presence of gramicidin or in the frozen-thawed preparations; (iii) Ca^{2+} was unable to elicit any further increase in ATPase activity in the presence of gramicidin or in the frozen-thawed preparations; (iv) Calcium did not affect the H^+ transport properties of the vesicles *per se*, but decreased the time of exposure to potassium required for a given amount of H^+ uptake. The results presented in this paper clearly demonstrate that calcium is able to induce a change in K^+ permeability of the vesicular membrane with a consequent enhancement of the ATP hydrolyzing activity.

Some of the effects of Ca^{2+} on the ATPase activity could be explained by an increase in H^+ permeability of the vesicles. However, this is not the case, since increasing H^+ permeability by using a H^+ ionophore does not enhance the K^+-ATPase [10]. On the other hand, an increase in H^+ permeability brings about a fast decay in the ATP induced H^+ gradient (Fig. 6 and Ref. 20). This is not the case for Ca^{2+}-treated vesicles.

This effect of calcium on potassium permeability is not unique to these membranes. The role played by calcium in regulating the potassium permeability of the red blood cell [21, 31] is well known today. Also, in liver cells, intracellular calcium concentrations play a role in the regulation of the passive permeability to potassium [41]. In nerve cell membranes, a rise in intracellular Ca^{2+} appears to increase the potassium permeability [3, 11, 23, 24].

Although the intracellular Ca^{2+} concentrations in the oxyntic cell are not known, they are probably similar to those found in other tissues, around 10^{-7}–10^{-6} M. Since the microsomes are inside-out, the outer side of the vesicle would be equivalent to the cytoplasmic side in the intact cell. Therefore, the concentrations we have found to affect potassium entry into the microsomal vesicles in the present study (μM range) may be of physiological significance.

These effects of increased intracellular calcium concentrations in different kinds of cells and in gastric microsomes appear to contrast directly

with the known effects of extracellular calcium in stabilizing cell membranes and maintaining a normal low permeability [5, 15, 38]. However, the asymmetry in the lipid composition of the membrane and the way the two different phases bind calcium could be responsible for this discrepancy. It is also interesting to note that an asymmetrical distribution of calcium across the two sides of phospholipid membranes is able to destabilize the membrane and induce an increase in cation permeability [26, 27, 28].

The effects of high calcium concentrations (1 mM) in inhibiting the potassium permeability of gastric microsomes may find explanation in the above findings in artificial systems. At high concentrations, some calcium would penetrate into the vesicle and have a stabilizing effect on the "extracellular side" of the membrane. Alternatively, there might be two binding sites for calcium with different affinities, each having an opposite stabilizing or destabilizing effect.

The findings reported herein may be of physiological importance. The presence of calcium in the extracellular fluid has long been known to be an essential requirement for the expression of many hormone-mediated processes [32] and particularly for the maintenance of acid secretion by the stomach [8, 12, 14, 16]. The requirement for calcium in the acid secretory process appears to be at different steps, both extracellular and intracellular [16]. Moreover, secretagogues are able to affect calcium movements across the cell membrane and modify intracellular calcium pools. Both influx and efflux of $^{45}Ca^{2+}$ are enhanced by secretagogues. At the intact epithelial level the enhancement of the $^{45}Ca^{2+}$ efflux has the same time course as the increase in secretory rate. This suggests that the mobilization of calcium and/or change in calcium membrane permeability may induce an increase in cytoplasmic calcium activity and, in turn, trigger the acid secretory process [17].

Based on the available physiological data and our present findings, it is tempting to propose a model for the action of calcium in mediating stimulus-secretion coupling in the parietal cell: Gastric hormones would increase intracellular cAMP levels [13, 33], and/or change Ca^{2+} permeability of the cell membrane. These elevated levels of cAMP or the change in Ca^{2+} permeability would lead to an enhancement of cytoplasmic calcium activity [17]. This increase in cell calcium, in turn, produces, as pictured in Fig. 7, an enhanced K^+ permeability of the tubulovesicular and/or apical membrane. Potassium enters the vesicle as KCl and is then exchanged by protons in the ATP driven process, leaving HCl inside the vesicle to be expelled outside the cell. These phenomena do

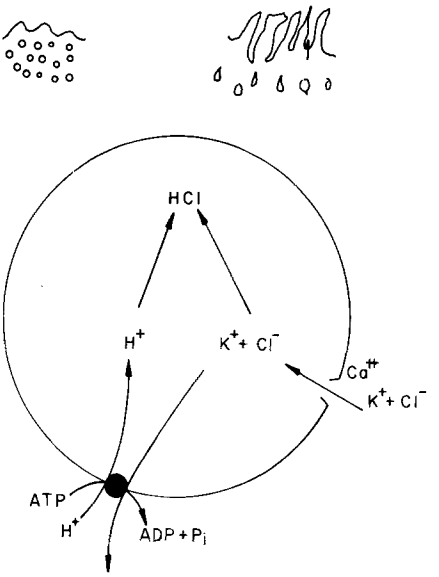

Fig. 7. Schematical model depicting the sequence of events involved in H^+ uptake and subsequent HCl formation in isolated microsomal vesicles. These events in the intact cell may take place either in closed vesicles such as the tubulo-vesicular system or at the infoldings of the apical membrane of the secreting parietal cell

not actually need to take place in an enclosed vesicle. They may happen in a diffusion-restricted space, such as the infoldings of the apical membrane of the secreting parietal cells (*see* upper part of Fig. 7).

Thus, according to this hypothesis, calcium plays a role as the final messenger in mediating stimulus-secretion coupling in the parietal cell.

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