Inhibitory Effects of Cations on the Gastric H\textsuperscript{+},K\textsuperscript{+}-ATPase

A POTENTIAL-SENSITIVE STEP IN THE K\textsuperscript{+} LIMB OF THE PUMP CYCLE*

Pia Lorentzon‡†, George Sachsg, and Björn Wallmark†

From the ‡Department of Biology, Hässle Gastrointestinal Research Laboratories, S-431 83 Mölndal, Sweden and the †Center for Ulcer Research and Education, Wadsworth Veterans Administration Hospital, University of California at Los Angeles, Los Angeles, California 90073

The presence of a cation inhibitory site on the dephosphoform of the H\textsuperscript{+},K\textsuperscript{+}-ATPase was confirmed by comparing the effects of K\textsuperscript{+} and NH\textsubscript{3} on overall activity and on phosphorylation and dephosphorylation. Inhibition of ATPase activity was pronounced at high cation/ATP ratios, but NH\textsubscript{3} was much less effective. At 60 mM cation, although the ATPase activity was greater in the presence of NH\textsubscript{3} (17.1 μmol/mg·h) as compared to K\textsuperscript{+} (5.1 μmol/mg·h), dephosphorylation of preformed phosphoenzyme was faster with K\textsuperscript{+} (2101 min\textsuperscript{-1}) than with NH\textsubscript{3} (1401 min\textsuperscript{-1}). Increasing K\textsuperscript{+} concentrations at the cytosolic face of the enzyme, at constant ATP, decreased the rate of phosphorylation from 1343 to 366 min\textsuperscript{-1} at 25 mM K\textsuperscript{+}. Increasing ATP concentrations in the presence of constant K\textsuperscript{+} concentrations accelerated ATPase activity and increased the steady-state phosphoenzyme level. Therefore, inhibition by cations was due to cation stabilization of a dephospho form of the enzyme at a cytosolically accessible cation-binding site. ATP promoted cation dissociation from this site. In ion-permeable vesicles, increasing K\textsuperscript{+} concentrations, at constant ATP, activated and then inhibited ATPase activity, with a K\textsubscript{2/3}(I) of 22 mM. In intact, ion-impermeable inside-out vesicles, in the presence of an interior potential positive potential induced by the presence of valinomycin, ATPase activity increased up to 175 mM K\textsuperscript{+}. Collapse of this potential by the addition of the electrogenic protonophore 3,3',4',5'-tetrachlorosalicylanilide restored the K\textsuperscript{+} inhibition of ATPase activity. Thus, the cation inhibition of the ATPase activity appears to be voltage-sensitive; and hence, its connection to the voltage sensitivity of acid secretion demonstrated in intact gastric mucosa is discussed.

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†To whom correspondence should be addressed.

*The abbreviations used are: E-P, phosphorylated enzyme intermediate; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; TCS, 3,3',4',5-tetrachlorosalicylanilide.

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the cytosolic face of the enzyme was inhibiting phosphorylation.

Measurement of potentials generated by the vesicular H⁺,K⁺-ATPase as well as the effects of ionophores such as TCS or nigericin showed that the pump was electroneutral. This meant that the forward and reverse steps of the pump transported equal numbers of H⁺ and K⁺ ions. An estimate of minimal pump stoichiometry was that two H⁺ and two K⁺ ions were transported per molecule of ATP hydrolyzed (22, 23). However, in amphibian and mammalian mucosa, H⁺ secretion was accelerated by lumen-negative voltage (24). These conflicting data could be reconciled if both the H⁺- and K⁺-transporting steps of the pump were charge-carrying.

Accordingly, we have re-examined the interaction of K⁺ with the enzyme in broken and intact vesicles and found kinetic evidence for a K⁺-stabilized dephospho form that is voltage-sensitive.

**EXPERIMENTAL PROCEDURES**

**Materials**

H⁺,K⁺-ATPase-containing vesicles were prepared from hog stomachs according to previously published methods (25). In short, the enzyme was prepared from homogenized mucosa by differential and gradient centrifugation. The 100,000 × g microsomal pellet was further purified by zonal density gradient centrifugation. The microsomal sample was applied on top of a step gradient consisting of 7.5% Ficoll/8% sucrose (tight vesicles) and stored at -70°C prior to use. ATP was purchased from Sigma. [γ-32P]ATP was obtained from Amersham Corp. All other reagents were of the highest purity available.

**Methods**

**Protein Determination**—Protein was determined either by the method of Lowry et al. (26) with bovine serum albumin as a standard or by the Bio-Rad microassay procedure using γ-globulin as a standard.

**ATPase Activity**—The enzyme was incubated with [γ-32P]ATP or unlabeled ATP at 22 or 37°C in a final volume of 1 ml under the conditions specified in the figure legends. The release of inorganic phosphate was determined as described by Yoda and Hokin (27).

**Determination of Phosphoenzyme Levels**—The content of the assay medium (1-ml final volume) is outlined in the figure legends relating to each experiment. The incubation was started by addition of [γ-32P]ATP and, after 15 s at 22°C, stopped by the addition of 1 ml of 10% perchloric acid containing 5 mM unlabeled ATP and 10 mM P. The precipitated protein was collected on Whatman GF/C glass fiber filters and then washed with 76 ml of 5% perchloric acid containing 10 mM P. The filters were air-dried and dissolved in 10 ml of Lumagel (Lumac, Schaesberg, The Netherlands) containing 70 μl of glacial acetic acid.

**Determination of Rates of Phosphorylation and Dephosphorylation**—The experiments were carried out at 22°C by means of a rapid mixing apparatus (28) using one or two mixers in series. This allowed the rates of formation and breakdown of the phosphoenzyme intermediate to be studied independently. The reaction was stopped by expelling the mixture into 5 ml of 10% perchloric acid containing 5 mM unlabeled ATP and 10 mM P. Further processing was as described under "Determination of Phosphoenzyme Levels."

**RESULTS**

**Effect of K⁺ on ATPase and Phosphoenzyme Levels**—K⁺ was found to affect biphasically the H⁺,K⁺-ATPase activity (Figure 1, upper; and Table I), in accordance with previously published results (11). In the concentration range up to approximately 3 mM, a stimulatory phase was found, whereas at higher concentrations, the activity was inhibited. In contrast, increasing K⁺ concentrations monophasically decreased the steady-state levels of phosphoenzyme (Fig. 1, lower). Such an effect of K⁺ on the phosphoenzyme levels is in accordance with its stimulatory effect on the dephosphorylation reaction (11). However, a stimulation of the breakdown of E-P would invariably lead to a concomitant stimulation of the ATPase activity. Since the activity was inhibited at high K⁺ concentrations, an additional site of action of K⁺ has to be postulated (19, 11). K⁺ binding at this site would then lead to a decreased phosphoenzyme formation and thereby decreased activity.

**Effect of ATP on ATPase and Phosphoenzyme Levels**—As shown in Fig. 2 (upper), the basal ATPase activity in the absence of K⁺ was barely increased by increasing ATP concentrations. However, in the presence of 1 mM K⁺, increasing ATP concentrations to 100 μM resulted in an 18-fold increase in activity as compared to 5 μM. Hence, the inhibitory effect

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**TABLE I**

| Parameter | K⁺ | NH4⁺: 50 μM ATP |
|-----------|----|-----------------|
| Vₜₜₑₓ (μmol/mg·h) | 4.1 | 18 |
| Kₜₑₓ(A) (mM) | 0.25 | 0.18 |
| Kₜₑₓ(I) (mM) | 27 | 1.3 |

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**FIG. 1.** K⁺ dependence of steady-state ATPase activity (upper) and phosphoenzyme levels (lower). Upper, 0.5 μg of membrane protein was incubated at 22°C with 5 μM [γ-32P]ATP in 5 mM Pipes/Tris, pH 7.4, 2 mM MgCl₂, and 0.01-150 mM KCl. The reaction was terminated after 15 min, and the amount of released 32Pi was determined. Lower, the reaction mixture was as described (upper), except that 40 μg of enzyme was used (100% E-P = 0.686 nmol of E-P/mg of protein).
of K⁺ was counteracted by increasing ATP concentrations. Approximately similar data were found with respect to the steady-state level of E-P. Figure 2 (lower) shows that the steady-state level of phosphoenzyme was hardly affected by increasing ATP concentrations up to 100 μM. However, in the presence of K⁺, there was a 12-fold increase of E-P levels, within the range found for the increase of ATPase activity. Thus, the increase in enzyme activity could be explained by an increase in the formation of E-P, with a constant rate of breakdown. This implies that the effect of ATP is to reduce the K⁺-induced inhibition of the formation of E-P. This is suggestively of a form of enzyme in the presence of K⁺ kinetically similar to the K⁺-occluded form of the Na⁺,K⁺-ATPase (13, 14). The first evidence for this form of the Na⁺ pump was obtained by comparing the effects of K⁺ and Rb⁺ on Na⁺,K⁺-ATPase activity; hence, similar experiments were performed on the H⁺,K⁺-ATPase, using NH₄⁺ instead of Rb⁺.

**Comparison of Effects of K⁺ and NH₄⁺ on ATPase Activity**—The effect of the two different cations on the dephosphorylation reaction was studied by first allowing formation of steady-state levels of phosphoenzyme in the absence of cations. 60 mM K⁺ or NH₄⁺ resulted in near-maximum activity (17.1 pmol/mg·h) (indicated with arrows in Fig. 3). This biphasic dephosphorylation has previously been reported for K⁺ (11). The rate constant for the fast phase was found to be larger when 60 mM K⁺ was used (Table I) than when 60 mM NH₄⁺ was used (Table II). The rate constant for
the slow phase was the same for the two ions. Thus, despite the fact that a much lower rate of ATP hydrolysis was obtained with 60 mM K⁺ than with 60 mM NH₄⁺, K⁺ induced faster dephosphorylation than NH₄⁺.

Comparison of Effects of K⁺ and NH₄⁺ on Phosphorylation—The effects of K⁺ and NH₄⁺ on the phosphorylation reaction were investigated by the addition of [γ-³²P]ATP to cation-free enzyme or enzyme equilibrated in 60 mM solutions of K⁺ and NH₄⁺, respectively (Fig. 5). A decrease in steady-state phosphoenzyme concentrations (data at 15 s; Fig. 5) was obtained for both K⁺ and NH₄⁺ compared with control. However, the steady-state level in the presence of K⁺ was lower than with NH₄⁺ (11 and 31% of the control, respectively). In the absence of cation, the rate of phosphorylation was found to be 1831 min⁻¹. At 60 mM NH₄⁺, a similar rate constant was found, namely 1712 min⁻¹. This small effect of 60 mM NH₄⁺ on phosphorylation is consistent with the steady kinetics of Fig. 3, which shows that 60 mM NH₄⁺ did not inhibit the ATPase activity. Due to the very low levels of phosphoenzyme obtained in the presence of 60 mM K⁺, the corresponding rate constant could not be determined.

One way of increasing the phosphoenzyme levels so that a reliable determination of the phosphorylation rate constant can be made is to use a vesicular preparation of the H⁺,K⁺-ATPase. In these tight vesicles, K⁺ penetrates the membranes only slowly. Hence, in the time course of the experiment, no access of K⁺ to the luminal, stimulatory site is allowed. The results of a determination of the phosphorylation rate constants at different K⁺ concentrations in a tight vesicle preparation are shown in Table III. Under these conditions, a clear effect of cytosolic K⁺ was found, with increasing K⁺ concentrations resulting in decreasing phosphorylation rate constants, as has been reported previously (11).

Effect of K⁺ on ATPase in Tight Vesicles—Since the tight vesicle preparation represents a more intact model of the H⁺,K⁺-ATPase, it was of interest to compare the K⁺ dependence of the ATPase activity in this system with the results obtained in the leaky vesicles. Fig. 6 shows the effect of K⁺ on the ATPase activity in tight vesicles at 2 mM ATP. K⁺ alone did only affect the activity slightly since under these conditions only the fraction of the preparation consisting of leaky vesicles was stimulated. However, even though the effect was small, it was clearly biphasic. Inclusion of the potassium ionophore valinomycin, permitting access of K⁺ to the lumen of the tight vesicles, resulted in stimulation of the ATPase activity, even at the highest concentrations of K⁺ (Fig. 6). This result was thus in contrast to that in the leaky vesicles, where K⁺ concentrations above 10 mM resulted in inhibition (Fig. 6). The increased K⁺(A) value found for activation by K⁺ in the tight as compared to the leaky vesicles can be explained by the rate-limiting entry of Cl⁻ into the vesicle lumen. Hence, even though K⁺ was present at 175 mM on the extravesicular, cytosolic side of the enzyme, no inhibition of the ATPase activity was obtained in the presence of valinomycin. However, inclusion of the protonophore TCS in the

![Table II](image)

| Quantity of fast reacting phosphoenzyme | Dephosphorylation rate constant |
|----------------------------------------|---------------------------------|
|                                        | %                               | Fast phase | Slow phase |
| 60 mM KCl                              | 62                              | 2101       | 227        |
| 60 mM NH₄Cl                            | 56                              | 1401       | 221        |

![Table III](image)

| Phosphorylation rate constant | min⁻¹ |
|------------------------------|-------|
| No K⁺                        | 1343  |
| 10 mM K⁺                     | 521   |
| 25 mM K⁺                     | 360   |

Fig. 5. Effect of 60 mM K⁺ or NH₄⁺ on phosphorylation of H⁺,K⁺-ATPase. Both the membrane protein and [γ-³²P]ATP were suspended in 5 mM Pipes/Tris, pH 7.4, 2 mM MgCl₂, and 60 mM KCl or NH₄Cl. The reaction was started by addition of 50 μM [γ-³²P]ATP to the enzyme and was allowed to proceed for up to ~150 ms. The steady-state level of phosphoenzyme (E-P) was determined by expelling the reaction mixture into an empty test tube and waiting 15 s until stopping the reaction. Values are the means of three determinations in one preparation and two determinations in the second. For values of time, mean ± S.E. (n = 5) is shown. Φ, KCl; ○, NH₄Cl; ■, no cation. Inset, semilogarithmic plot of the phosphorylation data. E-P is the steady-state level of phosphoenzyme found at 15 s in Fig. 4.
The above experiment resulted in a recovery of the inhibitory effect of high concentrations of K⁺ (Fig. 7). The sustained stimulation by high concentrations of K⁺, seen in the presence of valinomycin, could thus be explained by a build-up of an interior positive potential over the vesicular membrane which overcomes the inhibitory action of cytosolic K⁺. Under valinomycin conditions, a positive potential has been shown to develop in these vesicles due to the inward, maintained K⁺ gradient, either using SCN⁻ or anilinonaphthosulfonic acid as the lipid-permeable anion with which to monitor potential over the vesicular membrane which could thus be explained by a build-up of an interior positive potential over the vesicular membrane which overcomes the inhibitory action of cytosolic K⁺. Under valinomycin, could thus be explained by a build-up of an interior positive potential over the vesicular membrane which overcomes the inhibitory action of cytosolic K⁺. Under valinomycin, cytosolic face of the enzyme is also dependent on the concentration of ATP. When 50 μM ATP rather than 2 mM ATP (Fig. 6) was used to energize the pump in the presence of valinomycin, high concentrations of K⁺ resulted in inhibition (Fig. 8). Hence, in line with the results presented above (see Fig. 2), increasing concentrations of ATP result in a decreased inhibitory effect of K⁺ at its cytosolic site.

**DISCUSSION**

The existence of a cytosolic, inhibitory K⁺ site on the H⁺,K⁺-ATPase has been postulated earlier (10, 11). In this study, this hypothesis was further confirmed by comparing the effects of K⁺ on different reactions of the enzyme with the effects of another cation, NH₂⁺. From the results presented here, it seems likely that there is a step in the H⁺,K⁺-ATPase between dephosphorylation and rephosphorylation that is rate-limiting at low ATP/K⁺ ratios. In other words, as is the case for the Na⁺,K⁺-ATPase, there is a rate-limiting, cation-stabilized dephospho form of the H⁺,K⁺-ATPase. From the experiments performed in tight vesicles, for instance, where extravesicular, cytosolic K⁺ resulted in decreased phosphorylation rate constants, it follows that this form of the enzyme inhibits or slows phosphorylation. Moreover, the effect of increasing ATP concentrations at constant K⁺ concentrations was to increase the rate of formation of E-P, rather than to accelerate the breakdown of E-P, consistent with the interpretation that the cation-stabilized form of the H⁺,K⁺-ATPase is a dephospho form of the enzyme. In this respect, the NH₂⁺ form of the enzyme is less stable than the K⁺ form.

Physiologically, the enzyme is present in the parietal cell in a form equivalent to ion-tight vesicles, where the luminal concentration of K⁺ is low, the cytosolic concentration of K⁺ is high, and the luminal face is at a positive potential relative to the cytosolic face (30). Furthermore, the concentration of ATP in parietal cells is about 3 mM (31). These conditions were mimicked by studying the effect of increasing K⁺ concentrations in ion-tight vesicles in the presence of valinomycin, which allowed ATP turnover in the continuing presence of a K⁺ gradient and an extracytosolic positive potential. Under these conditions, only an increment of ATPase activity was found as a function of increasing K⁺ concentrations. At a lower ATP concentration of 50 μM, however, K⁺ concentrations above 100 mM resulted in inhibition. Thus, ATPase activity in situ would not be affected by the physiological ATP/K⁺ ratio, although if cytosolic ATP fell, inhibition of
acid secretion would be expected due to this K⁺ and ATP interaction.

There are several differences between the ion-tight vesicles studied in the presence of valinomycin at increasing K⁺ concentrations and the leaky vesicles or the vesicles in the presence of nigericin. Under the latter conditions, K⁺ has relatively free access to the luminal or extracytotoxic face of the enzyme. Under the valinomycin conditions, the internal K⁺ would be lower than in the case of the leaky vesicles or the vesicles in the presence of nigericin. However, this site has been shown to stimulate the ATPase by increasing the rate of dephosphorylation of phosphorynzyme. Even at high K⁺ concentrations, only stimulation of this step was observed, and much of the above data show that the K⁺ inhibition is due to interaction with a dephosphoform of the enzyme. Thus, the lack of inhibition in the ion-tight vesicles under valinomycin conditions is not likely to be due to the lower K⁺ concentration at the stimulatory extracytotoxic site of phosphorynzyme. In the absence of phosphorynzyme, no H⁺ gradient is formed under the conditions studied here. Since H⁺ is transported into the vesicles, accumulation of H⁺ would be expected to slow enzyme activity, but no inhibition was observed. Moreover, it is difficult to suggest a mechanism whereby the development of an H⁺ gradient would reduce the effect of K⁺ on a dephosphoform of the enzyme.

In the presence of valinomycin and ATP, these vesicles develop an interior positive diffusion potential (2, 29), which does not occur in leaky vesicles or in vesicles where the ion gradient is dissipated by nigericin. The inhibitory K⁺ form of the dephosphoenzyme could be an E₁,K⁻ form where the K⁺ site has free access to the cytosolic solution. Such a site would be relatively unaffected by potential and should be saturated at the K⁺ levels used in these experiments. Alternatively, the K⁺ site could be in a well accessed by K⁺ from the cytosolic face, which could then be affected by transmembrane potential as originally suggested for the F₁,F₀-ATPase in terms of H⁺ transport (32). On the other hand, the inhibitory K⁺ form could be a form analogous to the K⁺-occluded form, E(K⁺), of the Na⁺,K⁺-ATPase, but the barrier to de-occlusion would be relatively unaffected by potential and should be saturated as originally suggested for the F₁,F₀-ATPase in terms of H⁺ transport (32). In this case, the E(K⁺) to E₁,K⁻ step would be accelerated by the interior positive potential. The data presented here do not allow discrimination between an energy well or an occluded form that is potential-sensitive, but it does appear that the rate-limiting step at low ATP/K⁺ ratio is sensitive to transmembrane potential since the effect of valinomycin in removing K⁺ inhibition is lost when the potential is collapsed by the addition of TCS. It can be speculated that since a rate-limiting step in the K⁺ limb of the H⁺,K⁺-ATPase appears to be potential-sensitive, this step must be charge-carrying, even if it is only K⁺ exit from an energy well on the cytosolic face of the enzyme rather than a change from E₁,K⁻ to E₁,K⁺. Since the H⁺,K⁺-ATPase is electroneutral when exchanging H⁺ for K⁺, the implication is that there must be an equivalent charge-carrying step in the forward or H⁺-translocating steps of the enzyme. This idea is substantiated when the effects of voltage on acid secretion by the intact frog or dog mucosa are considered (24). Increasing the luminal negativity increased the rate of acid secretion. Conversely, luminal positivity decreased the rate of acid secretion. Thus, the H⁺,K⁺-ATPase in situ is voltage-sensitive and therefore charge-carrying. These data were also obtained at high concentrations of K⁺ in the luminal solution and therefore suggest that the rate-limiting, voltage-sensitive step is the electronic H⁺ limb of the H⁺,K⁺-ATPase. This is then consistent with a step in the K⁺ limb being equivalently electric and voltage-sensitive as shown here. This concept of coupled electrogenic steps for the H⁺,K⁺-ATPase has been discussed before in bilayer reconstitution and absorption experiments and helps to reconcile the differences found between vesicle and intact tissue experiments (33, 34).

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