Potassium Stimulation of Corn Root Plasmalemma ATPase

II. H+--Pumping in Native and Reconstituted Vesicles with Purified ATPase

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ABSTRACT

The stimulation by K+ of the initial rate of H+-pumping by ATPase was studied in native plasmalemma (Zea mays L. var Mona) vesicles and in reconstituted vesicles with enzyme purified on a gelonol gradient. In reconstituted vesicles, a very high H+-pumping rate (200,000% quenching per minute per milligram protein) was obtained with 9-amino-6-chloro-2-methoxyacridine provided that the pump was short-circuited by K+-valinomycin. A constant ionic strength was used to prevent indirect stimulation by the electrostatic effects of K+ salts. Indirect stimulation of H+-pumping by the short-circuiting effect of internal K+, could be abolished by using the per salts NO3- and Br- in native, but not in reconstituted vesicles. In both materials, half-stimula-
tion of the H+-pumping by K+ was observed at about 5 millimolar.

The same stimulation was obtained when K+ was present only in the external solution or when it was present both outside and inside the vesicles. It was concluded that the stimulation effect of K+ on the H+-pumping evidenced in these experiments on both native and reconstituted vesicles was due to a direct effect of the cation on the cytoplasmic face of the ATPase. These results are discussed within the context of the hypothesis of an active K+ transport driven by the ATPase through a direct H+/K+ exchange mechanism.

The vanadate-sensitive plasmalemma ATPase is typically stimulated by K+ (18), by both direct and indirect mechanisms. The direct stimulation of the H+-pumping ATPase by K+ can be properly studied only when indirect effects are eliminated. One of the indirect stimulating effects of K+ is to attenuate the repulsion by electrostatic forces, between the negative surface charge of the membrane, and the anion Mg-ATP (6, 7). This effect may be controlled by using a constant ionic strength. The other indirect stimulating effect of K+ is the dissipation of the H+ electrochemical gradient by K+ salts through secondary transport systems (8). This effect may be eliminated by using protonophores. In these conditions (constant ionic strength plus protonophores), K+ in the range around 1 mM specifically increased the maximum ATP hydrolysis activity and decreased the vanadate inhibition constant in native plasmalemma vesicles (7). These effects were attributed to a direct interaction of the cation with plasmalemma proteins. The response of vanadate inhibition to K+ was not modified after ATPase purification and solubilization, whereas the stimulation of ATP hydrolysis decreased significantly. This can result either from the elimination of some other plasmalemma protein which can regulate the ATPase activity (i.e. kinase, already evidenced) (14, 17), or from modifications of the lipid-protein interactions in the micelles.

Indeed, the significance of the stimulation of plasmalemma ATPase by K+ remains controversial (2). Two alternative hypotheses are currently proposed, namely a K+-stimulated ATPase which only transports protons, and/or an ATPase capable of exchanging H+/K+, and which is, thus, truly activated by K+. Purified ATPases from yeast and oat roots were shown to pump H+ even in the absence of K+ (9, 21), whereas there is a lack of strong evidence of coupling between H+ and K+ transport by ATPase (19). According to the direct coupling hypothesis, it is expected that the provision of K+ to the extracellular side of the enzyme (i.e. to the interior of the vesicle) should stimulate H+-pumping.

In this paper, we examine the stimulation of H+-pumping by K+ in both native corn root plasmalemma and reconstituted vesicles with purified ATPase. Adenosine triphosphatase has been purified from phase-partitioned plasmalemma according to Nagao et al. (10), who have reported, for the first time, an absolute K+ requirement of the Mg-ATP hydrolysis. We failed to find such an absolute K+ requirement of the Mg-ATP hydrolysis (7). We examine now the stimulatory effect of K+ on the H+-pumping when this cation acts on either the cytoplasmic, or on the extracellular side, or both sides of native plasmalemma and reconstituted vesicles. The effect of reconstituting the purified ATPase into a membrane bilayer on the stimulation of ATP hydrolysis by K+ is also presented.

MATERIALS AND METHODS

Membrane Preparation and Purification of the ATPase

Corn roots (Zea mays L. var Mona) were grown hydroponically for 5 d on aerated solution containing 0.1 mM CaSO4. Two methods were used to obtain plasmalemma vesicles. Tightly sealed plasmalemma vesicles were isolated according to De Michielis and Spanswick (3) with minor modifications: BTP2-Mes was replaced by BTP-CI, and KI was replaced by KCl.

Abbreviations: BTP, 1,3-bis(tris(hydroxymethyl)amino)propane; ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, fluoro-
carbonyl-cyanide-phénylhydrazone.

This work was supported by an Action Incitative Programmée grant from Institut National de la Recherche Agronomique.
by NaI for washing membranes to avoid K⁺ contamination. Microsomes were washed in 250 mM NaI and suspended in a buffer containing 2 mM BTP-Cl (pH 7.0), 250 mM sucrose, and 1 mM DTT. This suspension was then layered over a 30% sucrose (w/w) cushion and centrifuged for 90 min at 90,000g as described previously (7). The plasmalemma vesicles passed through the sucrose layer, and were pelleted. The supernatant was discarded and the plasmalemma-enriched pellet was suspended in 2 mM BTP-Cl (pH 7.0), 250 mM sucrose, 10% glycerol (v/v), 1 mM DTT, and 0.2% BSA and stored in liquid N₂.

ATPase was purified from plasmalemma isolated by phase partition according to Nagao et al. (10) and as described in our previous paper (7).

**Reconstitution of Proteoliposomes**

Soybean lipids (Sigma type II-S, 50 mg in 1 mL buffer containing 10 mM BTP-Cl, pH 6.5) were dispersed by vigorous mixing on a Vortex mixer in the presence of glass beads, for 15 min under Argon. Afterward, the liposome suspension was clarified by sonication for 15 min under Argon in a Bransonic sonicator bath. Concentrated aliquots of BTP-Cl or KCl were added both to the liposome preparation and to the purified ATPase fraction (0.5 mL containing 10 μg protein), to obtain final concentrations of 100 mM BTP-Cl (noted ‘K⁺’ preparations), or 10 mM BTP-Cl and 200 mM KCl (noted ‘K⁺-K⁻’ preparations). The liposome preparation was sonicated again as indicated above. Thereafter, liposomes and protein solutions were mixed on a Vortex mixer, then frozen for 15 min at -80°C, thawed at room temperature and sonicated for 1 min. The freeze-thaw sonication procedure was repeated once.

**Proton Transport Assay**

The initial rate of fluorescence quenching of quinacrine or ACMA was used to monitor the initial H⁺-pumping activity of native plasmalemma and reconstituted vesicles, respectively. In both cases, initial rate of quenching was linear with protein concentration, and thus could be expressed in specific units (percent quenching·min⁻¹·mg⁻¹ protein). Fluorescence was measured with a Jobin-Yvon JY3-CS apparatus fitted out with a stirred and thermostated cell, using excitation/emission wavelengths of 423/500 nm or 415/485 nm for quinacrine and ACMA, respectively. The assay medium contained K⁺ salts and BTP salts buffer (pH 6.5, 230 mM total ionic strength) as indicated in the legends, 10 μM quinacrine or 1 μM ACMA, 5 mM BTP-ATP and 100 μg proteins·mL⁻¹ (native vesicles), or 0.1 μg proteins·mL⁻¹ (reconstituted vesicles). Ionic strength (230 mM) was maintained constant by supplementing media containing various KCl (or NaCl) concentrations with BTP-Cl (the mean valency of BTP is +1.6 at pH 6.5). The reaction was started with concentrated aliquot of MgSO₄ (5 mM final concentration), and assayed at 30°C.

**ATP Hydrolysis Assay by Purified or Reconstituted ATPase**

The ATPase activity was defined as the Mg-dependent ATP hydrolysis (5). The standard incubation medium (0.5 mL final volume) contained 3 mM Tris-ATP, 3 mM MgSO₄, various KCl and BTP-Cl (pH 6.5) concentrations as a constant ionic strength (140 mM, see above). The assay medium was supplemented with 50 μg·mL⁻¹ lysophosphatidylcholine for glycerol gradient ATPase fraction, or with 2 μM gramicidin to dissipate the electrochemical H⁺ gradient in reconstituted preparations (–K⁺ preparations obtained as indicated above). The reaction was started by addition of 0.5 μg protein, and allowed to proceed for 15 min at 30°C. The released Pi was assayed by the method of AMES (1). SDS (0.75% w/v) was added to prevent interference with high lipid concentrations. The control was run without MgSO₄ and subtracted from the assay in the presence of MgSO₄ to calculate ATPase activity. Triplicate samples were run for each assay.

**Proteins Assay**

Protein was determined according to Schaffner and Weissman (16).

**RESULTS**

**Potassium Stimulation of H⁺-Pumping in Native Plasmalemma Vesicles**

The potassium stimulation of H⁺-pumping in native plasmalemma vesicles was studied at a constant ionic strength...
POTASSIUM STIMULATION OF H⁺-PUMPING BY CORN ROOT

(240 mM), as indicated in "Materials and Methods." This prevents indirect electrostatic stimulation due to the screening effect of K⁺ salt, which decreases the repulsion of the anion Mg-ATP by the negative surface charge, as previously demonstrated (6, 7). In Cl⁻ containing medium and in the presence of 50 mM K⁺, a 60% increase of the initial rate of H⁺-pumping (V₉⁺, estimated by quinacrine quenching) was observed upon valinomycin addition, and V₉⁺ reached 67% quenching-min⁻¹·mg⁻¹ protein (Fig. 1A). In a medium containing 50 mM NO₃⁻ and 50 mM Br⁻ as permeant anions, and 50 mM K⁺, V₉⁺ (60% quenching·min⁻¹·mg⁻¹ protein) was not augmented by valinomycin (Fig. 1B). A twofold decrease was observed when K⁺ was omitted. It has been shown, by using membrane potential probes, that addition of the permeant anions NO₃⁻ and Br⁻ totally depolarizes plasmalemma vesicles (4, 12). This depolarizing effect of NO₃⁻ and Br⁻ should explain the absence of stimulation of V₉⁺ by valinomycin.

When Mg²⁺ (to start the reaction) and K⁺ were added simultaneously, the half-stimulation of V₉⁺ was observed at about 5 to 7 mM K⁺ (Fig. 2). The presence of valinomycin prior to addition of K⁺ and Mg²⁺ did not modify V₉⁺. The stimulating effect of K⁺ was not higher when membranes were incubated in the presence of K⁺ for 20 min under stirring to load the vesicles, before starting the reaction with Mg²⁺. When K⁺ was replaced by Na⁺, no stimulation was observed.

**Potassium Stimulation of ATP Hydrolysis in Reconstituted Vesicles with Purified ATPase**

ATP hydrolysis by ATPase purified on glycerol gradient, before and after reconstitution into soybean liposomes was assayed as a function of K⁺ concentration, at constant ionic strength and in the presence of gramicidin (Fig. 3). For K⁺ concentrations above 50 mM, the specific activities of both preparations were nearly the same (4.3 µmol·min⁻¹·mg⁻¹ protein at 30°C). However, the activity in the absence of K⁺ was lower in reconstituted preparation and thus the maximum K⁺ stimulation was higher (65%) than before reconstitution (20%). The half-stimulation was observed at about 5 mM K⁺ in reconstituted, and about 1 mM in solubilized preparations.

**Effect of K⁺ on Passive H⁺ Transport in Reconstituted Vesicles with Purified ATPase**

When vesicles from +Kᵩ₀ preparations were introduced in pumping assay medium in the absence of Mg-ATP, a high rate of passive H⁺ transport was observed (Fig. 4). The initial rate of H⁺ transport reached 50,000% quenching·min⁻¹·mg⁻¹ protein, when the assay medium contained 2 mM K⁺ (curve a), and was threefold higher in the presence of valinomycin (curve b). Furthermore, a large pH gradient was instantaneously created upon FCCP addition (curve c). In every case, the pH gradient rapidly vanished upon elimination of the K⁺ gradient by addition of 200 mM K⁺ in the assay medium (all curves in Fig. 4). Similar results were obtained when vesicles contained only soybean lipids (data not shown). These results strongly suggest that a passive H⁺ transport may be caused by a K⁺ diffusion membrane potential, negative inside. Indeed, the proton is one of the most permeant ionic species, 10⁶-fold more permeant than K⁺ (11, 13). Consequently, H⁺ is likely accumulated inside the vesicles, according to the Nernst equilibrium. Addition of valinomycin to augment the K⁺ diffusion potential increased the passive pH gradient (curve b). The passive pH gradient was virtually instantaneously created when movement toward equilibrium was facilitated by FCCP (Fig. 4, curves a and c). These results show that, depending on ionic conditions and membrane potential, the addition of FCCP (and other protonophores) can favor the creation of a passive transient pH gradient, rather than its dissipation.

In conclusion, the membrane potential is determinant in H⁺ transport studies. When K⁺ is present only inside the vesicles, a passive H⁺/K⁺ exchange prevents study of the

![Figure 2. Effect of K⁺ concentrations on the initial rate of plasmalemma vesicles H⁺-pumping. Quinacrine quenching was assayed in medium (pH 6.5) containing 30 mM BTP-NO₃, 30 mM BTP-Br and various concentrations of BTP-Cl, and KCl (curves a and b) or NaCl (curve c), as indicated in "Materials and Methods." Curves a and c were obtained by incubating the vesicles for 20 min in the presence of the indicated K⁺ (C) or Na⁺ (A) concentrations, to load the vesicles (see Fig. 7), before starting the reaction with Mg²⁺. Curve b was obtained by adding simultaneously Mg²⁺ and the appropriate K⁺ concentration in the absence (D) or in the presence of valinomycin (A).](https://www.plantphysiol.org/content/115/7/1185/F2)

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Simultaneous addition of K⁺ and Mg²⁺ (to start the reaction) caused a 6-fold stimulation of V_H⁺, half the effect being observed at about 5 mM K⁺ (Fig. 6, curve a). The presence of valinomycin prior the addition of K⁺ and Mg²⁺ did not significantly modify V_H⁺ (Table I). A further increase of V_H⁺ was observed as a function of incubation time of −Kᵦ preparations in medium containing both 200 mM K⁺ and valinomycin prior to Mg²⁺ addition (Fig. 7). A plateau was reached after 20 min, and the maximum V_H⁺ value (about 200,000% quenching-min⁻¹-mg⁻¹ protein) was the same as the one obtained with +Kᵦ preparations assayed in the same conditions (Table I). A 20 min incubation of −Kᵦ preparations in the presence of valinomycin was used to equilibrate the vesicles with various K⁺ concentrations. At K⁺ concentration below 10 mM, the V_H⁺ values were nearly the same (Fig. 6, curve b) as the previous ones obtained when K⁺ was present only outside (curve a). At higher K⁺ concentrations, the

stimulation of the H⁺-pumping by K⁺. Consequently, the effect of K⁺ inside the vesicles can be estimated only by comparing the H⁺-pumping rates (a) with K⁺ present both outside and inside the vesicles, in the absence and in the presence of valinomycin, and (b) with K⁺ only outside the vesicles.

Effect of K⁺ on the H⁺-Pumping in Reconstituted Vesicles with Purified ATPase

Lipophilic anions have been used to short-circuit the purified and reconstituted ATPase to stimulate H⁺-pumping (15). However, in the presence of SCN⁻ (thiocyanate) and TPB⁻ (tetraphenylborate), we were unable to obtain high rates of H⁺-pumping (data not shown). The best pump short-circuiting was ensured by valinomycin and high K⁺ concentrations inside the vesicles (Figs. 5, 6, and Table I). When ATPase was reconstituted in the presence of 200 mM K⁺ salt (+Kᵦ preparations), V_H⁺ reached 200,000% quenching-min⁻¹-mg⁻¹ protein, provided that 200 mM K⁺ and valinomycin were also present in the assay medium (Fig. 5). A threefold decrease of V_H⁺ was observed when valinomycin was omitted, both in medium containing only Cl⁻ salts, or NO₃⁻ and Br⁻ salts (Fig. 5A). However, V_H⁺ values were 50% lower in NO₃⁻ and Br⁻ containing medium (Fig. 5B), probably due to chelotropic effects of these anions (18). Thus, chloride medium was retained in the following experiments. When ATPase was reconstituted in the absence of K⁺ (−Kᵦ preparations), V_H⁺ values were only about 10,000% quenching-min⁻¹-mg⁻¹ protein in the absence of K⁺ in the assay medium (Fig. 6).

Figure 3. Effect of K⁺ on the ATP hydrolysis activity of the solubilized (■) and reconstituted (○) ATPase, purified on glycerol gradient. The ATP hydrolysis was assayed as described in "Materials and Methods."
Added, the same $V_{H^+}$ values were obtained (close to 60,000% quenching min$^{-1}$ mg$^{-1}$ protein) (Table I).

**DISCUSSION**

**Effect of the ATPase Reconstitution on the ATP Hydrolysis Stimulation by K**

After lysophosphatidylcholine solubilization and purification of the ATPase on glycerol gradient, K$^+$ stimulation of ATP hydrolysis was appreciably less than in native vesicles (7). But K$^+$ stimulation was fully restored when the purified enzyme was reconstituted in membrane vesicles (Fig. 3). Similar results have been published for red beet (20). Thus, the decrease of the K$^+$ stimulation in purified and solubilized preparation seems not to result from the elimination of some ATPase related plasmalemma proteins (such as plasmalemma kinases) (14, 17). Since gramicidin dissipated the H$^+$ electrochemical gradient in these experiments (Figs. 1 and 5), the stimulating effect of K$^+$ on ATP hydrolysis probably results from a direct interaction with the ATPase itself in both native and purified preparations.

**Direct Effect of K$^+$ on Membrane Proteins in Native and Reconstituted Vesicles; Influence of Assay Condition**

Indirect electrostatic effects of K$^+$-salts were eliminated by using constant ionic strength (6, 7). The ATPase activity is expected to be slowed down by the transmembrane electrochemical H$^+$ gradient it creates. Thus, K$^+$ may indirectly stimulate the ATPase activity in native vesicles, by dissipating the electrochemical H$^+$ gradient, through secondary ionic transport systems of plasmalemma (8). It may, for example, dissipate the pH gradient through H$^+$/K$^+$ symport or antiport systems. It may also dissipate the transmembrane potential difference through specific channels or electrogenic H$^+/K^+$ carriers. Concerning the dissipation of the pH gradient, it is worthwhile noting that K$^+$ effects were determined on the initial rate of H$^+$ transport ($V_{H^+}$). The rate of net H$^+$ transport

**Figure 5.** Effect of anions on K$^+$-valinomycin stimulation of the H$^+$-pumping in reconstituted vesicles with purified ATPase. Traces are the quenching of ACMA fluorescence ($t_0/t_f$), measured as described in "Materials and Methods," where $t_0$ and $t_f$ are the fluorescence before and after addition of MgSO$_4$ (closed arrows), respectively. The vesicles were equilibrated with the medium for 20 min before adding MgSO$_4$. Quenching was reversed by addition of 2 μM gramicidin (open arrows). (A), curve a: 100 mM BTP-Cl; curve b: 20 mM BTP-Cl, 200 mM KCl; curve c: 20 mM BTP-Cl, 200 mM KCl, 0.25 μM valinomycin. (B), curve d: 40 mM BTP-Cl, 30 mM BTP-Br, 30 mM BTP-NO$_3$; curve e: 20 mM BTP-Cl, 100 mM KCl, 50 mM KBr, 50 mM KNO$_3$; curve f: 20 mM BTP-Cl, 100 mM KCl, 50 mM KBr, 50 mM KNO$_3$, 0.25 μM valinomycin.

The difference between the two curves rapidly increased and reached a plateau at 200 mM, corresponding to a 20-fold stimulation of $V_{H^+}$. The effect of K$^+$ on the extracellular side of the ATPase (exposed to the interior of the vesicle) was further studied by comparing $+$K$_{in}$ (200 mM K$^+$ inside) and $-$K$_{in}$ (no K$^+$ inside) preparations in the presence of 200 mM K$^+$ in the assay medium. Provided that no valinomycin was

**Figure 6.** Effect of K$^+$ concentrations on the initial rate of H$^+$-pumping in reconstituted vesicles with purified ATPase. ACMA quenching was assayed in medium containing various BTP-Cl (pH 6.5), and KCl concentrations, at a constant ionic strength (240 mM) and about 0.1 μg mL$^{-1}$ protein, reconstituted in the absence of K$^+$ ($-$K$_{in}$ preparations), as indicated in "Materials and Methods." Curve a was obtained by adding simultaneously Mg$^{2+}$ and the appropriate K$^+$ concentration. Curve b was obtained by incubating the vesicles for 20 min in the presence of the indicated K$^+$ concentrations and 0.25 μM valinomycin, before starting the reaction with Mg$^{2+}$.
subsequently decreased to zero value (when the maximum quenching of the dye was reached). This corresponded to a steady state where the active H⁺ influx was counterbalanced by the passive H⁺ efflux. Thus, it is likely that V_H⁺ was not significantly affected by the transmembrane pH gradient, and can be used to estimate the H⁺-pumping rate.

Concerning the effect of K⁺ on the membrane potential, the efficiency of the indirect stimulation by the so-called short-circuiting effect is evidenced in our experiments by the effect of valinomycin on H⁺-pumping in Cl⁻ medium (Figs. 1 and 5; Table I). The high permeability of plasmalemma vesicles to NO₃⁻ and Br⁻ allows an efficient short-circuit of the pump, attested by the disappearance of the stimulating effect of valinomycin, as already evidenced (12).

However, the ATPase could not be short-circuited by NO₃⁻ and Br⁻ after purification and reconstitution of the enzyme, due to the low permeability of soybean vesicles to these anions (Fig. 5). The only way we have found to short-circuit the pump was precisely to use valinomycin and very high internal K⁺ concentration (Fig. 6). This agrees with direct measurement of the electrogenic H⁺-pumping of purified and reconstituted ATPase from Schizosaccharomyces pombe, which is totally dependent on the presence of valinomycin (22).

In the absence of valinomycin, a high membrane potential is expected from H⁺-pumping, due to the high electrogenicity of plasmalemma ATPase. This might explain the reduction to one third of the maximum rate of the pump when valinomycin was omitted (Fig. 6). Furthermore, a high K⁺ diffusion potential, positive inside, was expected when K⁺ was only present outside the vesicles (theoretically infinite cis-trans gradient), in the experiment in Figure 6 (curve a). The existence of diffusion potential, negative inside, was evidenced in K⁺-loaded vesicles by the creation of a passive H⁺ gradient (Fig. 4). When K⁺ was only present outside, the pump must face two (additive) electrical constraints, a passive K⁺ diffusion potential, and its own electrogenic activity.

Table I. Effect of K⁺ Accessibility to Cytoplasmic and/or Extracellular Face of ATPase on H⁺-Pumping in Reconstituted Vesicles with Purified Enzyme (see "Material and Methods").

<table>
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<tr>
<th>Preparation</th>
<th>K⁺</th>
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*ATPase reconstituted as −K⁺ in 10 mM BTP-Cl; thereafter, K⁺ was added in the presence of valinomycin 20 min prior starting the reaction.

Figure 7. Effect of incubation time in the presence of K⁺ and valinomycin on the H⁺-pumping activity of purified ATPase in vesicles reconstituted in the absence of K⁺. ACMA quenching was assayed as indicated in "Materials and Methods" in a medium containing 20 mM BTP-Cl (pH 6.5), 200 mM KCl, 0.25 μM valinomycin, and 0.1 μg/mL protein (−K⁺ preparations). Vesicles were incubated for the indicated time, in the presence of K⁺ and valinomycin, before starting the reaction with Mg²⁺.

Addition of K⁺ outside the vesicles caused an increase of the H⁺-pumping, and of the ATP hydrolysis, with an half effect at 5 mM (Fig. 6, curve a). Above this concentration, a plateau was reached, and the activities remained constant up to 400 mM K⁺. This result is puzzling because addition of external K⁺ concentration from 50 mM to 400 mM, as well as addition of valinomycin in the presence of 200 mM K⁺ in the assay medium (Table I), were expected to cause an increase of the positive inside membrane potential, and thus a decrease of the H⁺-pumping rate. Our overall data suggest that the pump can be slowed down only to one third of its maximum activity (observed in the presence of valinomycin) by increasing the positive inside membrane potential, and further remains insensitive to higher potential values. A direct measurement of the membrane potential should be required to discuss further this point.

In conclusion, the direct stimulating effect of K⁺ on V_H⁺ could not be quantitatively ascertained in reconstituted preparations, unlike native ones, because the ATPase activity could not be short-circuited independently of the presence of valinomycin and high K⁺ concentration inside the vesicles. In the absence of valinomycin, the H⁺-pumping activity was slowed down by a membrane potential. Nevertheless, the half-stimulating K⁺ concentration (about 5 mM) is the same as the one of native plasmalemma vesicles short-circuited by the permeant anions NO₃⁻ and Br⁻. Furthermore, from the absence of detectable contaminants in gel electrophoresis (7), it
is likely that the K⁺ stimulation of the H⁺-pumping is related to a direct effect of K⁺ on the ATPase.

**Orientation of the ATPase Site for K⁺ Stimulation**

The stimulation of H⁺-pumping by K⁺ in native and reconstituted vesicles, in the absence of K⁺ inside, was insensitive to valinomycin when K⁺ and Mg²⁺ were simultaneously added outside (Fig. 2, curve b; Table I). This strengthens the hypothesis that, in these conditions, K⁺ acts only on the external, i.e. cytoplasmic, side of the enzyme. When K⁺ (from 1 to 100 mM) was present both inside and outside native vesicles, no significant additional stimulation of V_H⁺ was observed (Fig. 2, curve a). The maximum threefold stimulation of V_H⁺ observed when K⁺ was present both inside and outside reconstituted vesicles, it likely due to an indirect short-circuiting effect because it vanished in the absence of valinomycin (Table I). Thus, the only direct stimulating effect of K⁺ on V_H⁺ evidenced in the experiments with both native and reconstituted vesicles depends on the accessibility of this cation to the cytoplasmic side of the ATPase. Since the half stimulation of the ATPase is attained for K⁺ concentrations (5–10 mM) one order of magnitude lower than those prevailing in the cytoplasm, the physiological significance of this mechanism remains unclear.

Finally, we have not detected an additional stimulation of the H⁺-pumping activity when K⁺ was present inside both native (from 1 to 100 mM), and reconstituted vesicles (from 1 to 200 mM). This result does not strengthen the hypothesis of a direct H⁺/K⁺ exchange by the pump, since a stimulation of the H⁺-pumping should be expected from the provision of K⁺ to the extracellular side of the enzyme (i.e. to the interior of the vesicle). Nevertheless, a direct study of the K⁺ transport in purified and reconstituted ATPase is now required. The present study indicates that the main difficulty will be to clamp to zero value the membrane potential in order to prevent passive H⁺/K⁺ exchange in purified and reconstituted ATPase preparations.

**ACKNOWLEDGMENT**

We thank Professor D. T. Clarkson for critical reading of the manuscript.

**LITERATURE CITED**