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Demonstration of the Electrogenicity of Proton Translocation During the Phosphorylation Step in Gastric H^+K^+-ATPase

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Summary. Membrane fragments containing the H^+K^+-ATPase from parietal cells have been adsorbed to a planar lipid membrane. The transport activity of the enzyme was determined by measuring electrical currents via the capacitive coupling between the membrane sheets and the planar lipid film. To initiate the pump currents by the ATPase a light-driven concentration jump of ATP from caged ATP was applied as demonstrated previously for Na^+K^+-ATPase (Fendler, K., Grell, E., Haubs, M., Bamberg, E. 1985. *EMBO J.* **4**:3079–3085). Since H^+K^+-ATPase is an electroneutrally working enzyme no stationary pump currents were observed in the presence of K^+. By separation of the H^+ and K^+ transport steps of the reaction cycle, however, the electrogenic step of the phosphorylation could be measured. This was achieved in the absence of K^+ or at low concentrations of K^+. The observed transient current is ATP dependent which can be assigned to the proton movement during the phosphorylation. From this it was concluded that the K^+ transport during dephosphorylation is electrogenic, too, in contrast to the Na^+K^+-ATPase where the K^+ step is electroneutral. The transient current was measured at different ionic conditions and could be blocked by vanadate and by the H^+K^+-ATPase specific inhibitor omeprazole. An alternative mechanism for activation of this inhibitor is discussed.

Key Words gastric H^+K^+-ATPase · caged ATP · pump currents · planar lipid films

Introduction

The H^+ and K^+ transporting ATPase from parietal cells in the gastric mucosa (H^+K^+-ATPase EC 3.6.1.36) is an electroneutral ion pump, which exchanges protons for potassium ions (Forte, Forte & Saltman, 1967; Lee, Simpson & Scholes, 1974; Sachs et al., 1976). The physiological role of the pump is to produce and to maintain a pH level of about 1 in the lumen of the mammalian stomach. The H^+K^+-ATPase is located in the secretory membrane system of the parietal cell, where the protons must be transported against a gradient of 6 pH units. Transport activity of the H^+K^+-ATPase in membrane liposomes or reconstituted proteoliposomes has been described previously. The energy for the active hydrogen transport against the enormous pH difference is obtained by ATP hydrolysis, with a stoichiometry of 1 to 4 H^+/1 ATP depending on the source (Lee et al., 1974; Sachs et al., 1976; Reenstra & Forte, 1981; Rabon, McFall & Sachs, 1982; Smith & Scholes, 1982; Skrabanja, De Pont & Bonting, 1984; Skrabanja, Van der Hijden & De Pont, 1987). From conformational studies it has been deduced that the H^+K^+-ATPase behaves like an E_1, E_2 type enzyme (Schrijen et al., 1980, 1981; Wallmark et al., 1980; Jackson, Mendlein & Sachs, 1983; Morii, Ishimura & Takeguchi, 1984; Helmich-de Jong, van Emst-de Vries & De Pont, 1987) as has been proposed for the ubiquitous Na^+K^+-ATPase by Albers (1967) and Post et al. (1969). Na^+K^+-ATPase and the H^+K^+-ATPase have a homology of more than 60% in the primary structure (Shull, Schwartz & Lingrel, 1985; Shull & Lingrel, 1986; Shull & Greeb, 1988). The Na^+K^+-ATPase, however, is an electrogenic pump with a stoichiometry of $3Na^+$ to $2K^+$. The electrogenicity of the Na^+K^+-ATPase was directly demonstrated in intact cells (Thomas, 1969; Abercrombie & De Weer, 1978; Lafaire & Schwarz, 1984; Gadsby, Kimura & Noma, 1985), in reconstituted proteoliposomes (Goldin & Tong, 1974; Goldschlegger et al., 1987), or in planar lipid membranes (Fendler et al., 1985) by measuring the stationary electrical pump current or by use of voltage-sensitive dyes in case of the reconstituted proteoliposomes. The H^+K^+-ATPase, however, as an electroneutral exchanging enzyme is not expected to elicit any stationary current, because the Albers-Post scheme predicts for the H^+K^+-ATPase a phosphorylation step concomitant or prior to the proton translocation, followed by dephosphorylation concomitant with the potassium ion translocation. The sum of these steps represents the electro-

neutral exchange across the membrane, meaning that both the proton translocation and the K^+ step are electroneutral, or that both steps are electrogenic, but oppositely directed. Recently, voltage sensitivity of the K-limb of the reaction cycle was shown on H^+K^+-ATPase containing vesicles (Lorentzon, Sachs & Wallmark, 1988).

Planar lipid membranes are an excellent tool by which to study the electrical properties of ion pumps. This was demonstrated for the first time for the light-driven proton pump bacteriorhodopsin by Skulachev and his associates (Drachev et al., 1974, 1976*a*,*b*).The method consists of the adsorption of bacteriorhodopsin proteoliposomes or of bacteriorhodopsin-containing purple membranes to a planar lipid bilayer, where either photopotentials or photocurrents are obtained via the planar lipid bilayer as a capacitive electrode. A quantitative description of the system for proteoliposomes and purple membranes adsorbed to the lipid film has been given (Herrmann & Rayfield, 1978; Bamberg et al., 1979).

The immediate application of light, as the energy-supplying substrate, synchronizes the activation of bacteriorhodopsin, permitting detailed studies of the stationary and kinetic properties of the pump currents to be performed (Dancshazy & Karvaly, 1976; Herrmann & Rayfield, 1978; Bamberg et al., 1979; Fahr, Läuger & Bamberg, 1981). Since for ATP-driven pumps like the Na^+K^+-ATPase or the Ca^{2+} ATPase from sarcoplasmic reticulum the time required to add ATP by mixing is at least a few seconds, the method described above cannot be applied. A photolabile analogue of ATP, caged ATP, which produces a concentration jump of ATP in the millisecond range following irradiation with UV light (Kaplan, Forbush & Hoffmann, 1978; McCray et al., 1980) was successfully applied to the Na^+K^+ ATPase, Ca^{2+} ATPase from sarcoplasmic reticulum and to the F_0, F_1 ATP synthase from photosynthetic bacteria on planar lipid films. By adsorption of membrane fragments (Na^+K^+-ATPase), membrane vesicles (Ca^{2+} ATPase from sarcoplasmic reticulum) or reconstituted proteoliposomes (F_0, F_1 ATP synthase) to a planar lipid film, the properties of the electrical pump currents were investigated (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987; Fendler, Grell & Bamberg, 1987; Hartung et al., 1987; Nagel et al., 1987; Christensen et al., 1988).

Based on these studies we applied this technique to gastric H^+K^+-ATPase. Here we show for the first time the generation of transient electrical currents by a pump that operates electroneutrally under stationary conditions. This was achieved by separation of the pump cycle into its phosphorylation phase and its dephosphorylation phase, respectively.

A preliminary report on these results was presented at the 5th International Conference on Na^+K^+-ATPase (Fugslo, Denmark, 1987) and has been published (Fendler et al., 1988).

Materials and Methods

Preparation of the H^+K^+-ATPase Membrane Sheets

H^+K^+-ATPase from pig stomach was prepared as previously described (Forte, Ganser & Tanisawa, 1974; Rabon et al., 1985) with a few modifications. Mucosal scrapings of the fundic part of the pig stomach were homogenized in a buffer containing 150 mM sucrose and 20 mM Tris/HCl (pH 7.2). The homogenates were centrifuged for 20 min, at 20,000 × *g* (Sorvall SS 34 rotor) yielding a supernatant that was centrifuged for 45 min at 100,000 × *g* (MSE, 8 × 50 rotor). The resulting pellet was resuspended in 25 mM Tris/HCl (pH 7.2) and centrifuged for 60 min at 100,000 × *g* on top of a gradient consisting of 7% Ficoll/250 mM sucrose in 20 mM Tris/HCl (pH 7.2) over 37% sucrose in 20 mM Tris/HCl (pH 7.2). The buffer-Ficoll interface consisted of closed vesicles and the Ficoll-sucrose interface of broken membrane sheets. Both fractions were diluted in 20 mM Tris/HCl (pH 7.2) before centrifuging 60 min at 10,000 × *g* (MSE 8 × 50 rotor). The resulting supernatant was once again centrifuged in 20 mM Tris/HCl (pH 7.2) at 38,000 × *g* (MSE 8 × 50 rotor). The four different pellets were resuspended in 20 mM Tris/HCl (pH 7.2) and frozen at −20°C. After two or three freezing and thawing steps the vesicles were broken, yielding activities ranging from 0.02–0.06 for the 10,000 × *g* fractions and from 0.06 to 0.12 mmol per mg per hr for the 38,000 × *g* fractions. In some experiments further purification was carried out by zonal electrophoresis (Walters & Bont, 1979). Both preparations of membrane sheets gave similar results on planar lipid membranes. Residual activity of Na^+K^+-ATPase in the preparation was suppressed by the addition of 1 mM ouabain to the membrane sheets prior to their addition to the planar lipid membranes. Enzymatic activity was determined in the presence of 0.0375 mg enzyme per ml and 5 mM Tris-ATP at 37°C employing the technique of Jorgensen (1974), with incubation times of 8 and 15 min. The enzymatic activity of the gastric H^+K^+-ATPase is calculated as the difference between the activities determined in a medium containing (in mM): 60 imidazole-HCl, 10 $MgCl_2$, 40 KCl and 0.2 ouabain at pH 7.5, and in a similar medium where KCl is replaced by 40 mM choline chloride. The activity of the investigated sample was 27 μmol P_i mg^{-1} hr^{-1}. The Tris salts of caged ATP and ATP were prepared employing a Dowex 50-W ion exchanger column.

Membrane Setup

Optically black lipid membranes with an area of 10^{-2} cm^2 were formed in a thermostated Teflon cell with 1.5 ml of an appropriate electrolyte solution in each compartment (Mueller et al., 1964). The membrane-forming solution contained 1.5% phosphatidylserine wt/vol in *n*-decane or 1.5% wt/vol diphytanoyl-

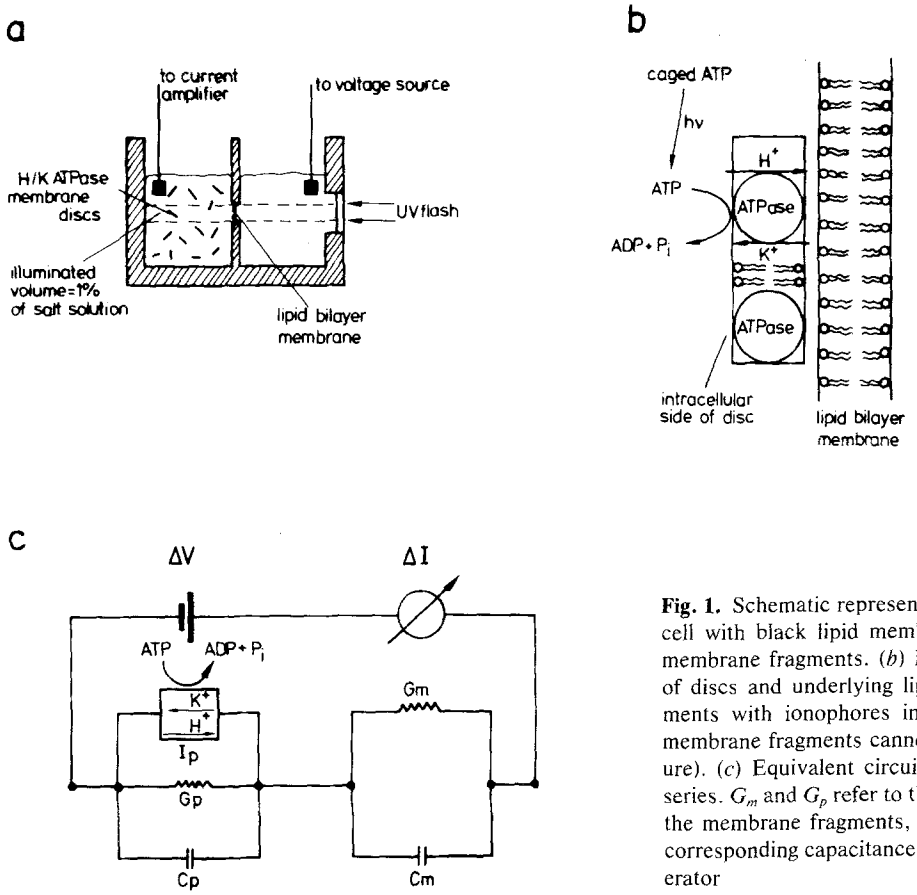

Fig. 1. Schematic representation of the bilayer setup. (*a*) Teflon cell with black lipid membranes and adsorbed H^+K^+-ATPase membrane fragments. (*b*) Proposed sandwich-like arrangement of discs and underlying lipid membrane. Note that in experiments with ionophores incorporation of ionophores into the membrane fragments cannot be excluded (not shown in the figure). (*c*) Equivalent circuit diagram of the two membranes in series. G_m and G_p refer to the conductance of the planar film and the membrane fragments, respectively. C_m and C_p refer to the corresponding capacitances. I_p designates the pump current generator

lecithin together with 0.025% wt/vol octadecylamine. To improve the adsorption of the negatively charged membrane sheets to the supporting lipid bilayer made from phosphatidylserine, about 10 μM free Ca^{2+} was added to the electrolyte. For the same purpose octadecylamine was added to the lipid-forming solution in case of diphytanoyllecithin yielding a positive surface charge on the lipid bilayer (Dancshazy & Karvaly, 1976).

The membrane was connected to an external measuring circuit via Ag/AgCl electrodes. To avoid artificial photoeffects the electrodes were separated from the aqueous compartments of the cell by agar-agar salt bridges. To prevent light pipe effects, the salt bridges were made from polyethylene tubing. In addition, the agar-agar in the bridges contained black ink to avoid any light conduction to the electrodes.

Caged ATP was photolysed by an UV light pulse of 125-msec duration with a maximal light intensity of 3.7 W/cm^2. The light intensity could be attenuated with appropriate UV light filters (Melles-Griot, Darmstadt, FRG). As light source a mercury high pressure lamp was used (200 W). Unless otherwise indicated, light pulses were applied in intervals of 10 min. After each measurement the stirrers in the cuvette were turned on, so that the liberated ATP was diluted within the path of the light beam and hydrolyzed by the enzyme in suspension. Consequently, the original concentration of caged ATP at the membrane surface was nearly recovered. Each flash liberated about 0.3% of the total amount of caged ATP in the cuvette (Nagel et al., 1987). Knowing the geometry of the light beam in the cuvette, the conversion rate of caged ATP was determined using a luciferin-luciferase assay (Fendler et al., 1985; Nagel et al., 1987). A schematic representation of the experimental situation is given in Fig. 1.

The two membranes in series are capacitatively and DC coupled, in the absence and the presence of the ionophores, respectively. The pump current can be described as follows (Bamberg et al., 1979):

$$I(t) = I_\infty + (I_0 - I_\infty)\exp(-t/\tau) \tag{1}$$

$$I_0 = I_{p0}\frac{C_m}{C_m + C_p} \tag{2}$$

$$I_\infty = I_{p0}\frac{G_m}{G_m + G_p + \frac{I_{p0}}{V^*}} \tag{3}$$

$$\tau = \frac{C_m + C_p}{G_m + G_p + \frac{I_{p0}}{V^*}} \tag{4}$$

where I_∞ is the current at $t \to \infty$, I_0 is the initial current at $t = 0$, I_{p0} is the pump current and V^* a constant. G_m and G_p are the conductances of the planar film and the adsorbed membrane sheets, respectively. C_m and C_p are the corresponding electrical capacitances. τ is the RC time of the system, which depends on the pump current I_{p0}. The discharging time is given by

$$\tau_{off} = \frac{C_m + C_p}{G_m + G_p} \quad (5)$$

which is equal to $\tau(I_{p0} \to 0)$.

Chemicals

P^3-1-(2-nitro)phenylethyladenosine-5-triphosphate (caged ATP) was prepared according to Kaplan et al. (1978) and Fendler et al. (1985). Phosphatidylserine from bovine brain and synthetic diphytanoyl-lecithin were purchased from Avanti Lipid Products, Birmingham, AL. The luciferin-luciferase assay kit was obtained from Boehringer, Mannheim, FRG. DTT (dithiothreitol) was obtained from Roth, Karlsruhe, FRG. The UV light-insensitive protonophore 1799 (1,1,1,7,7,7-hexafluoro-2,6-*bis* (trifluoromethyl) hepane-4-one was kindly provided by Dr. P. Heytler, DuPont de Nemours. Monensin was a gift from Dr. G. Szabo, Galveston, TX. Omeprazole was a gift from B. Wallmark, Mölndal, Sweden. All other reagents were analytical grade or suprapur standard (Merck, Darmstadt, FRG).

Results

Demonstration of ATP-Dependent Currents

H^+K^+-ATPase membrane discs were added together with caged ATP under stirring to one side of the lipid bilayer membrane. After 20 min the first UV light flash was applied in order to produce a concentration jump of ATP from caged ATP. Under physiological ion conditions (20 mM K^+, 120 mM Na^+, 3 mM Mg^{2+}, 50 mM imidazole (pH 7.0)) no electrical response was observed at all. A transient biphasic electrical current, however, was detected when K^+ was absent, indicating a charge movement during the phosphorylation process (Fig. 2, trace *a*). The sign of the transient current was the same in all experiments. The first phase corresponded to a movement of positive charges towards the protein-free side of the planar lipid membrane. The oppositely directed current phase disappeared after the addition of the proton-conducting system 1799 plus monensin, so that this component may represent the discharging of the membrane capacitors governed by the *RC* time constant (*see* Materials and Methods). The UV-insensitive protonophore 1799 together with the H^+, Na^+ exchanging carrier monensin was applied in previous work for the Na^+K^+-ATPase in order to increase the electrical permeability of the underlying lipid film in the sandwich-like structure as shown in Fig. 1 (Fendler et al., 1985; Nagel et al., 1987). This was necessary for the demonstration of the stationary pump currents. Trace *b* in Fig. 2 shows that in the presence of the ionophores the H^+K^+-ATPase does not produce a stationary pump current. Successive additions of K^+, which increases the speed of dephosphorylation, decreased the peak current but did not create stationary currents (*data not shown*), reflecting the electroneutral exchange of potassium ions and protons by the pump. The peak current disappears after addition of 50 mM K^+ which can be explained by the kinetics of the pump cycle (*see below*). The ATP-induced current is abolished completely by the addition of vanadate (trace *c* in Fig. 2). The blockade by vanadate occurs only when the agent is added to the protein-containing side of the membrane. Successive additions of caged ATP and vanadate to the protein free side had no effect on the signal, showing that the membrane fragments are adsorbed to the lipid film rather than integrated into it. In the latter situation, the oppositely oriented fraction of membrane fragments should show the usual signal in response to ATP and its blockade by vanadate.

As shown in previous papers, involving the Na^+K^+-ATPase, different control experiments were carried out to exclude possible artifacts by the photoreaction of caged ATP (Fendler et al., 1985; Nagel et al., 1987). In brief, the following controls also were made for the H^+K^+-ATPase:

(i) The pure lipid bilayer did not show any effect after a UV flash of 125 msec and 3.7 W/cm^2 light intensity.

(ii) The bilayer with adsorbed protein fragments showed no effect in the absence of caged ATP.

(iii) The bilayer in presence of 50 μM caged ATP alone showed a small (10 pA/cm^2) photoeffect, which is due to the protonation of the lipid bilayer and the concomitant capacitive current. One H^+ per molecule caged ATP was released during the photoreaction. This current depended strongly on the buffer concentration and disappeared at concentrations of about 100 mM imidazole at pH 7.0 (Christensen et al., 1988).

(iv) The addition of the ionophores 1799 and monensin did not cause further photoeffects.

(v) Prior addition of ATP (100 μM) in absence of K^+ to the lipid bilayer with adsorbed H^+K^+-ATPase abolished the electrical response after an ATP-releasing flash because under these conditions all of the enzyme was already in the phosphorylated form.

Ion Selectivity, ATP, ADP and P_i Dependency and the Inhibitory Effect of Caged ATP on the Transient Current

Figure 3 shows the dependence of the transient current on the concentration of ATP. The two traces were obtained in different ways. In trace *a*, increasing amounts of caged ATP were added to the protein-containing side, where the conversion rate of

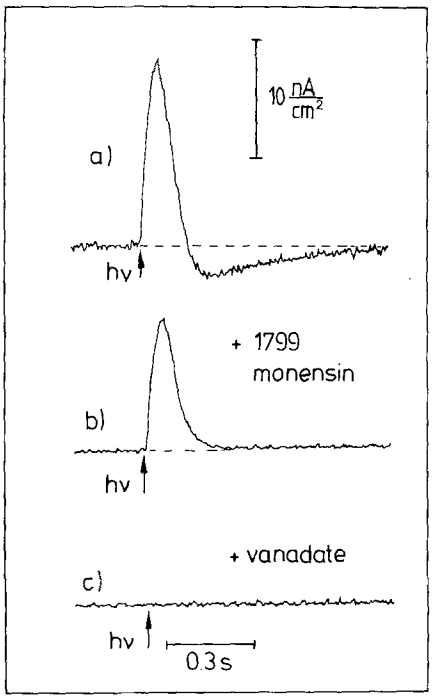

Fig. 2. Short-circuit current on the lipid bilayer system under different experimental conditions. The membrane bathing solution contained 3 mM $MgCl_2$, 50 mM imidazole pH 6.0 and 100 μM caged ATP (conversion rate 30%). The light intensity was 3.7 W/cm², and the flash duration was 125 msec. The arrow indicates the opening of the shutter. Trace *a*: Transient current after addition of 25 μl purified H^+K^+-ATPase 5, mg protein/ml. Trace *b*: Pump current after addition of 10 μm monensin and 1 μm 1799. Trace *c*: Blockade of the transient current by 20 μm vanadate

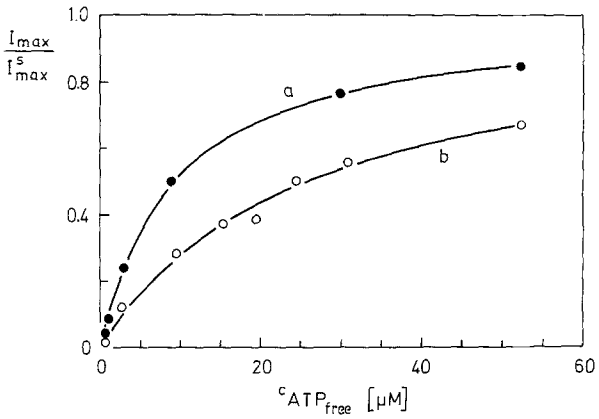

Fig. 3. ATP dependence of the peak current measured by two different procedures. Electrolyte composition was the same as in Fig. 2. Trace *a*: The conversion rate of caged ATP was kept at 30%; variation of released ATP was obtained by addition of caged ATP. Trace *b*: Initial concentration of caged ATP was kept at 100 μM. The release of ATP from caged ATP was varied by changing the rate of photolysis. UV light was attenuated with UV filters. The peak current was normalized to $I^s_{max} = 1$ which is the saturating current response at $c_{ATP} \rightarrow \infty$. Trace *a*: $K_{0.5} = 9.4$ μM. Trace *b*: $K_{0.5} = 25$ μM

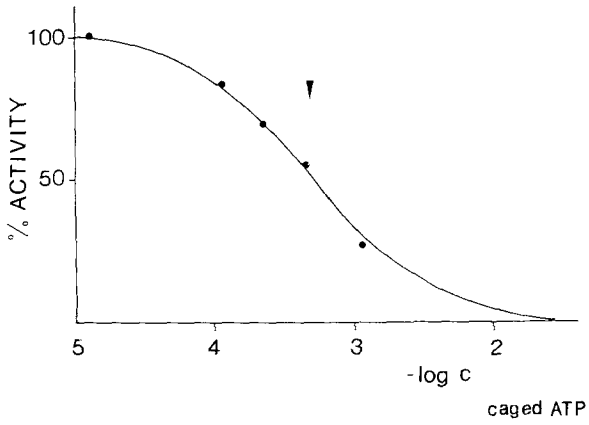

Fig. 4. Inhibition of enzymatic H^+K^+-ATPase activity at pH 7.5 by the Tris salt of caged ATP at 37°C: Plot of % activity in dependence of the negative logarithm of caged ATP concentration. The solid line corresponds to a theoretical inhibition curve with an equilibrium constant of 5×10^{-4} M at 5 mM ATP. Further details are given in Materials and Methods

ATP from caged ATP was kept constant at 30%. Trace *b* represents the ATP dependency when 100 μM caged ATP was added and the conversion rate increased by increasing light intensity. In the second of these two procedures the ratio of caged ATP to ATP was changed, so that an inhibitory effect of caged ATP, if present, should be detectable. As seen in Fig. 3, the two traces do not differ substantially so that an inhibitory effect of caged ATP on the enzyme appears neglegible. Depending on the experimental procedure, an apparent K_m for ATP of 9.4 μM (trace *a*) and 25 μM (trace *b*) was found. In parallel, the effect of caged ATP was studied in an enzymatic assay. The ATPase activity was measured at a fixed concentration (5 mM) and increasing amount of caged ATP. An inhibition constant of the enzyme activity with an $I_{0.5}$ of 5×10^{-4} M was found, which is 100 times bigger than the $K_{0.5}$ for ATP. (Fig. 4). This result indicates that the inhibition of the ATPase activity by caged ATP is neglegible for the H^+K^+-ATPase. The inhibitory effect, however, should be taken in account for a kinetic analysis as shown for the Na^+K^+-ATPase (Borlinghaus et al., 1987; Fendler et al., 1987).

The ion selectivity of the electrical signal with respect to monovalent cations is represented in Fig. 5. Sodium ions have no effect on the current response of the enzyme in the measured concentration range from 0–9 mM. K^+ and Rb^+ show a decrease of the peak current with an apparent inhibition constant of 3 mM at 30 μM released ATP.

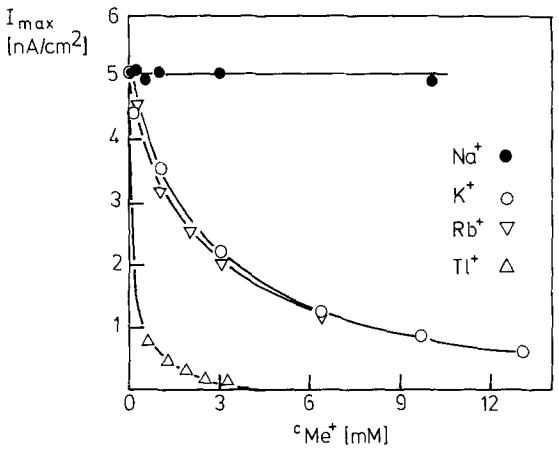

Fig. 5. Dependency of the peak current on monovalent cations. The cuvette contained 100 μM caged ATP, and the conversion rate was 30%. Purified membrane sheets (125 μg) containing H^+K^+-ATPase were added to the membrane. The electrolyte composition was the same as in Fig. 2 except for the additional monovalent cations Na^+ (●), K^+ (○), Rb^+ (▽) and Tl^+ (△) at the indicated concentrations

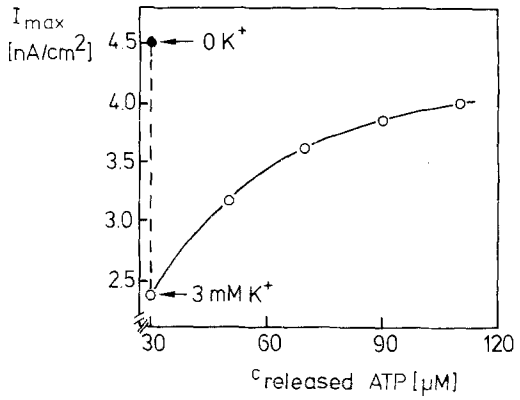

Fig. 6. Demonstration of the decrease in the affinity for ATP in presence of K^+. In the presence of 100 μM caged ATP, and a conversion rate of 30%, and in the absence of K^+, the peak current was measured (●); addition of 3 mM K^+ reduced the peak current (○). Thereafter the peak current was measured after subsequent additions of caged ATP. The initial electrolyte composition was the same as in Fig. 2

This effect is more pronounced for Tl^+ ions with an apparent inhibition constant of 100 μM.

The inhibition can partially be explained by a decrease in the affinity of the ATP-binding site for the substrate produced by cations like K^+, Rb^+ and Tl^+ (Schrijen et al., 1980). In the absence of K^+ and in presence of 30 μM ATP (from 100 μM caged ATP), saturating for phosphorylation, the peak current was measured (Fig. 6). Then 3 mM K^+ was added which reduced the peak amplitude to the half-maximal value. Further release of ATP from caged ATP up to 120 μM restored the current response almost completely (Fig. 6). When, however, a similar experiment was carried out with 50 mM instead of 3 mM K^+, the peak current was abolished completely and could not be restored by concentrations of released ATP up to 200 μM (*data not shown*). This can be understood on the basis of the kinetics of the proton pump cycle (*see* Discussion).

The H^+K^+-ATPase is a Mg^{2+}-dependent pump. Therefore the removal of Mg^{2+} should abolish the transient current response. In absence of Mg^{2+} no current was obtained. The dependency of the transient current on the free Mg^{2+} concentration which was adjusted with EDTA is shown in Fig. 7. An apparent K_m for Mg^{2+} was found to be 27 μM. At high concentrations of free Mg^{2+}, a slight decrease of the current could be observed, which might be related to lower affinity for ATP (Fig. 8*a*). It is interesting that the affinity for ATP ($K_{0.5}$ = 0.9 μM) is considerably higher in presence of low Mg^{2+} (10 μM) than at a concentration of 3 mM which gives a $K_{0.5}$ of 10.2 μM (Fig. 8*a*). The different ATP affinities at different Mg^{2+} concentrations can be tentatively explained, by assuming that ATP binds first and then Mg^{2+} or vice versa, but that MgATP is not bound to the enzyme or that it has a lower affinity than ATP.

The electrical signal is inhibited by the addition of ADP. The inhibition is slightly dependent on the free Mg^{2+} concentration such that at high Mg^{2+} a higher affinity for ADP is found compared to low Mg^{2+} concentrations (Fig. 8*b*).

That the electrical signal represents a part of the pump cycle of the H^+K^+-ATPase is further demonstrated by the inhibition of the signal by inorganic phosphate (P_i). Figure 9 shows the decrease of the electrical current after successive addition of P_i. This inhibition may result from the ability of P_i to convert the enzyme into its phosphorylated state (E_2P). For further details, *see also* Discussion and Fig. 12.

Finally, the pH dependency of the electrical signal was measured. A maximum for the current was obtained at pH 5.8 (Fig. 10) which is in agreement with the pH optimum of 6.0 for the phosphorylation reaction (Stewart, Wallmark & Sachs, 1981).

The Effect of the H^+K^+-ATPase Specific Inhibitor Omeprazole on the ATP-Induced Transient Current

Omeprazole is a specific inhibitor of the gastric H^+K^+-ATPase. Addition of omeprazole (30 μM) to

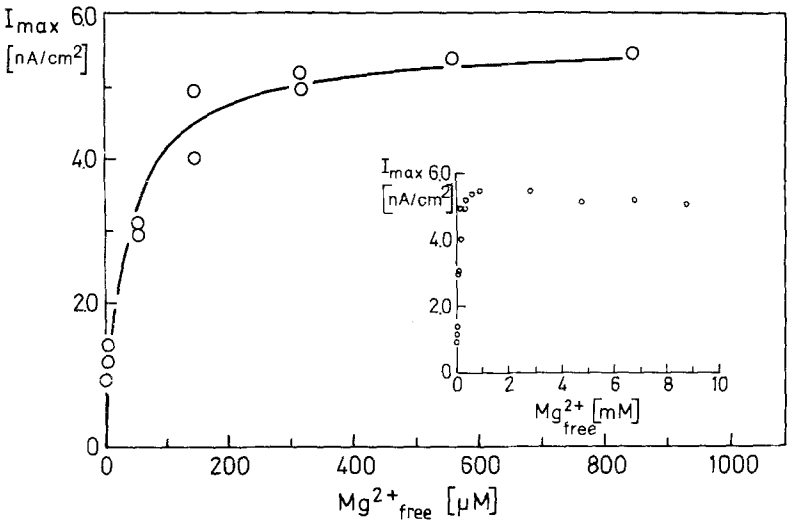

Fig. 7. Mg^{2+} dependency of the peak currents. Electrolyte composition: Imidazole 50 mM, pH 6.0. After adsorption of the membrane fragments to the planar lipid bilayer 10 mM EDTA was added. Thereafter 100 μM caged ATP (conversion rate 30%) was added and the peak current was measured at increasing concentrations of free Mg^{2+}. A $K_{0.5}$ of 27 μM was obtained. The inset shows the dependency up to 10 mM Mg^{2+}

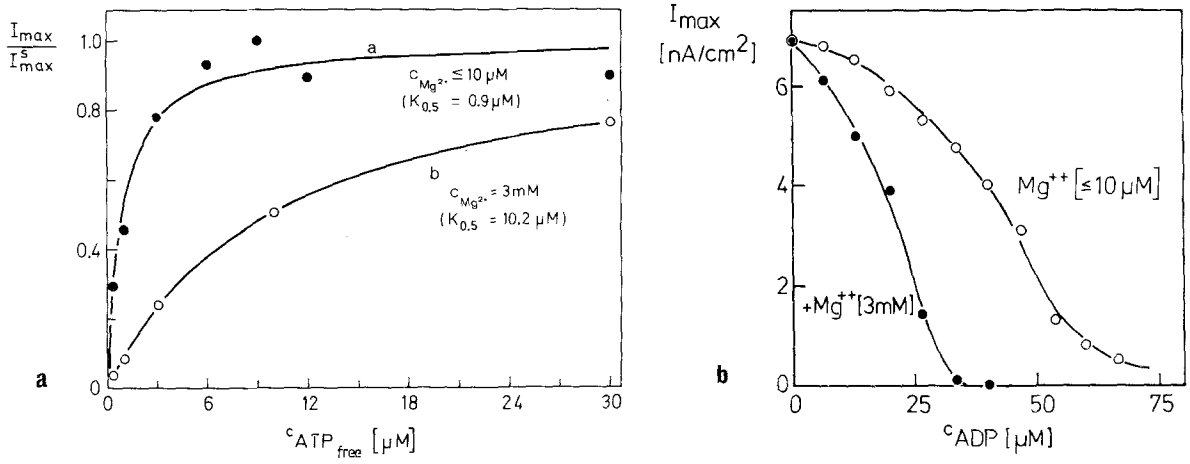

Fig. 8. (*a*) ATP dependency of the peak current at different concentrations of free Mg^{2+}. Trace *a*: ≤10 μM free Mg^{2+}. Trace *b*: 3 mM free Mg^{2+}. Other conditions as in Fig. 2. The peak current was normalized to $I^s_{max} = 1$, which is the saturating current response at $c_{ATP} \rightarrow \infty$. A $K_{0.5}$ of 0.9 μM (trace *a*) and 10.2 μM (trace *b*) was obtained. (*b*) Decrease of the peak current by ADP. Caged ATP (100 μM) was used (conversion rate 30%). Three mM $MgCl_2$ and 50 mM imidazole were present throughout pH 6.0; the peak currents were measured at ADP concentrations as indicated (●). In absence of added Mg^{2+} the experiment was repeated (○). The latter trace (○) was normalized to the first one (●)

the protein-containing compartment of the cuvette indeed abolished the peak current almost completely (Fig. 11). The lipid bilayer system offers the possibility of studying the mechanism of activation of the lipophilic compound. In the following experiments, a membrane-bound mechanism for the activation (protonation) of the inhibitor was tested. The possibility of activation by acid groups of the enzyme which can react with omeprazole during the H^+ pumping phase of the reaction cycle was considered. Such acid groups seem to be necessary because protons are released into the lumen, where the pH is 1. The groups should be located close to the extracellular side, which faces in our experiments the planar lipid film. According to the sign of the current the protons are moved into the interfacial space. In the absence of omeprazole and K^+, repetitive measurements of the transient current gave reproducible signals after 5 min with an accuracy of 5–10%. Then 30 μM omeprazole was added to the protein-free side of the membrane and the pump was activated by a light-induced concentration jump of ATP from caged ATP. After 27-min incubation with omeprazole, the current amplitude was reduced to 20–30% compared with the original value in the omeprazole-free medium. Further UV flashes releasing ATP increased the steepness of the time-dependent inactivation curve drastically. Fi-

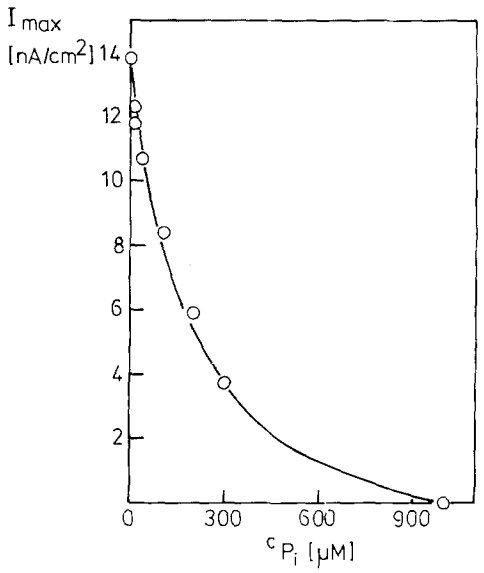

Fig. 9. Decrease of the peak current by inorganic phosphate (P_i). Using the conditions described in Fig. 2, the peak current was measured in presence of added P_i at the indicated concentrations

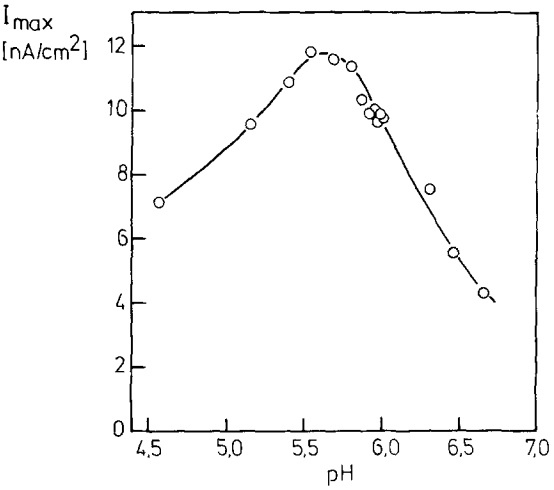

Fig. 10. pH dependence of the peak current in the presence of 100 μM caged ATP (conversion rate 30%). The data were obtained on the same membrane. The pH was varied in both compartments by addition of NaOH and H_2SO_4, respectively. Starting conditions: 50 mM imidazole, 3 mM Mg^{2+}, pH 5.99

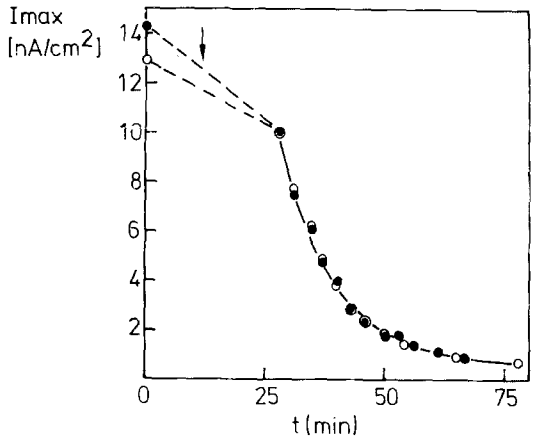

Fig. 11. Inhibition of the peak current in presence of 30 μM omeprazole, without (○) and with (●) the H^+, Na^+ exchanging carrier monensin (10 μM); 10 mM NaCl was present. The arrow indicates the addition of omeprazole. The curves are normalized to each other. Other conditions as in Fig. 2

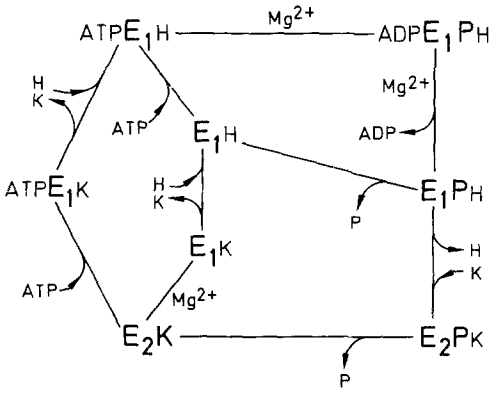

Fig. 12. Representation of the reaction cycle of the gastric H^+K^+-ATPase in a modified Albers-Post scheme according to Stewart et al., 1981; Sachs, Faller & Rabon, 1982

nally, after about 10 flashes, the current disappeared almost completely (Fig. 11). The repetition of the same experiment in presence of 3 mM K^+ did not show the increased inhibition. Addition of 1 mM DTT to the protein-containing compartment protected the enzyme partially from the inhibition by omeprazole (*data not shown*). Addition of monensin to both sides of the membrane in order to release transported protons from the interstitial space did not change the inhibition curve. These results suggest that a drop in the pH of the interstitial space is not the main reason for inactivation of the pump by omeprazole and that activation of the drug may occur at neutral pH.

Discussion

The pump cycle of the H^+K^+-ATPase can be described like the cycle of the Na^+K^+-ATPase by the Albers-Post scheme, shown in Fig. 12. Many similarities between the two enzymes are apparent. One of the main differences, however, is that the Na^+K^+-ATPase is an electrogenic pump, where the Na^+ transport contributes to the electrogenicity

(Fendler et al., 1985; Gadsby et al., 1985; Karlish, Raphaeli & Stein, 1985, Borlinghaus et al., 1987; Nagel et al., 1987). The potassium transport in the sodium pump is electroneutral (Karlish et al., 1985; Goldschlegger et al., 1987; Bahinski, Nakao & Gadsby, 1988). In this paper, the transient current generated during phosphorylation of the electroneutral H^+K^+-ATPase were studied. This was possible by separation of the H^+-dependent phosphorylation step from the K-dependent dephosphorylation step. The results show that phosphorylation of the pump by ATP in the absence of K^+ produces a transient biphasic electrical response, suggesting that proton translocation is electrogenic. As a direct consequence of these experiments, the transport of potassium must be electrogenic, too, since no net stationary current was obtained, when H^+ and K^+ were both present.

These experiments strongly support sequential transport of H^+ and K^+ ions and thus any model including the Albers-Post scheme, which is based on sequential transport. In principle, after a sudden release of ATP a transient electrical current either monophasic or biphasic should occur reflecting the H^+ and K^+ translocation which are oppositely directed. Considering the data obtained from the kinetics of enzymatic reactions it seems understandable that no current occurs in presence of high potassium and high ATP. At high potassium concentration the enzyme is in the E_2K or E_1K form with a low affinity for ATP. The transition of E_2K (E_1K) $\xrightarrow{ATP}$ E_2K ATP (E_1K ATP) is slow (250 min^{-1}), whereas the subsequent transition from E_1 ATP $\rightarrow$ E_1P + ADP is fast (4400–7900 min^{-1}) (Wallmark & Mårdh, 1979; Ljungström, Vega & Mårdh, 1984). A cycle starting with enzyme in the E_2K form will not give an electrical response, since a slow process (oppositely directed electrogenic or electroneutral) precedes the fast H^+-dependent electrogenic step. The demonstration of the proton transporting step in the absence or at low concentrations of potassium shows that the reaction under these conditions always starts in the E_1H form (Fig. 12).

Since only counter-transported ions like K^+, Rb^+ and Tl^+ in contrast to Na^+ have an effect on the electrical response and no stationary currents can be observed, one may conclude that the electrical signal represents proton transport and not a dipole movement during a conformational change within the protein. The ion specificity of the electrical current shown in Fig. 5 agrees well with data obtained by Ray and Forte (1976), and Sachs et al. (1976), who showed that the reduction of the phosphorylated intermediate decreased with the same specificity for Na^+, K^+, Rb^+, and Tl^+.

The Effect of Mg^{2+}: *ATP*, *ADP*, and P_i

Mg^{2+} influences the activation of the transient current with respect to the affinity of ATP (Fig. 8*a*). The high affinity for ATP at low concentrations of Mg^{2+} suggests that the affinity for ATP is higher than for MgATP. Since the enzyme contains tightly bound Mg^{2+} (50 nmol/mg protein) for steric reasons it is expected to bind ATP better to the enzyme than preformed MgATP. Similar results were obtained in ATP-dependent proton transport studies on membrane vesicles (Ljungström & Mårdh, 1985). These results agree within a factor of 3 with the electrical measurements presented above. The affinity of ADP shows the opposite Mg^{2+} dependence to that of ATP at high and low concentrations of Mg^{2+} (Fig. 8*b*). In presence of high Mg^{2+} (3 mM) the high affinity for ADP can be explained by a Mg^{2+}-dependent binding to the E_1P form. Then the inhibition of the current by ADP can be explained by two mechanisms: (i) competition of ADP with the ATP binding site E_1H^+ or (ii) binding of ADP to the E_1P form which drives the back reaction.

The inhibition of the electrical current by inorganic phosphate P_i reflects the formation of E_2P. The $K_{0.5}$ of 150 μM is in good agreement with a $K_{0.5}$ of 60 μM obtained from phosphorylation experiments (Jackson & Saccomani, 1984).

The Effect of Omeprazole

Omeprazole is a specific inhibitor of the gastric H^+K^+-ATPase. At neutral pH omeprazole has hydrophobic properties and is membrane permeable so that it can penetrate into the ATPase-rich vesicles. The inhibitory activity under these circumstances is low. Activation of the pump by ATP acidifies the intravesicular medium. Thereby omeprazole is decomposed at pH 4 to a hydrophilic compound (pK_a = 4) which is trapped within the vesicles. In its decomposed form omeprazole develops full inhibitory activity as an SH reagent. (Fellenius et al., 1981; Larsson et al., 1983; Im et al., 1984; Wallmark, Brandström & Larsson, 1984; Lindberg et al., 1986). Results of the present study suggest that inactivation of the H^+K^+-ATPase is not necessarily dependent on being in a low pH aqueous compartment. The experiment shown in Fig. 11 raises the question if omeprazole in addition to its efficacy at low pH can be activated by the pump itself. In an almost perfectly buffered system the inactivation increases the more the pump is inactivated. The addition of the H^+Na^+ exchanging carrier monensin which removes a possible H^+ gradient from the interstitial space did not change the

inhibition curve. Control experiments with the light-driven proton pump bacteriorhodopsin showed an inhibition of the pump by acidification of the interstitial space between the adsorbed purple membranes and the underlying lipid film. This effect could completely be removed by the addition of the ionophoric system monensin plus the protonophore 1799 (*data not shown*). As shown in Fig. 11 the addition of the same ionophoric system had no influence on the inactivation by omeprazole, so that in this particular experiment activation of the drug by acidification of the interstitial space might be excluded. In other words a blockade of the H^+K^+-ATPase by omeprazole close to neutral pH (pH = 6.4) is possible and can be explained as follows: In the inactive hydrophobic form omeprazole is expected to be located in the membrane. During the H^+ transport step of the H^+K^+-ATPase, possibly extremely acidic groups may be exposed to the membrane interface, which are not accessible to the well-buffered aqueous bulk phase. This is reasonable because the pump releases protons into a medium of pH 1. These groups might initiate the H^+-dependent decomposition of omeprazole, so that the inhibition occurs after activation of the pump during the formation of E_1P or E_2P. The experiments were carried out at zero K^+ which means that the phosphorylated form remains stable for a longer period of time than under physiological conditions so that an increased inhibition is probable. This is supported by experiments where in the presence of 3 mM K^+ an increased inhibition at pH 6.4 was not observed, where dephosphorylation decreases the concentration of the phosphorylated intermediates.

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