The basal Mg^{2+} -dependent ATPase activity is not part of the $(\text{H}^+ + \text{K}^+)$ -transporting ATPase reaction cycle

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Purified gastric $(\text{H}^+ + \text{K}^+)$ -transporting ATPase [$(\text{H}^+ + \text{K}^+)$ -ATPase] from the parietal cells always contains a certain amount of basal Mg^{2+} -dependent ATPase (Mg^{2+} -ATPase) activity. *lin*-Benzo-ATP (the prefix *lin* refers to the linear disposition of the pyrimidine, benzene and imidazole rings in the 'stretched-out' version of the adenine nucleus), an ATP analogue with a benzene ring formally inserted between the two rings composing the adenosine moiety, is an interesting substrate not only because of its fluorescent behaviour, but also because of its geometric properties. *lin*-Benzo-ATP was used in the present study to elucidate the possible role of the basal Mg^{2+} -ATPase activity in the gastric $(\text{H}^+ + \text{K}^+)$ -ATPase preparation. With *lin*-benzo-ATP the enzyme can be phosphorylated such that a conventional phosphoenzyme intermediate is formed. The rate of the phosphorylation reaction, however, is so low that this reaction with subsequent dephosphorylation cannot account for the much higher rate of hydrolysis of *lin*-benzo-ATP by the enzyme. This apparent kinetic discrepancy indicates that *lin*-benzo-ATP is not a substrate for the $(\text{H}^+ + \text{K}^+)$ -ATPase reaction cycle. This idea was further supported by the finding that *lin*-benzo-ATP was unable to catalyse H^+ uptake by gastric-mucosa vesicles. The breakdown of *lin*-benzo-ATP by the $(\text{H}^+ + \text{K}^+)$ -ATPase preparation must be due to a hydrolytic activity which is not involved in the ion-transporting reaction cycle of the $(\text{H}^+ + \text{K}^+)$ -ATPase itself. Comparison of the basal Mg^{2+} -ATPase activity (with ATP as substrate) with the hydrolytic activity of $(\text{H}^+ + \text{K}^+)$ -ATPase using *lin*-benzo-ATP as substrate and the effect of the inhibitors omeprazole and SCH 28080 support the notion that *lin*-benzo-ATP is not hydrolysed by the $(\text{H}^+ + \text{K}^+)$ -ATPase, but by the basal Mg^{2+} -ATPase, and that the activity of the latter enzyme is not involved in the $(\text{H}^+ + \text{K}^+)$ -transporting reaction cycle (according to the Albers-Post formalism) of $(\text{H}^+ + \text{K}^+)$ -ATPase.

INTRODUCTION

The basal Mg^{2+} -dependent ATPase (Mg^{2+} -ATPase) activity is a hydrolytic activity that always occurs in preparations of gastric $(\text{H}^+ + \text{K}^+)$ -transporting ATPase [$(\text{H}^+ + \text{K}^+)$ -ATPase] [1–3]. This activity is, like $(\text{H}^+ + \text{K}^+)$ -ATPase, dependent on Mg^{2+} , but is not activated by K^+ and is assumed not to be involved in active cation transport. The basal Mg^{2+} -ATPase activity can only be partially removed from the preparation by further purification. It is, however, impossible to isolate $(\text{H}^+ + \text{K}^+)$ -ATPase totally free of this basal Mg^{2+} -ATPase activity. An important question

is whether this activity originates from a phosphatase that is co-purified with the enzyme or is intrinsic to the enzyme itself. In the latter case the hydrolytic activity could be due to spontaneous dephosphorylation of the phosphorylated $(\text{H}^+ + \text{K}^+)$ -ATPase (Scheme 1) or to a phosphatase activity of the enzyme which is not part of the reaction cycle involved in cation transport.

Experiments with substrate analogues can give important information in a study directed towards the mechanism of an enzyme. For ATPases, such as $(\text{H}^+ + \text{K}^+)$ -ATPase, ATP analogues fulfil such a rôle [4,5]. *lin*-Benzo-ATP (Fig. 1), an analogue in which the adenine ring is laterally extended by the formal insertion of a benzene ring into the centre of the purine-ring system [6,7], has been shown to be an interesting substrate for several kinases [8,9] and for mitochondrial ATPase [10], primarily because of its fluorescent properties.

Scheme 1. Modified Post-Albers scheme of the reaction cycle of $(\text{H}^+ + \text{K}^+)$ -ATPase

a Depicts a possible route for K^+ -independent P_i formation.

Fig. 1. Structure of *lin*-benzo-ATP

Abbreviations used: the prefix *lin* refers to the linear disposition of the pyrimidine, benzene and imidazole rings in the 'stretched-out' version of the adenine nucleus in *lin*-benzo-adenine (systematic name 8-aminoimidazo[4,5-*g*]quinazoline); Mg^{2+} -ATPase, Mg^{2+} -dependent ATPase; $(\text{H}^+ + \text{K}^+)$ -ATPase, $(\text{H}^+ + \text{K}^+)$ -transporting ATPase; I_{50} , concentration causing 50% inhibition; $\text{K}_{0.5}$, concentration causing 50% stimulation; K_d , dissociation constant.

lin-Benzo-ATP was supposed to be a proper substrate analogue for $(H^+ + K^+)$ -ATPase. We therefore studied *lin*-benzo-ATP as substrate analogue for the hydrolysis and phosphorylation reaction of $(H^+ + K^+)$ -ATPase. Surprisingly, the pseudo-substrate, which is, apart from the spatial extension, equivalent to the natural substrate, had a disappointingly low affinity for this enzyme in the phosphorylation reaction. On the other hand, however, it could be used as a tool to distinguish between $(H^+ + K^+)$ -ATPase and the basal Mg^{2+} -ATPase activity accompanying the former enzyme. Surprisingly $(H^+ + K^+)$ -ATPase could be phosphorylated by *lin*-benzo-ATP, but the hydrolysis of the latter substrate must attributed to the Mg^{2+} -ATPase activity. This apparent contradictory phenomenon has been studied in further detail here.

MATERIALS AND METHODS

Preparation of the $(H^+ + K^+)$ -ATPase-containing vesicles and membrane sheets

$(H^+ + K^+)$ -ATPase from pig stomach was prepared as described previously [11] 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 20000 *g* (Sorvall SS 34 rotor). The resulting supernatant was centrifuged for 45 min at 100000 *g* (MSE 8 × 50 rotor). The pellet was resuspended in 25 mM-Tris/HCl, pH 7.2, and centrifuged for 60 min at 100000 *g* on top of a gradient consisting of 7% (v/v) Ficoll/250 mM-sucrose in 20 mM-Tris/HCl over 37% (w/v) sucrose in 20 mM-Tris/HCl. The buffer/Ficoll interface consisted of closed vesicles, and the Ficoll/sucrose interface consisted of broken membrane sheets. For transport studies the vesicle fraction was pelleted (60 min at 38000 rev./min in an MSE 8 × 50 rotor). Thereafter the pellet was resuspended in buffer solution, preincubated overnight in an appropriate medium and used in the transport assay the next day. For all other assays both fractions were diluted in 20 mM-Tris/HCl before centrifuging for 60 min at 10000 rev./min (MSE 8 × 50 rotor). The resulting supernatant was once again centrifuged at 38000 rev./min (MSE 8 × 50 rotor). The four different pellets were resuspended in 20 mM-Tris/HCl and frozen at -20°C . After two or three freezing-and-thawing steps the vesicles were broken and the ATPase activities of the 10000 *g* fractions ranged from 20 to 60 and those from the 38000 *g* fraction from 60 to 120 $\mu\text{mol/h}$ per mg.

Synthesis of *lin*-benzo-ADP and *lin*-benzo-ATP

The Tris salts of these components were synthesized by the method of Leonard *et al.* [6,9].

Synthesis of *lin*-benzo- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

This labelled compound was prepared by the method of Glynn & Chappell [12] as modified by De Meis [13] for preparation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. To 3.25 ml of a reaction mixture (containing 12 mM- MgCl_2 , 4 mM-cysteine, 2 mM-EGTA and 100 mM-Tris/HCl, pH 8.6) 3.5 mg of *D*-(-)-3-phosphoglycerate (7 μmol ; tricyclohexylammonium salt), 2.4 mg of NADH (3.6 μmol of the Tris salt) and 0.2 ml of 100 mM-Tris/*lin*-benzo-ATP solution were added, the final volume being adjusted to 5 ml with water. The enzymic reaction was started by addition of 75 μl of 2-mercaptoethanol, 10 μl of muscle glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) in 0.25 ml of water, 5 μl of yeast phosphoglycerate kinase (10 mg/ml) in 0.25 ml of water and 1 ml of $^{32}\text{P}]\text{P}_i$ (10 mCi in water). The reaction mixture was stirred during 16 h at room temperature. The reaction was stopped by heating the reaction mixture at 80°C for 1 min, followed by immediate cooling on ice. Subsequently the mixture was filtered (Millex-GS

0.22 μm -pore-size Millipore filter) and diluted with 25 ml of water. The *lin*-benzo- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was separated by a column of Dowex-1X8 resin as described by Glynn & Chappell [12]. About 42% of the ^{32}P was eluted in the fraction containing the labelled *lin*-benzo-ATP. H.p.l.c. analysis (reverse-phase column filled with ODS Hypersil; 5 μm particle size) showed a contamination with $18 \pm 1\%$ *lin*-benzo-ADP and $3 \pm 1\%$ $^{32}\text{P}]\text{P}_i$. The contamination of *lin*-benzo-ADP is due to the settlement of the equilibrium in the enzymic conversion. The ion-exchange column was apparently not able to remove the remaining *lin*-benzo-ADP formed during the enzymic conversion. However, in the experiments the labelled *lin*-benzo-ATP was considerably diluted with unlabelled *lin*-benzo-ATP so that the concentration of *lin*-benzo-ADP was less than 2%.

Phosphorylation experiments

Phosphorylation of the $(H^+ + K^+)$ -ATPase by *lin*-benzo-ATP and ATP was carried out at 22°C at pH values as indicated in the text. The labelled *lin*-benzo-ATP and ATP concentrations were as indicated in the legends to the Figures. The reaction was started by rapid mixing of 10 μl of $(H^+ + K^+)$ -ATPase suspension (preincubated with 0.2 mM-ouabain if not indicated otherwise) with 90 μl of the medium containing ATP or *lin*-benzo-ATP and the other ligands. The reaction was stopped after 30 s (unless indicated otherwise) by addition of 3 ml of 5% (w/v) trichloroacetic acid, containing 100 mM-phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm -pore-size Selectron filter (Schleicher und Schuell, Dassel, Germany), which was then washed three times with 3 ml of the stopping solution. Incorporated ^{32}P was determined by liquid-scintillation counting. For obtaining blank values, experiments were carried out in which the $(H^+ + K^+)$ -ATPase suspension was mixed with the stopping solution before addition of the substrate.

Phosphorylation of $(H^+ + K^+)$ -ATPase by $^{32}\text{P}]\text{P}_i$ was carried out by a method similar to that used by Helmich-de Jong *et al.* [14].

Dephosphorylation

After phosphorylation for 30 s at room temperature the dephosphorylation reaction was carried out as described before [14].

The sensitivity of the phosphoenzymes to hydroxylamine was determined as described previously [15].

Hydrolysis of the substrates

The ATP hydrolysis was determined as the release of $^{32}\text{P}]\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or *lin*-benzo-ATP [16]. To 10 μl of $(H^+ + K^+)$ -ATPase suspension 190 μl of a medium containing cations and buffer solution, ouabain (0.2 mM) and labelled ATP or *lin*-benzo-ATP were added, and this mixture was incubated at 37°C . Incubation times were short (< 1 min), and the amount of P_i formed was never more than 30% of the original substrate concentration. Several time points were used for sampling, and linearity was preserved in all cases. For obtaining blank values, experiments were performed in which $(H^+ + K^+)$ -ATPase was denatured with trichloroacetic acid before incubation with the assay medium. The $^{32}\text{P}]\text{P}_i$ formation was measured after stopping the reaction at a given time by the addition of 0.4 ml of 10% trichloroacetic acid, followed by mixing with 0.4 ml of aq. 20% (w/v) charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly for 10 s, and this was repeated after 5 and 10 min. Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 *g* at 4°C . Aliquots (0.2–0.5 ml) were taken from the supernatant and radioactivity was measured by Čerenkov counting in a liquid-scintillation analyser.

H⁺-uptake measurements

The uptake of protons from the extravesicular medium was measured at 20 °C by recording the changes in the medium pH after addition of the substrate, namely ATP or *lin*-benzo-ATP [17]. A 2 ml portion of gastric vesicles (2–3 mg of protein), equilibrated overnight at 4 °C in 5 mM-glycylglycine buffer, pH 6.11, containing 150 mM-K⁺, were placed in a small vessel. The suspension was adjusted to pH 6.11 and was magnetically stirred throughout the experiment while the pH was continuously recorded with a Amagruss combined electrode connected to a Radiometer PHM 75 Research pH-meter with a BD 41 recorder (Kipp, Delft, The Netherlands). The reaction was started by adding known volumes of an ATP or *lin*-benzo-ATP solution adjusted to pH 6.11. Nigericin was added from a solution in ethanol to a final concentration of 10 μM.

Binding assay

Binding of *lin*-benzo- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under non-phosphorylating conditions (free $[\text{Mg}^{2+}] = 0$) was measured by a filtration method, as described previously [18]. $[\text{C}^{14}]\text{sucrose}$ was used for correction of non-specific binding to the filter. The 100 μl incubation medium contained 50 mM-imidazole (pH 7.0), 3 mM-EDTA, 1 mM-sucrose and 2 mg of protein/ml and different concentrations of labelled *lin*-benzo-ATP (0.5–30 μM). Blanks containing 10 mM unlabelled ATP were subtracted.

Materials

ATP, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and Tris were purchased from Boehringer, Mannheim, Germany. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{P}^{32}]\text{P}_i$ were obtained from Amersham International, Bucks., U.K., nigericin and Dowex-1X8 resin from Sigma, St. Louis, MO, U.S.A., Ficoll from Pharmacia Fine Chemicals, Uppsala, Sweden, Omeprazole was kindly given by Dr. B. Wallmark, Hässle, Mölndal, Sweden, SCH 28080 was kindly provided by Dr. A. Barnett from Schering-Plough Corporation, Bloomfield, NJ, U.S.A. The monoclonal antibody against (H⁺ + K⁺)-ATPase was provided by Mr. T.J.F. van Uem (Department of Biochemistry, University of Nijmegen, Nijmegen, the Netherlands). All other chemicals were of reagent grade.

RESULTS

Formation of the phospho intermediate by *lin*-benzo-ATP

Phosphorylation of (H⁺ + K⁺)-ATPase with *lin*-benzo-ATP (Fig. 1) yielded a substrate-dependent phospho intermediate up to a steady-state level which reached about 60 % of that obtained with ATP under the same conditions [20 mM-imidazole/HCl (pH 6.0)/3 mM-Mg²⁺]. The steady-state phosphorylation level was not influenced by addition of 1 mM-ouabain, indicating that the observed phosphorylation was not due to phosphorylation of (Na⁺ + K⁺)-ATPase present in the preparation as an impurity. This suboptimal level could only partially be explained by the inhibition of the phosphorylation reaction by *lin*-benzo-ADP present in the substrate preparation. *lin*-Benzo-ADP inhibited the phosphorylation reaction with a concentration causing 50 % inhibition (*I*₅₀) of 150 μM in the presence of 300 μM-*lin*-benzo-ATP. The affinity for *lin*-benzo-ATP (Fig. 2) was very low compared with that for ATP [concentration causing 50 % stimulation (*K*_{0.5}) 110 μM for *lin*-benzo-ATP versus 0.016–0.1 μM for ATP] [14,19]. The dissociation constant (*K*_d) for *lin*-benzo-ATP derived by extrapolation from binding experiments with *lin*-benzo-ATP concentrations up to 100 μM was in the same range (200–400 μM). This extrapolation was based on a maximal binding capacity for ATP of 3 nmol/mg of protein [20].

The most striking deviation from the characteristics of the

Fig. 2. Substrate-dependence of the steady-state phosphorylation level of (H⁺ + K⁺)-ATPase phosphorylated with *lin*-benzo-ATP

A 100 μg portion of (H⁺ + K⁺)-ATPase was incubated at 20 °C in 100 μl of a medium containing 3 mM-Mg²⁺, 0.2 mM-EDTA, 25 mM-imidazole/acetic acid, pH 6.0, with various concentrations of ^{32}P -labelled *lin*-benzo-ATP, and stopped after 30 s with trichloroacetic acid as described in the Materials and methods section. A *K*_{0.5} value of 110 μM was obtained by Lineweaver–Burk analysis (inset). Values are means ± s.d. for five experiments performed in duplicate. (The continuous line was drawn by eye.) In this and subsequent Figures, E-P is the phosphorylation level.

Fig. 3. Time-dependence of the phosphorylation reaction of (H⁺ + K⁺)-ATPase with *lin*-benzo-ATP

(H⁺ + K⁺)-ATPase was incubated as described in Fig. 2 in the presence of 100 μM-*lin*-benzo-ATP. The reaction was stopped after the indicated time intervals. [The continuous line was drawn by eye. A *t*_{1/2} value of 7.45 s was calculated (program DISCRETE 1B; [40]) for a single exponential.]

phosphorylation reaction with ATP was the very low rate (Fig. 3) of the phosphorylation reaction with *lin*-benzo-ATP (*k*_{obs}, 0.1 s⁻¹ for *lin*-benzo-ATP versus 23–130 s⁻¹ for ATP [21,22]). The rate-limiting step in the phosphorylation reaction was not the binding of the substrate, but the phosphorylation step itself. This was shown in an experiment in which the (H⁺ + K⁺)-ATPase was preincubated with *lin*-benzo-ATP in the presence of EDTA and

Fig. 4. K^+ -dependence of the steady-state phosphorylation level

($H^+ + K^+$)-ATPase was phosphorylated in the presence of 100 μM *lin*-benzo-ATP and K^+ concentrations as indicated. Other conditions are as described in Fig. 2. (The continuous line was drawn by eye.)

Mg^{2+} added at zero time. The rate of phosphorylation was equal to that in the experiment in which the reaction was started with *lin*-benzo-ATP (results not shown). The presence of *lin*-benzo-ATP neither influenced the rate of phosphorylation nor that of dephosphorylation by [γ - ^{32}P]ATP (results not shown).

The pH optimum for the phosphorylation by *lin*-benzo-ATP was 6.0; at pH 8.0, however, the level was reduced to only 10% of that at pH 6.0. With ATP as substrate the steady-state phosphorylation levels at pH 6.1 and 8.0 were nearly the same [23]. K^+ , which increased the dephosphorylation rate, also reduced the steady-state phosphorylation level for *lin*-benzo-ATP with a half maximal effect at 90 μM (Fig. 4). The $K_{0.5}$ for Mg^{2+} (170 μM) in the phosphorylation reaction with *lin*-benzo-ATP was much higher than with ATP (< 5 μM ; [14]), which is in

Table 1. Residual steady-state phosphorylation level of ($H^+ + K^+$)-ATPase with ATP and *lin*-benzo-ATP as substrate

The ($H^+ + K^+$)-ATPase preparation was preincubated with the inhibitors SCH 28080 and omeprazole for 30 min at 37 °C at pH 6.0. With the monoclonal antibodies the (MAbs) incubation was at 20 °C for 30 min. Omeprazole and SCH 28080 were dissolved in a 2% (v/v) dimethyl sulphoxide solution. Omeprazole was not activated in acid medium. The phosphorylation reaction of 100 μg of ($H^+ + K^+$)-ATPase was carried out at 20 °C in the presence of 3 mM- Mg^{2+} , 0.2 mM-EDTA, 25 mM-imidazole/acetic acid, pH 6.0, and 0.3 mM-*lin*-benzo-ATP for 30 s. Under the same conditions, 10 μg of ($H^+ + K^+$)-ATPase was phosphorylated with 5 μM -ATP (10 s). Values are means $\pm$ S.D. for four experiments.

Inhibitor	Steady-state phosphorylation level (% of control)	
	ATP	<i>lin</i> -Benzo-ATP
SCH 28080 (1 μM)	10 $\pm$ 4	39 $\pm$ 4
Omeprazole (1 mM)	< 5	< 5
MAB 5B6 (100 mg/ml)	68 $\pm$ 8	70 $\pm$ 5

accordance with the idea of a 1:1 binding of Mg^{2+} and substrate.

When phosphorylation with 1 μM -[γ - ^{32}P]ATP was carried out after preincubation for 1 min with increasing concentrations of unlabelled *lin*-benzo-ATP, the level of labelled phosphorylated intermediate decreased by 80% (Fig. 5a). To obtain 50% of the phosphoenzyme level, 50 μM -*lin*-benzo-ATP was needed. This strongly suggests that ATP and *lin*-benzo-ATP phosphorylated ($H^+ + K^+$)-ATPase on the same site.

Effect of inhibitors on the phosphorylation of ($H^+ + K^+$)-ATPase by *lin*-benzo-ATP and ATP

Two rather specific inhibitors of the ($H^+ + K^+$)-ATPase, omeprazole [24] and the K^+ -competitive SCH 28080 [25–27],

Fig. 5. Effect of *lin*-benzo-ATP on the steady-state phosphorylation level with ATP and the basal Mg^{2+} -ATPase and K^+ -stimulated ATPase activity

(a) ($H^+ + K^+$)-ATPase (15 μg) was incubated at 22 °C in 100 μl of a medium containing 25 mM-imidazole/acetic acid, pH 6.0, 3 mM- Mg^{2+} , 0.1 mM-ouabain and the indicated *lin*-benzo-ATP concentrations for 1 min, whereafter [γ - ^{32}P]ATP (final concn. 1 μM) was added. The steady-state phosphorylation level (E-P) was determined after 3 s incubation, as indicated in the Materials and methods section. (b) ($H^+ + K^+$)-ATPase (1.9 μg) was incubated for 10 min at 37 °C in 200 μl of a medium containing imidazole/acetic acid, pH 7.4, 3 mM- Mg^{2+} , 0.1 mM-ouabain, 100 μM -[γ - ^{32}P]ATP and the indicated *lin*-benzo-ATP concentrations in the absence (○) or presence of K^+ . The K^+ -stimulated ATPase activity (●) was calculated by subtracting the basal Mg^{2+} -ATPase activity. The amount of ATP hydrolysis was determined as described in the Materials and methods section.

have been used to examine their effects on the phosphorylation level of (H⁺ + K⁺)-ATPase with *lin*-benzo-ATP and ATP.

SCH 28080 decreased the steady-state phosphorylation level for both substrates with an I_{50} of 10^{-8} M. The non-inhibitable level of phosphoenzyme after phosphorylation differed with the two substrates. With ATP a non-inhibitable level of less than 10% remained, whereas with *lin*-benzo-ATP a resting activity of 39% was found at 10^{-6} M-SCH 28080 (Table 1). The cause for this different behaviour was found in the phosphorylation by P_i . Like other P-type ATPases [28], (H⁺ + K⁺)-ATPase can be phosphorylated by P_i up to a maximal level of 2.5 nmol/mg [14,29,30]. Preincubation of the enzyme with SCH 28080 increased the phosphorylation level at suboptimal P_i concentrations. A similar behaviour was found for (Na⁺ + K⁺)-ATPase with its specific inhibitor ouabain [31]. Incubation of (H⁺ + K⁺)-ATPase with 0.1 mM-SCH 28080 increased the phosphorylation level with P_i by about 200%. The *lin*-benzo-ATP preparation contained about 3% P_i , which was enough to produce the observed residual activity under these conditions. The remaining level with ATP as substrate was much smaller, and could not be caused by P_i because the ATP preparation was practically free of P_i . P_i production during the 10 s incubation period was also too small to account for this level.

Omeprazole reduced the phosphorylation level with both substrates identically. The I_{50} for both substrates was about 0.1 mM, and at 1 mM-omeprazole only a negligible resting activity was left for both substrates (Table 1). In contrast with the SCH 28080-treated enzyme, the omeprazole-treated enzyme showed a decrease in the phosphorylation level with P_i over the same concentration range which inhibited the enzyme in the phosphorylation reaction with ATP. Therefore the P_i phosphorylation was negligible with the omeprazole-inhibited enzyme. The residual phosphorylation level with *lin*-benzo-ATP was practically zero.

Inhibition of the phosphorylation reaction with a monoclonal antibody 5B6 against (H⁺ + K⁺)-ATPase [32] was identical for the two substrates. With ATP a resting activity of $68 \pm 8\%$ and for *lin*-benzo-ATP of $70 \pm 5\%$ was observed (Table 1).

Properties of the phosphorylated intermediate

The phosphorylated intermediate formed by *lin*-benzo-ATP proved to be identical with the one generated by ATP. This phospho intermediate had a sensitivity to hydroxylamine similar to that of ATP-generated phospho intermediate [19], indicating that the same mixed anhydride was formed. The kinetic parameters of the dephosphorylation reaction of the phosphorylation product of *lin*-benzo-ATP matched those of the phosphorylated intermediate formed by ATP. The rate constants for both substrates were 3.0, 1.2 and $> 50 \text{ min}^{-1}$ in the presence of 10 mM-EDTA, 10 mM-EDTA + 1 mM-ADP and 10 mM-EDTA + 1 mM-K⁺ respectively. This shows that an identical K⁺-sensitive phospho intermediate as with ATP had been formed.

Hydrolysis

Some of the properties of the hydrolysis reaction with *lin*-benzo-ATP matched those of the K⁺-stimulated hydrolysis of ATP, but some were rather different from those of the K⁺-stimulated ATPase reaction of (H⁺ + K⁺)-ATPase. The K_m for *lin*-benzo-ATP (0.22 mM; Fig. 6) in the absence of K⁺ for the hydrolysis reaction was comparable with that for ATP (50 μM ; [33]). A small difference was observed in the need for Mg²⁺. With *lin*-benzo-ATP (1 mM) the half-maximal stimulatory effect of Mg²⁺ was at 0.2 mM, compared with 0.8 mM with ATP (3 mM) as the substrate [34].

Remarkable is the absence of the stimulatory effect of K⁺ on the rate of the hydrolysis reaction. K⁺ in the concentration range

Fig. 6. Determination of the K_m of the hydrolytic activity of the (H⁺ + K⁺)-ATPase preparation

A 10 μg portion of (H⁺ + K⁺)-ATPase was incubated at 37 °C in 100 μl of a medium containing 3 mM-Mg²⁺, 0.2 mM-EDTA, 25 mM-imidazole, pH 7.4, and labelled *lin*-benzo-ATP at the indicated concentrations. Incubation times were chosen so that not more than 30% of the substrate was hydrolysed. The $K_{0.5}$ value of 214 μM was obtained by Lineweaver-Burk analysis (inset). (The continuous line was drawn by eye.)

Fig. 7. K⁺-dependence of the hydrolysis of *lin*-benzo-ATP by the (H⁺ + K⁺)-ATPase preparation

A 10 μg portion of (H⁺ + K⁺)-ATPase was incubated for 10 min at 37 °C in 100 μl of a medium containing 1 mM labelled *lin*-benzo-ATP in the presence of the indicated K⁺ concentrations (○). Results are means $\pm$ S.D. for three experiments performed in duplicate. The hydrolytic activity of (H⁺ + K⁺)-ATPase with ATP under the same conditions is shown (●) for comparison. (The continuous and broken lines were drawn by eye and should be referred to similarly drawn axes.)

1 μM –30 mM had no effect on the rate of hydrolysis of *lin*-benzo-ATP (Fig. 7). In the presence of 100 mM-K⁺, even a slight ($< 10\%$) inhibition was observed. This could be due to an ionic-strength effect.

The pH-dependence of the enzymic hydrolysis reaction of *lin*-

Fig. 8. Hydrolytic activity of the $(H^+ + K^+)$ -ATPase preparation, with *lin*-benzo-ATP as the substrate, as a function of the pH

$(H^+ + K^+)$ -ATPase was incubated in the presence of 1 mM labelled *lin*-benzo-ATP and 50 mM-Mes/Tris buffer adjusted to the indicated pH under otherwise the same conditions as in Fig. 7 with 20 mM-KCl (○). The pH-dependence of $(H^+ + K^+)$ -ATPase with ATP (from [32]) is added for comparison (●). (The solid and dotted lines were drawn by eye and should be referred to similarly drawn axes.)

benzo-ATP did not resemble the pattern of that for ATP, but was almost identical with that of the basal Mg^{2+} -stimulated hydrolysis (Fig. 8).

The effect of *lin*-benzo-ATP on the basal Mg^{2+} -ATPase activity and the $(H^+ + K^+)$ -ATPase activity (with suboptimal K^+ and ATP concentrations of 1 and 0.1 mM respectively) was also studied. Fig. 5(b) shows that only the basal Mg^{2+} -ATPase was inhibited (I_{50} 100 μ M), whereas the K^+ -stimulated activity was not affected.

When $(H^+ + K^+)$ -ATPase was incubated with 3 mM- Mg^{2+} , 25 mM-imidazole/HCl, pH 7.4, and 1 mM-*lin*-benzo-ATP in the presence or absence of K^+ , the substrate was hydrolysed at a rate which varied in different preparations from 5 to 15 μ mol of P_i /h per mg, which is 60–70 % of the Mg^{2+} -ATPase activity determined under the same conditions. The rate of hydrolysis of 20 different $(H^+ + K^+)$ -ATPase preparations using *lin*-benzo-ATP

Table 2. Inhibition of the hydrolytic activity of $(H^+ + K^+)$ -ATPase by monoclonal antibodies 5B6 and specific inhibitors SCH 28080 and omeprazole

The hydrolytic activities were determined at 37 °C in the presence of 3 mM- Mg^{2+} , 0.2 mM-EDTA, 25 mM-imidazole/HCl, pH 7.5, and 1 mM labelled substrate. For determination of the K^+ -stimulated ATPase activity, 3 mM- K^+ was added to the medium. The hydrolytic activity with *lin*-benzo-ATP was also carried out in presence of 3 mM- K^+ . This did not influence the inhibition. In the experiment where the monoclonal antibodies (MAbs) or inhibitors are used, the enzyme was preincubated with the antibodies or the inhibitors for 30 min at 37 °C. The hydrolysis reaction was carried out as described in the Materials and methods section. The hydrolytic activity in absence of the inhibitors were taken as 100 %. Values are means $\pm$ S.D. for four experiments.

Inhibitor	ATPase activity (% of control)		
	K^+ -stimulated ATPase	Mg^{2+} -ATPase	Hydrolysis of <i>lin</i> -benzo-ATP
SCH 28080 (10 μ M)	8.5 $\pm$ 2.5	103 $\pm$ 11	100 $\pm$ 4
Omeprazole (0.1 mM)	4.5 $\pm$ 1.5	77 $\pm$ 6.0	74 $\pm$ 9
MAb 5B6 (100 μ g/ml)	28 $\pm$ 5	104 $\pm$ 9	103 $\pm$ 8

as substrate correlated with the basal Mg^{2+} -stimulated ATPase activity with a correlation coefficient (r) of 0.94 (derived by linear regression) and much less with the K^+ -stimulated $(H^+ + K^+)$ -ATPase activity ($r = 0.66$). These findings strongly indicate that the hydrolysis of *lin*-benzo-ATP has no direct relationship with the reaction cycle of $(H^+ + K^+)$ -ATPase.

Effect of inhibitors on the hydrolysis of *lin*-benzo-ATP

Incubation of $(H^+ + K^+)$ -ATPase with 0.1 mM-omeprazole resulted in an almost complete reduction of K^+ -stimulated hydrolytic activity with ATP. Under identical conditions the basal Mg^{2+} -stimulated ATPase was only inhibited by 23 %. The inhibition of the hydrolysis of *lin*-benzo-ATP resembled the latter one; 26 % inhibition was found (Table 2). After incubation with 0.1 mM-SCH 28080 the K^+ -stimulated hydrolysis of the $(H^+ + K^+)$ -ATPase was almost completely inhibited, whereas the basal activity was not inhibited at all. Under the same conditions the hydrolysis of *lin*-benzo-ATP was not inhibited either (Table 2).

In the presence of the monoclonal antibody 5B6, 72 ± 5 % inhibition of the K^+ -stimulated ATPase was observed, whereas the Mg^{2+} -ATPase kept its full activity. The hydrolysis rate of *lin*-benzo-ATP remained unchanged too (Table 2). The effect of the inhibitors on the hydrolysis rate of *lin*-benzo-ATP was not influenced by the presence of K^+ . Thus the hydrolysis of *lin*-benzo-ATP by the $(H^+ + K^+)$ -ATPase preparations matched the properties of the Mg^{2+} -ATPase activity rather than those of the K^+ -stimulated activity.

H^+ transport

The active transport of H^+ into gastric vesicles preloaded with K^+ is due to the exchange of K^+ against H^+ by the pumping activity of the $(H^+ + K^+)$ -ATPase. After addition of ATP to a vesicle suspension the active uptake of H^+ could be monitored by an increase of the medium pH by use of a sensitive pH-electrode. Upon addition of the ionophore nigericin the medium pH decreased quickly a few minutes after the addition of ATP as a result of H^+ leaking out of the vesicles. In presence of vanadate, no pH jump apart from a small pH artefact was observed upon addition of ATP.

Upon addition of several concentrations of *lin*-benzo-ATP (0.1–1.0 mM) to the medium containing gastric vesicles, no active pumping could be registered. Only a small artefact similar to that for ATP in presence of vanadate was observed. In the presence of vanadate or nigericin the artefact remained exactly the same. Addition of nigericin after addition of *lin*-benzo-ATP had no effect on the measured pH, indicating that no H^+ was accumulated in the vesicle.

DISCUSSION

It is an old dispute in the field of gastric ATPase whether the basal Mg^{2+} -stimulated ATPase activity is an intrinsic part of the $(H^+ + K^+)$ -ATPase reaction cycle (Scheme 1) or is due to an impurity in the enzyme preparation. An argument for the second possibility is the decrease in the Mg^{2+} -ATPase activity upon further purification by zone or free-flow electrophoresis [34,35]. The observation that the Mg^{2+} -ATPase activity and the $(H^+ + K^+)$ -ATPase activity were immunologically different [36] also supports the latter possibility. The observation of Wallmark *et al.* [33], however, that the rate of spontaneous dephosphorylation is high enough to cause the basal hydrolytic activity of the enzyme and the fact that it is impossible to discard the basal activity totally from the enzyme preparation rather support the first possibility. The fact that the amount of basal Mg^{2+} -ATPase increases upon aging of the enzyme could also be an argument in favour of the first possibility. In this view the

basal activity could originate from partially inactivated enzyme. The effects of treatment of a (H⁺ + K⁺)-ATPase preparation with phospholipase A₂, namely a stimulation of the basal Mg²⁺-ATPase activity together with a decrease in the K⁺-stimulated ATPase activity [37], argue also in favour of the first possibility.

With *lin*-benzo-ATP we have a tool which can distinguish between the two activities: K⁺-stimulated ATPase and basal Mg²⁺-stimulated ATPase. This substrate is hydrolysed well by the Mg²⁺-stimulated ATPase, whereas it is not a substrate for the K⁺-stimulated ATPase. On the other hand, *lin*-benzo-ATP can phosphorylate the (H⁺ + K⁺)-ATPase, although the rate of phosphorylation is very slow.

The product of phosphorylation of (H⁺ + K⁺)-ATPase by *lin*-benzo-ATP strongly resembled the phospho intermediate generated by ATP. The differences, such as the lower affinity for the substrate and the higher K_{0.5} value for Mg²⁺, are probably due to the different pK_a value and steric hindrance of the extended adenosine ring of the synthetic substrate. It is remarkable that a slight modification of the adenosine-ring system [an extension of 0.24 nm (2.4 Å)] has such severe implications for the affinity to the enzyme. The lower steady-state phosphorylation level obtained with *lin*-benzo-ATP compared with that of the natural substrate was only partly due to substrate inhibition, but is more likely due to the low phosphorylation rate, which is in the same range as that of the dephosphorylation step, so that a maximal level cannot be reached.

The extreme low rate of phosphorylation of *lin*-benzo-ATP (200 times lower than with ATP [21,22]) made it kinetically impossible that the observed rate of hydrolysis was due to the phosphorylation and subsequent dephosphorylation of (H⁺ + K⁺)-ATPase. The observed initial velocities of the phosphorylation reactions indicate that maximally 4 % of the P_i formed could be attributed to phosphorylation and subsequent dephosphorylation of (H⁺ + K⁺)-ATPase. The argument of Wallmark *et al.* [33] that the dephosphorylation reaction in absence of K⁺ is fast enough to be responsible for the basal hydrolysis (and therefore the basal activity may be an intrinsic one) of ATP only holds for low ATP concentrations and for preparations with low amounts of basal Mg²⁺-ATPase activity.

The formation of a steady-state phosphorylation level, however, demands that the rate of dephosphorylation is slower than that of the phosphorylation reaction. A strong increase in the rate of dephosphorylation by addition of K⁺ indeed reduced the steady-state phosphorylation level, but had no effect at all on the rate of hydrolysis of the synthetic substrate. This means that, in the presence of K⁺, when the dephosphorylation reaction is not rate-limiting, the rate of the phosphorylation must be the slowest step in the cycle. Because it is very unlikely that the phosphorylation rate will increase in the presence of K⁺ [23], only a minor part of the hydrolysis of *lin*-benzo-ATP could be due to the action of (H⁺ + K⁺)-ATPase via the phospho-enzyme cycle. The major part (> 95 %) of the hydrolysis of *lin*-benzo-ATP must be due to another hydrolysing activity.

Further evidence for the latter hypothesis comes from experiments with inhibitors, where the inhibition pattern of the hydrolysis matched that of the Mg²⁺-stimulated ATPase and not that of the K⁺-stimulated ATPase. In addition, the pH-dependence of the hydrolysis of *lin*-benzo-ATP was very similar to that of the Mg²⁺-ATPase activity [34] and did not resemble that of the K⁺-stimulated ATPase activity. The fact that *lin*-benzo-ATP inhibits the basal Mg²⁺-ATPase but not the K⁺-stimulated ATPase activity, the positive correlation between the hydrolysis of *lin*-benzo-ATP and the Mg²⁺-ATPase activity and the lack of transport capacity provide further evidence for the notion that the hydrolysis of *lin*-benzo-ATP is catalysed by the Mg²⁺-ATPase. Finally the Mg²⁺-dependence with *lin*-benzo-ATP as substrate is

a strong indication that the phosphorylated intermediate is not responsible for the observed rate of hydrolysis. Phosphorylation with *lin*-benzo-ATP needs more Mg²⁺ than the hydrolysis, whereas the opposite is true for phosphorylation and hydrolysis with ATP as substrate.

Wallmark *et al.* [33] found a biphasic substrate-dependency for the basal Mg²⁺-ATPase activity with K_{0.5} values of 0.4 and 50 mM. They cannot distinguish between two ATP sites on one enzyme or on two different enzymes. We found with *lin*-benzo-ATP only one K_m value for the substrate. Supposing that the high-affinity site for ATP is due to (H⁺ + K⁺)-ATPase and the low affinity one is due to the basal Mg²⁺-ATPase activity, then the affinity of the latter site for *lin*-benzo-ATP is only 5 times lower than for ATP, whereas the difference in affinities for the phosphorylation site is more than 200. This accords with the notion that the basal Mg²⁺-ATPase activity has a less specific substrate site.

The dispute about whether the Mg²⁺-ATPase activity is due to another protein co-purified with (H⁺ + K⁺)-ATPase or to catalysis of the hydrolysis of the substrate by a reaction apart from the reaction cycle of the (H⁺ + K⁺)-ATPase itself cannot be answered by the present study. The characteristics of the basal Mg²⁺-ATPase activity described here are definitely not the same as the Mg²⁺-ATPase in the low-density membranes as described by Nandi *et al.* [38]. The separation of an impurity (which was Ca²⁺- and Mg²⁺-dependent ATPase) from a (H⁺ + K⁺)-ATPase preparation has been reported [39]. Testing the properties of this impurity with *lin*-benzo-ATP would further elucidate this question.

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