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A monoclonal antibody against pig gastric H^+/K^+-ATPase, which binds to the cytosolic $E_1 \cdot K^+$ form

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Monoclonal antibodies were raised against a purified membrane fraction from hog gastric mucosa containing H^+/K^+-ATPase. The properties of one of these monoclonal antibodies (5B6) were further evaluated. On immunoblot it recognized the 95 kDa peptide of the H^+/K^+-ATPase-rich membrane fraction. The K^+-ATPase activity was inhibited by 65% under standard assay conditions (pH 7.0). At pH 6.0 and 8.0 this enzyme activity was inhibited by 40% and 100%, respectively. The maximal inhibition in inside-out vesicles was also 65% at pH 7.0. The inhibition was uncompetitive with respect to K^+ and noncompetitive with respect to ATP. Mg^{2+}-ATPase activity and K^+-dependent *p*-nitrophenylphosphatase activity were not influenced. The monoclonal antibody lowered the steady-state phosphorylation level at pH 6.0, 7.0 and 8.0 by 30%, 40% and 60% respectively. The rate of the K^+-stimulated dephosphorylation step was not inhibited. These findings demonstrate that 5-B6 recognizes the $E_1 \cdot K^+$ dephosphoenzyme at the cytosolic side.

Introduction

The H^+/K^+-ATPase of the gastric mucosa catalyzes the exchange of H^+ and K^+ upon hydrolysis of ATP which results in a pH difference of more than 6 between the cytoplasm and gastric lumen [1,2]. The catalytic subunit of this membrane-bound enzyme is related to a number of other cation transport ATPases, including the Na^+/K^+-ATPase and the Ca^{2+}-ATPase [3,4]. These enzymes belong to the class of P-type ATPases as they all have a stable phosphoenzyme intermediate in the enzyme cycle [5,6]. Recently the amino acid sequences of the catalytic subunits of rat and hog gastric H^+/K^+-ATPase have been deduced from the nucleotide sequence of their cDNA [3,7] and the molecular weight of the protein was calculated to be 114000 in either case. There is a 60% homology between the primary sequences of the catalytic subunits of H^+/K^+-ATPase and Na^+/K^+-ATPase [3]. Hydropathy analysis revealed that the H^+/K^+-ATPase subunit probably has eight transmembrane domains as is the case for Na^+/K^+-ATPase and Ca^{2+}-ATPase [3]. Besides these structural similarities, the H^+/K^+-ATPase also has many functional aspects in common with other P-type ATPases. Phosphorylation is on an aspartate residue [4] and hydrolysis of ATP is inhibited by vanadate [8]. The existence of two conformational states, E_1 and E_2 which have reciprocal affinities for H^+ and K^+ at cytosolic and luminal side, respectively, has been proved by limited proteolytic digestion [9] and by fluorescent probes [10,11]. Cycling of the enzyme between these two conformational states, during ATP hydrolysis, gives rise to the translocation of H^+ to the lumen and of K^+ to the cytosol.

So far relatively little is known about the structure of the enzyme. It is not known, for example, which part of the enzyme is localized at the luminal and which at the cytosolic side; which amino acids contribute to the binding sites for ions and inhibitors as omeprazole [12] and SCH 28080 [13].

Monoclonal antibodies against the H^+/K^+-ATPase could be used as probes to investigate the structure and function of the enzyme. They could clarify whether the gastric enzyme is an oligomer of identical or nonidenti-

Abbreviations: H^+/K^+-ATPase (EC 3.6.1.36), magnesium-dependent hydrogen ion transporting and potassium-stimulated adenosine triphosphatase; K^+-ATPase, K^+-dependent adenosine triphosphatase; Mg^{2+}-ATPase, Mg^{2+}-dependent adenosine triphosphatase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Tween 20, polyoxyethylene sorbitan monolaurate.

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cal 114 kDa subunits [14] and whether it has any immunological similarity to the Na^+/K^+-ATPase [15].

Monoclonal antibodies against the subunit of the pig gastric H^+/K^+-ATPase are already available [16–18]. These monoclonal antibodies have been used for histological studies on H^+/K^+-ATPase [16] and to suggest that the Cl^- channel is part of the function of H^+/K^+-ATPase [18].

In order to get more information about structure and function of H^+/K^+-ATPase we prepared monoclonal antibodies against the enzyme. In this paper we describe the characteristics of one of these monoclonal antibodies which inhibits the gastric H^+/K^+-ATPase.

Materials and Methods

Preparation of gastric H^+/K^+-ATPase

Isolation of the H^+/K^+-ATPase has been carried out according to the procedure previously reported by Skrabanja et al. [19] with some modifications. Stomachs of freshly slaughtered pigs were transported to the laboratory on ice. After flushing with tap water the fundic region was placed during 30 min in homogenization buffer containing 250 mM sucrose and 20 mM Tris-HCl (pH 7.4). Mucus was removed by wiping the tissue with paper towels and the mucosa was scraped off from the underlying muscular layer. The scraped material was placed in homogenization buffer and homogenized with a Braun teflon-glass homogenizer by three up-down strokes of the rotating pestle (250 rev./min). The homogenate was centrifuged for 30 min at $20\,000 \times g$. The resulting supernatant was centrifuged for 60 min at $100\,000 \times g$. The pellet was resuspended in homogenization buffer and centrifuged on top of a discontinuous gradient of 7% (w/v) Ficoll-250 mM sucrose in 20 mM Tris-HCl (pH 7.4) over 37% (w/v) sucrose in 20 mM Tris-HCl (pH 7.4). After 60 min centrifugation at $150\,000 \times g$ the 250 mM sucrose/7% (w/v) Ficoll-250 mM sucrose interface containing the vesicular H^+/K^+-ATPase fraction and the 7% (w/v) Ficoll-250 mM sucrose/37% (w/v) sucrose interface containing the open membrane H^+/K^+-ATPase fraction were diluted in 20 mM Tris-HCl (pH 7.4) and centrifuged for 60 min at $150\,000 \times g$. The final pellets were resuspended in 20 mM Tris-HCl (pH 7.4) and frozen at $-20\,°C$.

Hybridoma production

The antigen was H^+/K^+-ATPase purified from hog stomach and emulsified in complete Freund's adjuvant. Several 6-week-old female BALB/c mice were immunized intraperitoneally with 200 μg of enzyme. After 3 weeks, sera were drawn for evaluation of titer against gastric H^+/K^+-ATPase. One mouse was selected on the basis of the highest titer and boostered by tail vein injection of 100 μg antigen in 0.3 ml of 0.9% (w/v) NaCl on three subsequent days. On the fourth day the spleen was removed and spleen cells ($5 \cdot 10^7$) were fused with mouse Sp2/0 myeloma cells ($2 \cdot 10^7$) in the presence of 40% (v/v) polyethylene glycol 4000. The cells were washed and cultured in RPMI 1640 medium (Dutch modification, Flow Laboratories, Irvine, Scotland) supplemented with 20% (v/v) fetal calf serum (Flow Laboratories), 2 mM glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate and 50 mg/l gentamycin. After 3 hours a solution containing hypoxanthine, aminopterin and thymidine (final concentrations 0.1 mM, 0.4 μM and 16 μM) was added to the medium and cells were plated out.

Production and partial purification of monoclonal antibodies

Hybridomas producing antibodies were cloned by limited dilution and injected ($5 \cdot 10^6$ cells) intraperitoneally into BALB/c mice primed with pristane (0.5 ml i.p.) 2–4 weeks before. After 10 days antibody-rich ascites fluid was obtained every 2–3 days and centrifuged for 10 min at $2000 \times g$ to remove cell debris. The supernatant was precipitated with ammonium sulfate (pH 7.4, adjusted with Tris) at 50% saturation. The $12\,000 \times g$ precipitate was resuspended in a volume of 20 mM Tris-HCl (pH 7.4) equal to the original volume of the ascites, dialyzed against 100 volumes of 20 mM Tris-HCl (pH 7.4) at 4 °C with three changes of the dialysate and stored at $-20\,°C$. The subclasses of the monoclonal antibodies were determined with subclass-specific antibodies according to Ouchterlony [20].

ELISA

Reagents per well and incubation times were as follows: 0.5 μg of gastric H^+/K^+-ATPase in 50 μl PBS was immobilized by overnight evaporation in wells of flat-bottomed, 96-well microtiter plates (Costar, Cambridge, MA, U.S.A.). Blocking was done by incubating with a solution of 1% (w/v) gelatin in PBS for 2 h at room temperature whereafter the plates were washed with PBS containing 0.05% (v/v) Tween 20. Incubation for 1 h at room temperature with antibodies from hybridoma culture media was followed by washing with PBS/Tween and subsequent incubation with peroxidase conjugated rabbit-anti-mouse immunoglobulins (Dakopatts, Denmark) diluted 1 : 800 in PBS with 1% gelatin. After washing a substrate solution containing 0.8 mg/ml 5-aminosalicylic acid in 50 mM phosphate buffer (pH 6.0) and 0.024% (w/v) hydrogen peroxide was added. Absorption at 492 nm was measured after 30 min using a Titertek Multiscan plate reader (Flow Laboratories).

Gel electrophoresis and immunoblotting

Purified H^+/K^+-ATPase was electrophoresed on 10% (w/v) SDS-polyacrylamide slab gel according to Laemmli [21]. The separated proteins were transferred to

nitrocellulose by electroblotting [22]. The blots were incubated first for 2 h with PBS-1% (w/v) gelatin-0.05% (w/v) Tween 20 (Buffer I) to block nonspecific reactive sites, then for 16 h with 1 : 100 dilutions of monoclonal antibody 5-B6 in Buffer I. After washing five times in PBS-0.05% (w/v) Tween 20 (Buffer II) the blots were incubated for 1 h with 1 : 250 dilutions of rabbit-anti-mouse immunoglobulin (Dakopatts, Denmark) in Buffer I, washed again with Buffer II and incubated for another hour with 1 : 250 dilution of mouse peroxidase-antiperoxidase immunoglobulin (Dakopatts, Denmark) in Buffer I. Finally the blots were washed with Buffer II and substrate solution (60 mg 4-chloronaphthol, 60 μl 30% (w/v) hydrogen peroxide, 20 ml methanol, 100 ml PBS) was added.

ATPase and p-nitrophenylphosphatase assay

K^+-ATPase activity and K^+-*p*-nitrophenylphosphatase activity of the enzyme were determined according to Schrijen et al. [23]. Gastric H^+/K^+-ATPase, 100 μl with a concentration of 60 μg protein/ml, was preincubated for 10 min at 37 °C with 100 μl of a diluted monoclonal antibody solution with concentrations as indicated in the figures. Dilutions of enzyme and monoclonal antibody were made in 30 mM imidazole-HCl (pH 7.0). The reaction was started by adding 200 μl medium containing 10 mM Na_2ATP or 10 mM *p*-nitrophenyl phosphate, 10 mM Mg^{2+}, 0.2 mM ouabain, 30 mM imidazole-HCl (pH 7.0) and 40 mM KCl or 40 mM choline chloride. Stopping of the reaction and further treatment of the samples were carried out as described before [24]. The K^+-ATPase and K^+-*p*-nitrophenylphosphatase activities were obtained from the difference in activity with KCl and choline chloride, respectively. Protein was determined according to Lowry et al. [25], using bovine serum albumin as standard.

Radioactive ATPase assay

Aliquots of 50 μl enzyme (0.08 mg/ml) were preincubated for 10 min at 37 °C with 50 μl monoclonal antibody solution at various concentrations and 50 μl medium containing 60 mM imidazole-HCl (pH 7.0), 0.4 mM ouabain and 80 mM KCl or 80 mM choline chloride. The reaction was initiated by adding 50 μl of a solution containing [γ-^{32}P]ATP and Mg^{2+} at a ratio of 0.5 and at various concentrations. The reaction was stopped by adding 800 μl 5% (w/v) trichloroacetic acid/10% (w/v) Norit. After centrifugation for 10 min at 1500 × *g* (Heraeus Christ) 200 μl of the supernatant was counted in a Packard 2200CA liquid scintillation analyzer.

Enzyme phosphorylation and dephosphorylation

The phosphorylation and dephosphorylation reactions were carried out as described by Helmich-de Jong et al. [26]. Aliquots of 50 μl H^+/K^+-ATPase (0.3 mg/ml) were preincubated for 10 min at room temperature with 50 μl monoclonal antibody solution at various concentrations and 4 mM $MgCl_2$. The phosphorylation reaction was started by adding 100 μl 10 μM [γ-^{32}P]ATP. After 10 s at 20 °C the reaction was stopped by adding 5 ml 5% (w/v) trichloroacetic acid/0.1 M H_3PO_4.

The effect of the monoclonal antibody on the rate of the potassium stimulated dephosphorylation was studied at 0–4 °C. Phosphorylation was first initiated by adding 50-μl aliquots of 10 μM [γ-^{32}P]ATP, 10 μM $MgCl_2$ to 50 μl aliquots of H^+/K^+-ATPase (0.2 mg/ml). After 10 s dephosphorylation was started by adding ATP (final concentration 1 mM), KCl at various concentrations and monoclonal antibody. The reaction was stopped by adding 5 ml 5% (w/v) trichloroacetic acid/0.1 M H_3PO_4. Further processing was carried out as described by Schuurmans Stekhoven et al. [27].

Results

Preparation of monoclonal antibodies

By ELISA screening of the produced hybridomas six monoclonal antibodies were obtained which showed a positive reaction against the purified hog gastric H^+/K^+-ATPase. Four of them inhibited the K^+-ATPase activity. One of them, called 5-B6, gave the strongest inhibition and was further characterized.

Double immuno-diffusion with Ig subclass specific anti-mouse immunoglobulins showed that 5-B6 is of subclass IgG_1. By Western blot analysis of the purified H^+/K^+-ATPase membrane fraction, 5-B6 recognized a 95 kDa peptide (Fig. 1). There was no cross reactivity with rabbit kidney Na^+/K^+-ATPase.

Effect on H^+/K^+-ATPase

The gastric H^+/K^+-ATPase preparation contains K^+-ATPase, K^+-*p*-nitrophenylphosphatase as well as a Mg^{2+}-ATPase activity. The effect of monoclonal antibody 5-B6 on these enzyme activities was studied with a non-inhibitory monoclonal antibody, directed against H^+/K^+-ATPase and also partially purified, as control.

Fig. 2 shows the inhibition of K^+-ATPase activity with various amounts of 5-B6 (0.05 to 150 μg/ml) and with three different H^+/K^+-ATPase concentrations (5, 15 and 50 μg/ml) in the reaction mixture. The maximal inhibition obtained was 65% for all three H^+/K^+-ATPase concentrations used. Variation of the preincubation time had no effect on the degree of inhibition. Activities of K^+-*p*-nitrophenylphosphatase and Mg^{2+}-ATPase were not influenced (data not shown).

The maximal extent of inhibition of K^+-ATPase activity by 5-B6 varied with the pH in the reaction mixture (Fig. 3). At pH 6.0, 7.0 and 8.0 the maximal inhibition of the K^+-ATPase activity was 40%, 65% and 100%, respectively. The half-maximal inhibitory con-

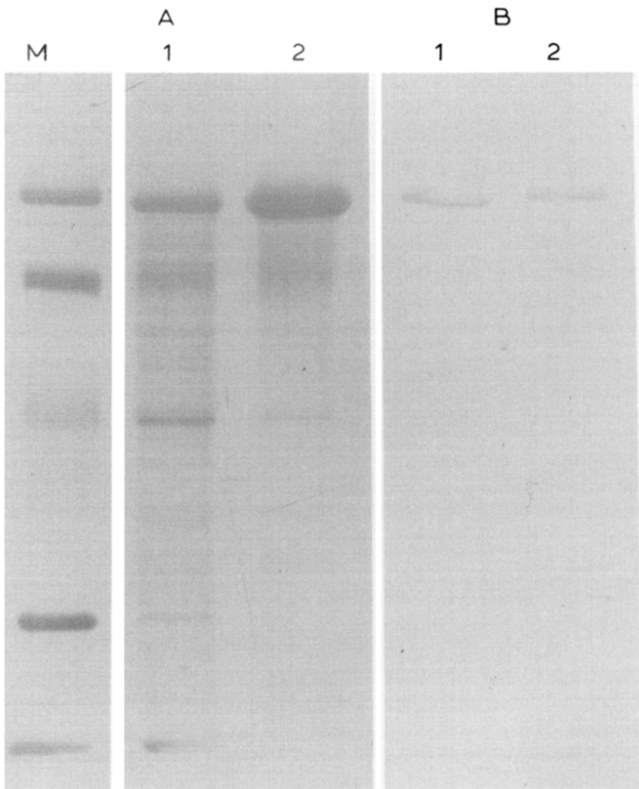

Fig. 1. Western blot of pig gastric H^+/K^+-ATPase stained with monoclonal antibody 5-B6. (A) Amido Black staining of proteins on nitrocellulose after electroblotting of a 10% SDS polyacrylamide gel. Lane 1, 5 μg gastric microsomal membrane proteins. Lane 2, 5 μg purified H^+/K^+-ATPase. Molecular mass markers are 94, 67, 43, 30 and 20.1 kDa. (B) Western blot stained with the anti-H^+/K^+-ATPase monoclonal antibody 5-B6. Lane 1, 3 μg gastric microsomal membrane proteins. Lane 2, 1 μg purified H^+/K^+-ATPase.

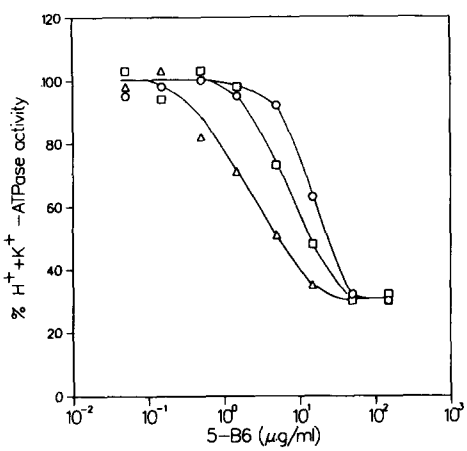

Fig. 2. Inhibition of K^+-ATPase activity of H^+/K^+-ATPase by monoclonal antibody 5-B6. Density gradient purified and leaky H^+/K^+-ATPase vesicles. 5 μg/ml (△), 15 μg/ml (□) and 50 μg/ml (○), were preincubated with different concentrations of monoclonal antibody 5-B6 at 37 °C for 10 min. K^+-ATPase activity at pH 7.0 was determined as described in Materials and Methods (representative of three experiments). The 100% activity in the absence of 5-B6 was 64.1 μmol P_i per mg per h.

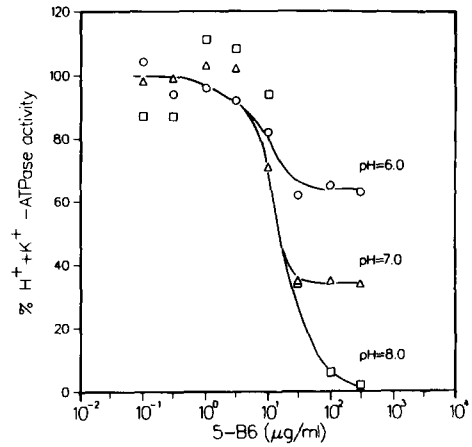

Fig. 3. Inhibition of K^+-ATPase activity of H^+/K^+-ATPase by monoclonal antibody 5-B6 as a function of pH. 15 μg/ml leaky H^+/K^+-ATPase vesicles were preincubated with different concentrations of monoclonal antibody 5-B6 in 30 mM imidazole-HCl at pH 6.0 (○), pH 7.0 (△) or pH 8.0 (□) at 37 °C for 10 min. K^+-ATPase activity was determined as described in Materials and Methods (representative of three experiments). The 100% activity at pH 6.0, 7.0 and 8.0 was 29.8, 79.8 and 19.3 μmol P_i per mg per h, respectively.

centration of the antibody did not change with pH and was 15 μg of antibody/ml when 15 μg/ml H^+/K^+-ATPase was used.

The inhibition of the K^+-ATPase activity by 5-B6 was measured as function of both K^+ as well as ATP concentration. Fig. 4 shows that 20 μg/ml 5-B6 in an assay medium containing 15 μg/ml H^+/K^+-ATPase lowered the V_{max} value from 80 to 35 μmol P_i per mg per h. The K_m value for K^+ was lowered from 4.8 to 1.9 mM. These results indicate that the interaction between K^+ and 5-B6 is uncompetitive. Variation of the ATP concentration at a fixed K^+ concentration (Fig. 5) showed that 20 μg/ml 5-B6 in an assay medium con-

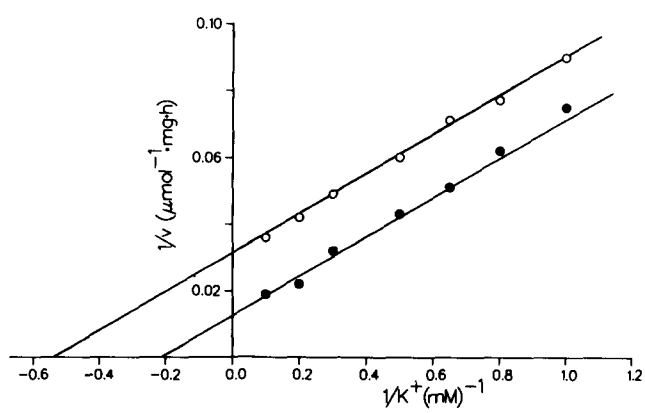

Fig. 4. Effect of K^+ concentrations on the inhibition of K^+-ATPase activity of H^+/K^+-ATPase by monoclonal antibody 5-B6. 15 μg/ml H^+/K^+-ATPase were preincubated with 20 μg/ml 5-B6 at 37 °C for 10 min. K^+-ATPase activity was determined in assay media containing 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM ouabain, 30 mM imidazole-HCl (pH 7.0) and KCl concentrations varying between 1 and 10 mM. P_i was measured as described in Materials and Methods. ●, Control; ○, 5-B6 (representative of three experiments).

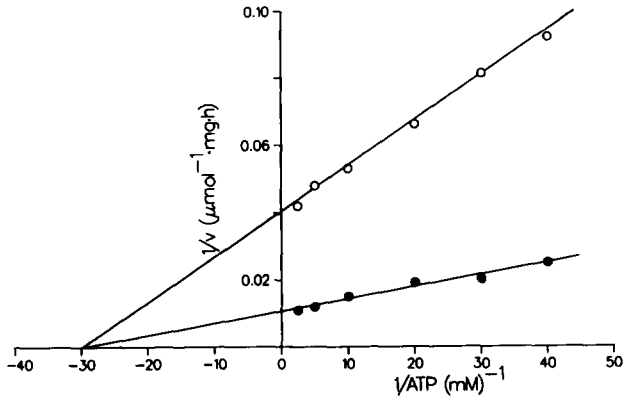

Fig. 5. Effect of ATP concentrations on the inhibition of K^+-ATPase activity of H^+/K^+-ATPase by monoclonal antibody 5-B6. 15 μg/ml H^+/K^+-ATPase were preincubated with 20 μg/ml 5-B6 at 37 °C for 10 min. K^+-ATPase activity was determined as described in Materials and Methods with assay media containing 20 mM KCl, 0.1 mM ouabain, 30 mM imidazole-HCl (pH 7.0), [γ-^{32}P]ATP concentrations varying between 0.025 and 0.5 mM and $MgCl_2$ concentrations varying between 0.05 and 1 mM, thereby maintaining an ATP/Mg^{2+} ratio of 1:2. ●, Control; ○, 5-B6 (representative of three experiments).

taining 15 μg/ml H^+/K^+-ATPase decreased the V_{max} value from 100 to 25 μmol P_i per mg per h. The K_m values did not change. The effect of 5-B6 on the K^+-ATPase reaction with respect to ATP was therefore noncompetitive.

Effects on phosphoenzyme

The effect of monoclonal antibody 5-B6 on the steady-state level of phosphoenzyme in the absence of K^+ and at pH 6.0, 7.0 and 8.0, respectively, is shown in Fig. 6. The steady-state phosphorylation level at pH 6.0,

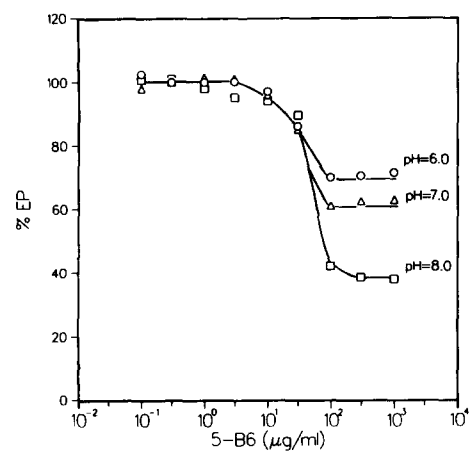

Fig. 6. Effect of monoclonal antibody 5-B6 on steady-state phosphoenzyme levels of H^+/K^+-ATPase at different pH. 75 μg/ml purified H^+/K^+-ATPase were preincubated in 30 mM imidazole-HCl pH 6.0 (○), pH 7.0 (△) and pH 8.0 (□) with various concentrations of 5-B6 and 4 mM $MgCl_2$. Initiation of the phosphorylation and determination of phosphoenzyme levels were carried out as described in Materials and Methods. The phosphoenzyme levels, in the absence of 5-B6 (100%), were 0.75 nmol/mg protein (representative of three experiments).

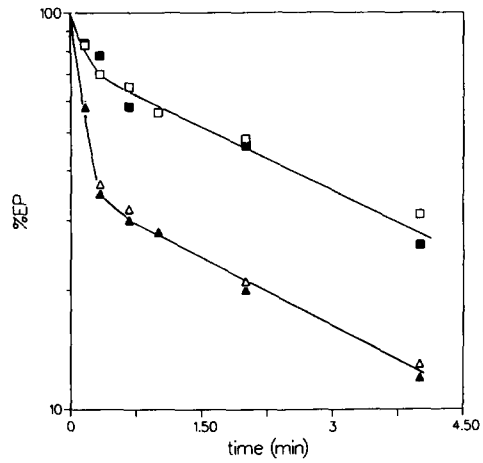

Fig. 7. Effect of monoclonal antibody 5-B6 on K^+-stimulated dephosphorylation of H^+/K^+-ATPase. Phosphorylation at 0 °C of 100 μg/ml H^+/K^+-ATPase was carried out as described in Materials and Methods. After 10 s dephosphorylation of the phosphoenzyme was started with addition of cold ATP (final concentration 1 mM), 5-B6 (final concentration 100 μg/ml) and KCl to a final concentration of 0.1 mM (■) or 1 mM (▲). Blank symbols represent control values. Stopping of the phosphorylation reaction and determination of phosphoenzyme levels were carried out as described in Materials and Methods. The phosphoenzyme levels, in the absence of 5-B6 (100% values), were 0.92 nmol/mg protein (representative of three experiments).

7.0 and 8.0 was concentration dependently reduced to a maximal extent of 30%, 40% and 60%, respectively. The half-maximal inhibitory concentration of 5-B6 in all cases was 30 μg/ml with 75 μg/ml H^+/K^+-ATPase used.

When the effect of 5-B6 on the steady-state phosphorylation level was studied without preincubating with 5-B6 (the monoclonal antibody was added at the same time as phosphorylation was started) the same extent of inhibition was seen (data not shown). These results indicated a very rapid binding of monoclonal antibody to gastric H^+/K^+-ATPase.

TABLE I

Inhibition of K^+-ATPase activity by 5-B6 in inside-out and in leaky vesicles

80 μg/ml H^+/K^+-ATPase (specific activity 85 μmol P_i/mg per h) was preincubated for 15 min at room temperature with 10 μM nigericin (inside-out vesicles) or with 0.3% (w/v) saponin (leaky vesicles). The K^+-ATPase reaction was started by adding 100 μl enzyme solution to 300 μl isotonic assay medium with a final concentration of 100 μg/ml 5-B6. The values are given as percentages relative to K^+-ATPase activity with control mouse IgG. Each value is the average of three separate determinations, each of which is done in duplicate.

	Percentage residual K^+-ATPase activity
1. Inside-out vesicles	34 ± 7
2. Leaky vesicles	28 ± 4

The effect of 5-B6 on the K^+-stimulated dephosphorylation rate is shown in Fig. 7. In this experiment dephosphorylation was initiated after 10 s by adding cold ATP (final concentration 1 mM) along with the indicated concentrations of KCl and 5-B6. The monoclonal antibody did not affect the rate of the K^+-stimulated dephosphorylation, nor the second phase in the dephosphorylation, which is interpreted as interconversion from $E_1 \cdot P$ to $E_2 \cdot P$ [28].

Sidedness of inhibition of gastric H^+/K^+-ATPase by 5-B6

For determination of the sidedness of binding of monoclonal antibody 5-B6 on gastric H^+/K^+-ATPase freshly isolated inside-out vesicles were used. Table I shows that the maximal level of inhibition of K^+-ATPase activity was identical in inside-out vesicles as well as in leaky H^+/K^+-ATPase vesicles. These results indicate that 5-B6 binds to the cytosolic side of gastric H^+/K^+-ATPase.

Discussion

In the present study, the interactions of monoclonal antibody 5-B6 with H^+/K^+-ATPase have been investigated. To clarify interpretation of the interactions of 5-B6 with H^+/K^+-ATPase the following enzymatic mechanism of gastric H^+/K^+-ATPase has to be considered (Fig. 8) [28–30]. In this mechanism, which requires several steps related to binding of substrate and cationic ligands, E_1 and E_2 represent conformational states of the enzyme with cation binding sites facing the cytosolic and luminal side, respectively.

When considering the phosphorylation reaction, the E_1 conformation can interact competitively with either H^+ or K^+. The $E_1 \cdot K^+$ form relatively slowly reacts with ATP, forming an $E_1 \cdot ATP \cdot K^+$ complex. At higher H^+ concentrations K^+ may also be displaced from the

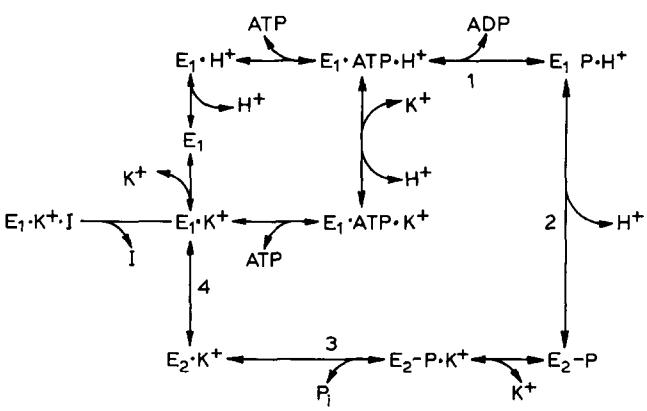

Fig. 8. A reaction cycle of H^+/K^+-ATPase. E_1 and E_2 represent conformational states of the enzyme with cation binding sites facing the cytosol and lumen, respectively. I represents the inhibitory monoclonal antibody 5-B6.

enzyme and $E_1 \cdot H^+$ is formed. This form reacts with ATP and the $E_1 \cdot ATP \cdot K^+$ with H^+ to a $E_1 \cdot ATP \cdot H^+$ complex which is subsequently phosphorylated (step 1). The $E_1 \cdot P$ form then generated, is ADP-sensitive [26] and converts spontaneously to E_2P with concomitant transport of H^+ (step 2) [31]. This form has a high affinity for K^+ at the luminal side and is rapidly hydrolyzed (step 3) [28]. The $E_2 \cdot K^+$ form then converts to the $E_1 \cdot K^+$ form with consequent countertransport of K^+ (step 4) [32].

The monoclonal antibody 5-B6, which binds to the 95 kDa peptide in the purified H^+/K^+-ATPase fraction, inhibited the K^+-ATPase activity in a concentration dependent way. The maximal extent of inhibition was pH dependent and was maximal at pH 8.0. Lineweaver-Burk plots showed that inhibition of the K^+-ATPase by 5-B6 at pH 7.0 was noncompetitive with respect to ATP and uncompetitive with respect to K^+. Since the stimulation of the ATP hydrolysis by K^+ is mediated through an increase of the dephosphorylation rate of $E_2 \cdot P$, these findings would suggest an interaction of 5-B6 with the $E_2 \cdot K^+$ or the $E_1 \cdot K^+$ form. A distinction between these two dephospho-forms can not be made since the $E_2 \cdot K^+$ to $E_1 \cdot K^+$ conversion is not a rate-limiting step in the enzyme cycle at the high ATP/K^+ ratio used [28]. The finding however, that 5-B6 recognizes an epitope on the cytosolic side, would favour binding of 5-B6 to the $E_1 \cdot K^+$ form. The varying degree of maximal inhibition of the K^+-ATPase activity at different pH can be explained by the influence of $[H^+]$ on step 1 and step 3 of the enzyme cycle [29–30]. An alkaline pH increases the rate of the K^+-stimulated dephosphorylation and stabilizes the $E_1 \cdot K^+$ form of the enzyme which has low affinity for ATP [29]. This results in an accumulation of the $E_1 \cdot K^+$ form during ATP hydrolysis and therefore increases the maximal extent of inhibition. An acidic pH on the other hand, stabilizes the acid-stable phosphoenzyme by reducing the affinity for luminal K^+ and increases the rate and extent of phosphorylation. As a consequence, the maximal extent of inhibition by 5-B6 decreases.

The finding that the K^+-*p*-nitrophenylphosphatase activity is not inhibited by 5-B6 is consistent with binding of 5-B6 to the $E_1 \cdot K^+$ form, since hydrolysis of *p*-nitrophenylphosphate requires a dephosphoenzyme with a cytosolic K^+ stimulation site ($E_1 \cdot K^+$) [30] or a luminal K^+ stimulation site ($E_2 \cdot K^+$) [33].

An alternative explanation might be that 5-B6 binds to a form of the enzyme which is intermediate between $E_1 \cdot K^+$ and $E_2 \cdot K^+$. This form $E(K^+)$ would represent an enzyme form which occludes K^+. Such a form has not yet been demonstrated for H^+/K^+-ATPase in contrast to Na^+/K^+-ATPase [34,35] where such a form easily can be found.

The steady-state phosphorylation level of H^+/K^+-ATPase was reduced by 5-B6 in a concentration depen-

dent way. The maximal extent of reduction was also pH dependent. A reduction of the steady-state level of phosphoenzyme does not indicate whether inhibition of formation, or stimulation of breakdown has occurred. Since 5-B6 neither accelerated the spontaneous (data not shown) and the K^+-stimulated dephosphorylation nor influenced the interconversion from $E_1 \cdot P$ to E_2-P, it seems possible that binding of 5-B6 prevents the phosphoenzyme formation. Normally, the steady-state level of phosphoenzyme does not change with pH, as does the rate of phosphorylation in the absence of K^+ [29]. In the case of reduction of the EP level by 5-B6 however, an alkaline pH might cause an inhibition of the phosphorylation rate by 5-B6 through stabilizing an enzyme form which inhibits or slows down phosphorylation. This is probably an E_1 form without H^+ bound to it. An acidic pH on the other hand would favour the $E_1 \cdot H^+$ form, and as a result a smaller maximal extent of reduction of EP level by 5-B6 would be reached. The difference in the maximal extent of inhibition of the K^+-ATPase activity, and of reduction of the steady-state phosphoenzyme level at each pH value, can be explained by the absence of the $E_1 \cdot K^+$ form during phosphorylation and for which 5-B6 has a greater affinity than for the E_1 form without H^+ bound to it. Therefore the enzyme conformation differs in the $E_1 \cdot K^+$ or E_1 and $E_1 \cdot H^+$ in such a way that effective binding with monoclonal antibody 5-B6 is selective for the $E_1 \cdot K^+$ form.

References

1 Sachs, G., Chang, H.H., Rabon, E., Schackman, R., Lewin, M. and Saccomani, G. (1976) J. Biol. Chem. 251, 7690–7698.
2 Ganser, A.L. and Forte, J.G. (1973) Biochim. Biophys. Acta 307, 169–180.
3 Shull, G.E. and Lingrel, J.B. (1986) J. Biol. Chem. 261, 16788–16791.
4 Walderhaug, M.O., Post, R.L., Saccomani, G., Leonard, R.T. and Briskin, D.P. (1985) J. Biol. Chem. 260, 3852–3859.
5 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–151.
6 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 186–189.
7 Maeda, M., Ishizaki, J. and Futai, M. (1988) Biochem. Biophys. Res. Commun. 157, 203–209.
8 Faller, L.D., Rabon, E. and Sachs, G. (1983) Biochemistry 22, 4676–4685.
9 Helmich-de Jong, M.L., Van Emst-de Vries, S.E. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 905, 358–370.
10 Helmich-de Jong, M.L., Van Duynhoven, J.P.M., Schuurmans Stekhoven, F.M.A.H. and De Pont, J.J.H.H.M. (1986) Biochim. Biophys. Acta 858, 254–262.
11 Jackson, R.G., Mendlein, J. and Sachs, G. (1983) Biochim. Biophys. Acta 731, 9–15.
12 Lorentzon, P., Jackson, R., Wallmark, B. and Sachs, G. (1987) Biochim. Biophys. Acta 897, 41–51.
13 Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E. and Sachs, G. (1987) J. Biol. Chem. 262, 2077–2084.
14 Faller, L.D., Smolka, A. and Sachs, G. (1985) in The Enzymes of Biological Membranes, 2nd Edn., Vol. 3 (Martonosi, A.N., ed.), pp. 431–438.
15 Peters, W.H.M., Ederveen, A.G.H., Salden, M.H.L., De Pont, J.J.H.H.M. and Bonting, S.L. (1984) J. Bioenerg. Biomembr. 16, 223–232.
16 Smolka, A., Helander, H.F. and Sachs, G. (1983) Am. J. Physiol. 245, G589–G596.
17 Smolka, A. and Weinstein, W. (1986) Gastroenterology 90, 532–539.
18 Asano, S., Inoie, M. and Takeguchi, N. (1987) J. Biol. Chem. 262, 13263–13268.
19 Skrabanja, A.T.P., Van der Hijden, H.T.W.M. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 903, 434–440.
20 Ouchterlony, O. (1959) Lancet 1, 346–348.
21 Laemmli, U.K. (1970) Nature 227, 680–685.
22 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
23 Schrijen, J.J., Luyben, W.A.H.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 331–344.
24 Schoot, B.M., Schoots, A.F.M., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1977) Biochim. Biophys. Acta 483, 181–192.
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
26 Helmich-de Jong, M.L., Van Emst-de Vries, S.E., De Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1985) Biochim. Biophys. Acta 821, 377–383.
27 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 100–111.
28 Wallmark, B., Stewart, H.B., Rabon, E., Saccomani, G. and Sachs, G. (1980) J. Biol. Chem. 255, 5313–5319.
29 Stewart, H.B., Wallmark, B. and Sachs, G. (1981) J. Biol. Chem. 256, 2682–2690.
30 Ljungström, M., Vega, F.V. and Mårdh, S. (1984) Biochim. Biophys. Acta 769, 220–230.
31 Fendler, K., Van der Hijden, H.T.W.M., Nagel, G., De Pont, J.J.H.H.M. and Bamberg, E. (1988) in The Na^+,K^+-pump (Skou, J.C., Nørby, J.G., Maunsbach, A.B. and Esmann, M., eds.), Part A, pp. 501–510, A.R. Liss, New York.
32 Schackman, R., Schwartz, A., Saccomani, G. and Sachs, G. (1977) J. Membr. Biol. 32, 361–381.
33 Saccomani, G., Barcellona, M.L. and Sachs, G. (1981) J. Biol. Chem. 256, 12405–12410.
34 Beaugé, L.A. and Glynn, I.M. (1979) Nature 280, 510–512.
35 Glynn, I.M., Howland, J.L. and Richards, D.E. (1985) J. Physiol. London 368, 453–469.