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K^+-stimulated *p*-nitrophenyl phosphatase is not a partial reaction of the gastric ($H^+ + K^+$)-transporting ATPase

Evidence supporting a new model for the univalent-cation-transporting ATPase systems

Tushar K. RAY and Jyotirmoy NANDI
Department of Surgery, The State University of New York, Upstate Medical Center, Syracuse, NY 13210, U.S.A.

Studies with intact and lysed gastric microsomal vesicles demonstrate that there are two *p*NPP (*p*-nitrophenyl phosphate)- and one ATP-hydrolytic sites within the gastric H^+,K^+-ATPase [($H^+ + K^+$)-transporting ATPase] complex. Whereas the ATPase site is located exclusively on the vesicle exterior, the *p*NPPase sites are distributed equally on both sides of the bilayer. Competition by ATP for the *p*NPPase reaction on the vesicle exterior suggests that both ATP and *p*NPP are hydrolysed at the same catalytic site present at the outside surface of the intact vesicles. However, a biphasic inhibition of the K^+-*p*NPPase (K^+-stimulated *p*NPPase) by ATP in the lysed vesicles suggest the *p*NPPase site of the vesicle interior to have very low affinity ($K_i \approx 1.2$ mM) for ATP compared with the vesicle exterior ($K_i \approx 0.2$ mM). Studies with spermine, which competes with K^+ for the K^+-*p*NPPase reaction without inhibiting the H^+,K^+-ATPase, suggest there are two separate K^+ sites for the *p*NPPase reaction and another distinct K^+ site for the ATPase reaction. In contrast with the K^+ site for the ATPase, which is located opposite to the catalytic site across the bilayer, both the K^+ and the catalytic site for the *p*NPPase are located on the same side. The data clearly demonstrate that the *p*NPPase is not a manifestation of the phosphatase step of the total H^+,K^+-ATPase reaction. The K^+-*p*NPPase associated with the Na^+,K^+-ATPase also has properties strikingly similar to the gastric K^+-*p*NPPase system, suggesting a resemblance in the basic operating principle of the two ion-transporting enzymes. A unified model has been proposed to explain the present data and many other observations reported in the literature for the ATPase-mediated transport of univalent cations.

INTRODUCTION

The reaction mechanism of the univalent-cation-transporting ATPase(s) such as H^+,K^+-ATPase and Na^+,K^+-ATPase can be broadly divided into two principal steps: a kinase step, involving ATP for the generation of enzyme–phosphate (E ~ P) complex, and a K^+-requiring phosphatase step responsible for the hydrolysis of E ~ P into E and P_i. The latter step is thought to be a representation of the K^+-pNPPase that is always co-purified with the ATPase system. Primary evidence for such belief is that both the ATPase and *p*NPPase reactions are influenced by similar cations such as Tl^+, K^+, Rb^+, Cs^+ and NH_4^+ and the orders of activation are also the same (Skou, 1965; Forte *et al.*, 1976; Ray & Fromm, 1981).

The present paper deals with the orientation of the *p*NPP-hydrolytic sites across the bilayer and assessment of the relationship between the ATPase and *p*NPPase activities. To this end the ATPase and *p*NPPase activities were measured in the intact and broken gastric microsomal vesicles; competition between ATP and *p*NPP was studied and differential inhibition of the *p*NPPase by spermine was investigated. The data demonstrate that the *p*NPPase is not a partial reaction of the ATPase. Furthermore, separate sites having different affinities for K^+ were suggested to be involved in the regulation of the ATPase and *p*NPPase reactions. A model for the univalent-cation-transporting ATPase is proposed to explain these and other reported data.

METHODS AND MATERIALS

Isolation of plasma membranes

Purified pig gastric microsomal vesicles highly enriched in H^+,K^+-ATPase and K^+-*p*NPPase activities were isolated by the method of Ray (1978). No oubain-sensitive Na^+,K^+-ATPase is detected in these membranes. The preparation is also free from the mitochondrial marker enzymes such as succinic dehydrogenase and HCO_3^--stimulated ATPase. The microsomes are derived primarily from the apical and tubulovesicular membranes of the parietal cells (Forte *et al.*, 1980; Ray & Fromm, 1981). Isolated microsomes appear in the form of tightly sealed vesicles with the ATP-hydrolytic site facing the vesicle exterior and the K^+ effector site for the ATPase facing the interior of the vesicles (Forte *et al.*, 1980).

Assay of ATPase and *p*NPPase

The ATPase and *p*NPPase were assayed as previously described (Ray *et al.*, 1983). The incubation mixture for the ATPase contained, in a total volume of 1 ml, 50 μmol of Pipes, pH 6.8, 1 μmol of $MgCl_2$, 2 μmol of Tris/ATP, 10 μg of membrane in the presence and absence of appropriate amount of KCl and/or NaCl as shown in the Results section. After 10 min pre-incubation at 37 °C, the reactions were started with ATP and, after further incubation for 15 min, P_i was assayed as described by Sanui (1974).

For *p*NPPase, the incubation mixture contained, in a total volume of 1 ml, 50 μmol of Tris buffer, pH 7.5,

Abbreviations used: *p*NPP, *p*-nitrophenyl phosphate; *p*NPPase, *p*-nitrophenyl phosphatase; K^+-*p*NPPase, K^+-stimulated *p*-nitrophenyl phosphatase; H^+,K^+-ATPase, ($H^+ + K^+$)-transporting ATPase; Na^+,K^+-ATPase, ($Na^+ + K^+$)-transporting ATPase; SDS, sodium dodecyl sulphate.

2 μmol of $MgCl_2$, 5 μmol of *p*NPP, 25 μg of membrane protein, with and without the designated amount (see the Results section) of KCl. After a pre-incubation period of 2 min the reactions were started with *p*NPP and continued for 5 min at 37 °C. Preliminary experiments demonstrated strict linearity of *p*NPP hydrolysis by the gastric microsomal vesicles (under iso-osmotic conditions) within such (5 min) an incubation time, suggesting negligible penetration of *p*NPP and K^+ to the vesicle interior during this period. The reactions were stopped by the addition of 1 ml of 1.5 M-NaOH. After a brief centrifugation, the A_{410} of the supernatant was read.

Intact and leaky vesicles

The gastric vesicles were tightly sealed to K^+ permeation, as demonstrated by stimulation of the ATPase activity by valinomycin. From the extent of valinomycin stimulation it was estimated that 15–20% of the vesicles were ruptured in the microsomal population from various preparations. For studies with intact vesicles, the osmoticity of the enzyme assay medium was maintained at 250 mM with sucrose.

The leaky vesicles were prepared by treatment of gastric microsomes (2 mg) with 0.01% SDS at room temperature for 30 min in a medium consisting of 2 mM-Pipes, 0.25 M-sucrose and 0.2 mM-EDTA, pH 6.8. After incubation, the membranes were diluted with the same buffer as above without any SDS under ice-cold conditions and stored at 0 °C for use on the same day. Higher concentrations (over 0.015%) of SDS under otherwise identical conditions inhibited the K^+-stimulated enzyme activities. Subsequent treatment of the SDS (0.01%)-lysed vesicles with hypo-osmotic medium (2 mM-Pipes containing 0.2 mM-EDTA, pH 6.8) at 0–4 °C overnight did not generate any additional K^+-stimulated activity, suggesting the generation of totally leaky vesicles. No detectable solubilization of the membrane protein was observed after 0.01% SDS treatment.

RESULTS

Table 1 shows the effects of various treatments in generating the leaky vesicles from gastric microsomes. It is noteworthy that the K^+-stimulated ATPase activity associated with the totally leaky vesicles is higher, by about 10%, than that obtained with intact vesicles in the presence of K^+ and valinomycin (Table 1). This phenomenon has been attributed to the limited Cl^- conductance of the gastric microsomal vesicles, since the (valinomycin + K^+)-stimulated ATPase activity in vesicles pre-loaded with Cl^- (as NaCl) is nearly identical with the K^+-stimulated activity associated with the leaky vesicles (Table 1). Na^+ itself does not have any effect on the H^+, K^+-ATPase activity under similar or other conditions (Ray & Nandi, 1985*a*).

Orientation of the hydrolytic sites for ATP and *p*NPP across the gastric microsomal vesicles was studied (Table 2) by using the intact and broken vesicle preparation. Taking into consideration the limited Cl^- conductance of the intact vesicles (see above), the data (Table 2) show that the H^+,K^+-ATPase activity associated with the leaky vesicles is about the same as that of the intact vesicles in presence of valinomycin. The data suggest that nearly all of the ATP-hydrolytic sites associated with the intact vesicles are facing the vesicle exterior. Since K^+ is essential in the vesicle interior for the ATPase activation, the extent of stimulation by K^+ in the presence and absence of valinomycin gives a measure of the degree of leakiness of the microsomal vesicles (Sen & Ray, 1981). Taking into account the fact that 18% of the vesicles were leaky, 50% of the total K^+-stimulated *p*NPPase was located on the vesicle exterior and 50% on the vesicle interior. It may be argued, however, that the vesicle-interior-located *p*NPPase activity may be due to some contaminating protein of non-H^+,K^+-ATPase origin entrapped within the vesicles. The following facts would argue against such possibility. Thus all the microsomal K^+-*p*NPPase activities are always associated with the gastric H^+, K^+-ATPase during and after purification in a highly active form showing a single major 100 kDa catalytic subunit on SDS/polyacrylamide-gel-electrophoresis (Chang *et al.*, 1977; Meng-Ai *et al.*, 1984). In contrast with *p*NPPase, all of the ATP-hydrolytic activity was located on the vesicle exterior (Table 2).

ATP inhibits the K^+-*p*NPPase reaction associated with the intact vesicles in a competitive manner (Fig. 1) when assayed under iso-osmotic conditions. The K_m value for *p*NPPase is increased from 1.3 mM to 2.5 and 4 mM respectively in presence of 40 and 100 μM-ATP, whereas the $V_{max.}$ remains unaltered.

Effects of increasing concentrations of ATP on the K^+-*p*NPPase reaction associated with leaky vesicles show biphasic inhibition (Fig. 2). Also a rapid inactivation of the vesicle-exterior-located *p*NPPase (Fig. 2 below, inset) which is closely similar to the initial rapid inhibitory phase (Fig. 2 below) of the *p*NPPase associated with the leaky vesicles was observed. The linearity observed in the ATP inhibition of the *p*NPPase in the

Table 1. Gastric microsomal ATPase activities after various treatments

For 'freeze–thaw' experiments, the membranes were quickly frozen (ethanol/solid CO_2) and thawed (at 37 °C with shaking) four times within a period of 15–20 min. The 'Hypo-osmotic (overnight)' indicates treatment of the membranes at 0–4 °C overnight in a hypo-osmotic medium (2 mM-Pipes buffer containing 0.2 mM-EDTA, pH 6.8). Conditions for SDS (0.01%) lysis are given in the Methods and materials section. For the Cl^--loaded vesicles, the microsomes were incubated for 60 min at room temperature in a medium consisting of 5 mM-Pipes, pH 6.8, 250 mM-sucrose and 25 mM-NaCl. After pre-loading, portions of the vesicles were used for the ATPase assay. The 'K^+' and 'K^+ plus val' values were obtained by subtracting the corresponding Mg^{+2}-dependent rate from those in presence of 20 mM-K^+ and 20 mM-K^+ plus 10 μM-valinomycin (val). Nearly identical results were obtained in three separate membrane-vesicle preparations. Since the specific activity of the enzyme varies from preparation to preparation, the data from one typical experiment are provided.

Treatments	ATPase activity (μmol/h per mg)		
	Mg^{2+}	K^+	K^+ plus val
Iso-osmotic	20.4	14.6	61.1
Iso-osmotic (Cl^--loaded)	20.4	14.5	67.4
Freeze–thaw	23.8	62.0	67.5
Hypo-osmotic (overnight)	22.0	67.6	67.5
SDS (0.01%)-treated	20.4	67.5	67.5

Table 2. ATPase and *p*NPPase activities associated with intact and leaky gastric microsomal vesicles and their orientation across the vesicles

The basal (Mg^{2+}-dependent) activity has been subtracted from the ATPase and *p*NPPase values. Iso-osmoticity of the medium was maintained at 250 mM by using sucrose. The membranes for the hypo-osmotic assay was treated with 0.01% SDS (see the Methods and materials section) before assay in hypo-osmotic medium; 20 mM-K^+ was used for each enzyme assay. Details of the enzyme assay are given in the Methods and materials section. Data are means ±S.D. for three separate studies. Abbreviation used: val, valinomycin.

Conditions used	Activity (μmol/h per mg)
H^+,K^+-ATPase	
1. Iso-osmotic medium	11.5±2.2
2. Iso-osmotic + 10 μM-val	60.5±2.4
3. Hypo-osmotic medium (leaky membranes)	63.9±4.1
4. Contaminating leaky vesicles (%)...	18
5. Vesicle-exterior-associated ATPase (%)...	100
6. Vesicle-interior-associated ATPase (%)...	0
K^+-*p*NPPase	
7. Iso-osmotic medium	31.4±3.6
8. Iso-osmotic plus 10 μM-val	31.2±3.0
9. Hypo-osmotic (leaky membranes)	52.0±3.4
10. Leaky-vesicle-associated *p*NPPase (%) (18% of hypo-osmotic)...	9.4
11. Vesicle-exterior-associated *p*NPPase (7−10)(%)...	22
12. Vesicle-interior-associated *p*NPPase (9−7) (%)...	21

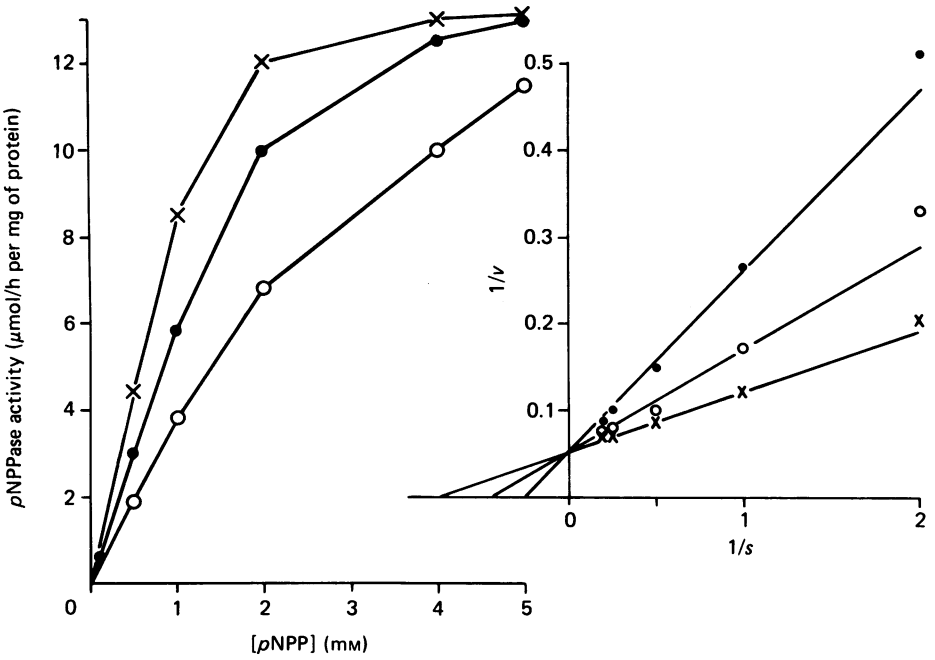

Fig. 1. Competitive effect of ATP for *p*NPP in the K^+-stimulated *p*NPPase reaction associated with intact vesicles

The Lineweaver–Burk plot of the data is also shown. The lines were drawn by eyeball estimates. Non-linear regression analysis of the data revealed that the K_m values are significantly altered in the presence of 0.04 mM and 0.1 mM-ATP, whereas there are no significant changes in the V_{max}. The K_m (mM) and V_{max}. (μmol/h per mg) values (±S.E.M.) for the control (×), 0.04 mM-ATP (●) and 0.1 mM-ATP (○) experiments are: (1) control: 1.26±0.5, 20.89±3.83; (2) 0.04 mM-ATP: 3.77±1.0, 26.17±5.81, and (3) 0.1 mM-ATP: 7.02±1.75, 29.71±6.76 respectively. The χ-square values showed that the data did not deviate significantly from the fit. The concentration of K^+ was 20 mM. Designated amounts of ATP and *p*NPP were premixed and used to initiate the *p*NPPase reaction for the competition study. Details of the *p*NPPase assay are given in the Methods and materials section.

intact vesicles is somewhat conspicuous in view of the fact that ATP competes with *p*NPP for the vesicle-exterior *p*NPPase (Fig. 1). Precise reasons for such unusual effects are not understood at present. The data in Fig. 2 suggest that the affinities for ATP for the vesicle-exterior or cytosolic *p*NPPase (*cis*-*p*NPPase) site is significantly different than the vesicle-interior-located *p*NPPase (*trans*-*p*NPPase) site. The affinities (K_i) for the *cis*- and *trans*-*p*NPPase for ATP were about 0.2 and 1.2 mM respectively.

Table 3. Effects of spermine and furosemide on the gastric K^+-stimulated ATPase and *p*NPPase activities

The K^+-stimulated ATPase and *p*NPPase reactions were performed under hypo-osmotic assay conditions with 0.01%-SDS-lysed vesicles in the presence of 10 and 20 mM-K^+ respectively. The basal (Mg^{2+}-dependent) activity has been subtracted from the ATPase and *p*NPPase values. Details of the vesicle lysis and enzyme assays are given in the Methods and materials section. Data are means ±S.D. for three separate studies.

	Enzyme activity (μmol/h per mg of protein)	
	K^+-ATPase	K^+-*p*NPPase
1. Control	68±2	35±2
Spermine		
0.5 mM	70±3	27±3
1.0 mM	68±3	19±4
2. Control	101±4	48±1
Furosemide		
0.5 mM	73±3	48±2
1.0 mM	50±5	52±3

The effects of two unrelated compounds, namely spermine and furosemide, on the gastric H^+, K^+-ATPase and associated K^+-stimulated *p*NPPase activities are shown in Table 3. Differential effects of these two compounds on the two gastric phosphatases were observed. Thus spermine inhibited the K^+-*p*NPPase without affecting the H^+,K^+-ATPase activity, whereas furosemide inhibited the H^+,K^+-ATPase without having any appreciable effect on the K^+-*p*NPPase activity. The latter compound was found to compete with ATP for the gastric H^+, K^+-ATPase reaction. The K_m value for the H^+,K^+-ATPase activity was increased 10-fold (from 83 μM to 830 μM) in the presence of 0.5 mM-furosemide. Spermine, on the other hand, was found to compete with K^+ for the K^+-*p*NPPase reaction. Since there are two separate *p*NPP-hydrolytic sites of opposite orientation within the gastric H^+, K^+-ATPase complex (see above), spermine was used as a tool to further explore whether the regulating K^+ site is common to both *p*NPPase activities across the bilayer or have separate K^+ sites for each.

The data in Fig. 3 show that spermine competes with K^+ responsible for the hydrolysis of *p*NPP at the vesicle exterior. The data in Table 2 show that, unlike the H^+, K^+-ATPase, the vesicle-exterior-located *p*NPPase is not sensitive to valinomycin; hence, both the K^+ site and *p*NPP-hydrolytic site must reside on the same side, i.e. the exterior of the microsomal vesicles. Spermine did not, however, have any effect on the H^+, K^+-ATPase activity, either in presence of valinomycin (not shown) or the lysed vesicles (Table 3). Also, the K^+ site necessary for the activation of the ATPase is located within the vesicles. Thus the data in Fig. 3 strongly suggest that the K^+ site for *p*NPPase is clearly different than that for the ATPase reaction.

Similar to the intact vesicles (Fig. 3), the K^+-*p*NPPase activity associated with the lysed vesicles also show a competition between K^+ and spermine (Fig. 4). The kinetic parameters, such as K_a values, for K^+ in the absence and presence of spermine (0.5 mM) were nearly the same in the intact (Fig. 3) and lysed (Fig. 4) vesicles. Thus the K_a values were 1.2 and 1.0 mM in the absence and 3.8 and 4.0 mM in the presence of spermine in the intact and lysed vesicles respectively. The data suggest that the nature of the two K^+ sites responsible for *p*NPP hydrolysis are oriented equally on both sides of the bilayer and are closely similar or possibly identical.

In view of the spermine (see above) and Na^+ (Ray & Nandi, 1985*a*) inhibition of the gastric K^+-pNPPase without its having any effect on the H^+, K^+-ATPase and similar reported effects on the Na^+, K^+-ATPase system (Tashima *et al.*, 1978; Formby & Clausen, 1958), it is important to study the bilayer orientation of the ouabain-sensitive K^+-*p*NPPase activity together with the K^+ regulatory site in the latter system. To this end studies were conducted with a population of highly enriched (about 85%) and viable (over 90%) gastric surface epithelial cells isolated from rabbit stomach. The cells prepared as described by Tanaka *et al.* (1982) had appreciable Na^+, K^+-ATPase, but lacked H^+, K^+-ATPase activity upon lysis. Preliminary studies (J. Nandi, R. Levine & T. K. Ray, unpublished work) with the intact cells demonstrated an ouabain-sensitive K^+-*p*NPPase but no Na^+,K^+-ATPase and insignificant Mg^{2+}-ATPase activity exposed to the cell exterior. After lysis, both the Na^+,K^+-ATPase and K^+-*p*NPPase activities sensitive to ouabain inhibition were observed. The extra- and intra-cellular distribution of the ouabain-sensitive K^+-*p*NPPase activity was found to be equal and was about 926±34 nmol/h per 10^6 cells. Also, spermine was found to compete with K^+ for the *p*NPPase activity. The observations point out the similarities involved in the

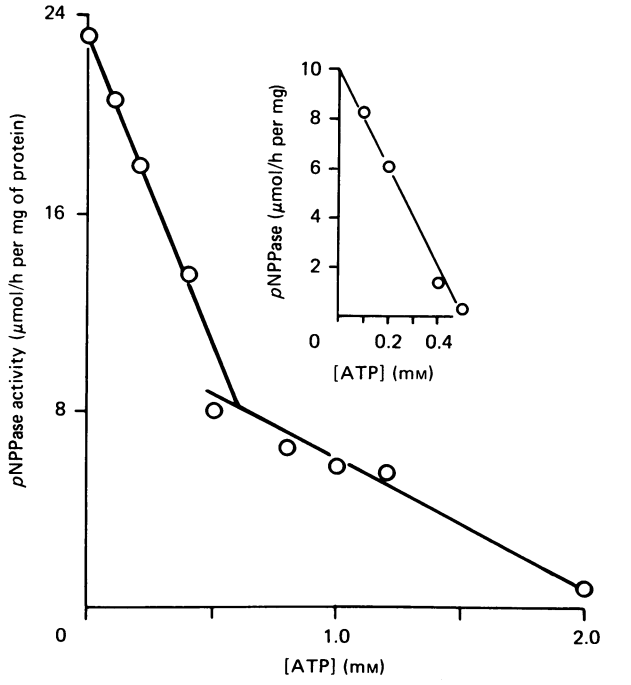

Fig. 2. Effects of increasing concentrations of ATP on the K^+-stimulated *p*NPPase activity associated with lysed and intact vesicles

Data for the intact vesicles are shown in the inset. Details for vesicle lysis and *p*NPPase assay are given in the Methods and materials section and Fig. 1.

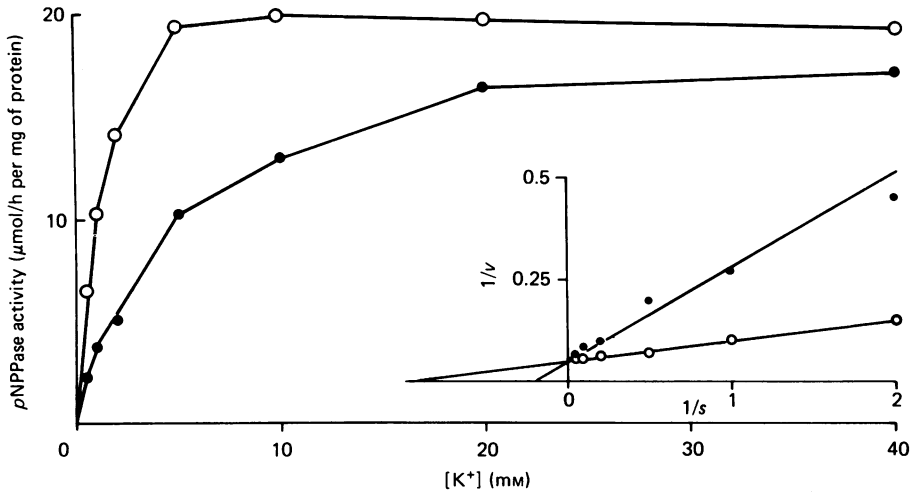

Fig. 3. Competition between K^+ and spermine in the K^+-stimulated *p*NPPase reaction associated with the intact vesicles

The inset shows the Lineweaver–Burk plot of the data. ○, without spermine; ●, with 0.5 mM-spermine. The lines were drawn by eyeball estimation. Statistical analysis of the data showed significant differences only in the K_m values between the two groups. The K_m (μM) and $V_{max.}$ (μmol/h per mg) values (±S.E.M.) were: (1) control; 1.18±0.68, 22.0±2.69; (2) 0.5 mM-spermine: 3.22±0.59, 15.99±3.33 respectively. The χ-square values showed no significant deviation of the data from the fit. Details of the *p*NPPase assay are given in Fig. 1 and in the Methods and materials section.

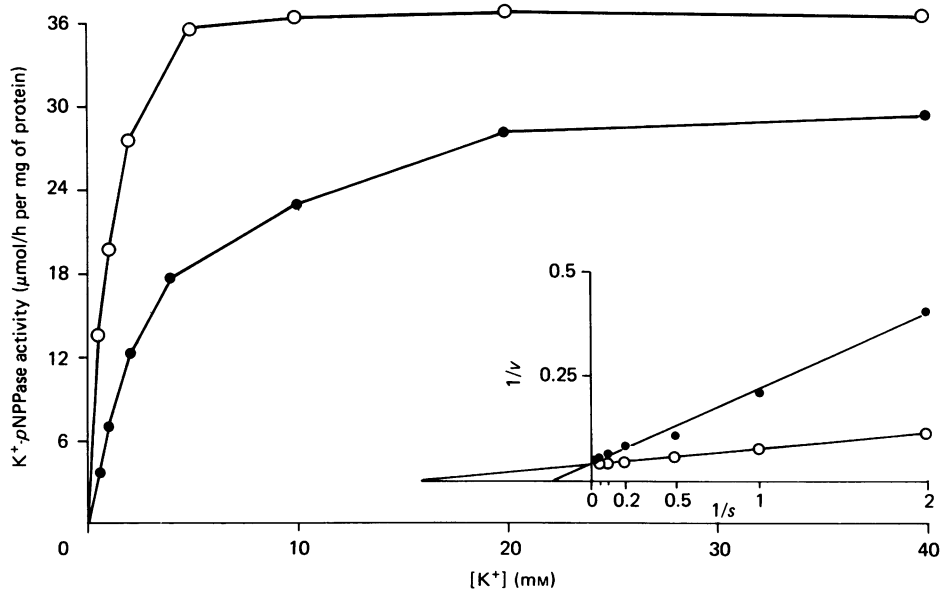

Fig. 4. Competition between K^+ and spermine in the K^+-stimulated *p*NPPase reaction associated with lysed vesicles

The inset shows the Lineweaver–Burk plot of the data. ○, without spermine; ●, with 0.5 mM-spermine. Details are given in Fig. 4 and in the Methods and materials section.

orientation of the K^+-*p*NPPase activities associated with two different univalent-cation-transporting ATPase systems, namely H^+,K^+-ATPase and Na^+,K^+-ATPase, across their respective membrane loci.

DISCUSSION

The data reveal that gastric microsomal K^+-*p*NPPase activity is distributed equally across the vesicle i.e. half of the activity is exposed to the exterior and half to the interior of the vesicles. In contrast with *p*NPPase, all of the ATP-hydrolytic sites were exposed to the vesicle exterior (Table 2).

Competition between *p*NPP and ATP for the *p*NPPase reaction associated with intact (Fig. 1) vesicles suggest both *p*NPP and ATP share a common hydrolytic site. Although the vesicle-interior-located *p*NPP-hydrolytic site can not hydrolyse ATP (Table 2), the *p*NPPase is inhibited by ATP in a dose-dependent manner (Fig. 2). Biphasic behaviour of the *p*NPPase (associated with leaky vesicles) inhibition by increasing concentrations of ATP suggests differential affinities of the two *p*NPP-hydrolytic

sites towards ATP. Thus the data (Fig. 3) indicate the vesicle-exterior-located *p*NPPase site to have higher ($K_i \approx 0.2$ mM) affinity for ATP than the one within ($K_i \approx 1.2$ mM) the vesicle interior. The K_m values for the *p*NPPase, however, are nearly the same for both the *p*NPPase-hydrolytic sites (Figs 3 and 4).

Differences in the nature of the K^+ sites responsible for the activation of the ATPase and *p*NPPase reactions were also observed. Thus spermine, which does not inhibit the H^+,K^+-ATPase (Table 3), inhibits the K^+-*p*NPPase in a dose-dependent manner. A competition between K^+ and spermine was observed both in lysed and intact vesicles (Figs 3 and 4). The data demonstrate that the K^+ site for the *p*NPPase activation is clearly different than that responsible for the ATPase stimulation. Like spermine, Na^+ also has recently been demonstrated to compete with K^+ for inhibiting the K^+-*p*NPPase without inhibiting the H^+,K^+-ATPase activity (Ray & Nandi, 1985*a*). It is noteworthy that a similar differential effect of spermine (Tashima *et al.*, 1978) and Na^+ (Formby & Clausen, 1958) has been noted in the case of Na^+,K^+-ATPase and associated K^+-*p*NPPase activities. Preliminary data (see the Results section) on the intact and lysed surface epithelial cells containing ouabain-sensitive Na^+,K^+-ATPase and no H^+,K^+-ATPase activity also demonstrated that, unlike the Na^+,K^+-ATPase, the associated *p*NPPase activity was distributed equally on the exterior and interior of the cells. Furthermore, competition with spermine suggested separate K^+ regulatory sites for the cell-exterior- and interior-located K^+-*p*NPPase activities.

In view of the fact that the K^+-*p*NPPase reaction is independent of the H^+,K^+-ATPase activation both in terms of orientation of catalytic and K^+ sites and inhibitor susceptibility (see above), we concluded that the *p*NPPase is not a partial step in the reaction sequence of the gastric H^+,K^+-ATPase. Such a conclusion would be consistent with our recent report (Ray & Nandi, 1985*b*). It is noteworthy that even though K^+ can be substituted for by various congeners in the sequence $Tl^+ > K^+ > Rb^+ > Cs^+ > NH_4^+$ in both the ATPase and *p*NPPase reactions, the cationic affinities for the ATPase is lower by an order of magnitude than that for the *p*NPPase (Forte *et al.*, 1976).

*p*NPP is not a natural substrate, and our data demonstrate that the pNPPase does not represent the partial reaction of the ATPase. In view of these observations, the question naturally arises as to what could be the functions of the two *p*NPPase-regulating K^+ sites demonstrated in the present study. It is noteworthy in this connection that the presence of spermine in the secretory solution inhibits gastric H^+ transport by chambered bullfrog gastric mucosa and the inhibition could be released by elevation of secretory K^+ (Ray *et al.*, 1982). In addition, studies with gastric microsomal vesicles suggested that spermine interacts with a low-affinity luminal K^+ site and thereby cause interference with the vectorial translocation of H^+ (Ray *et al.*, 1982; Nandi & Ray, 1982). Whether the said low-affinity luminal K^+ site is the same as the low-affinity luminal K^+ site responsible for the *p*NPPase reaction remains to be seen.

The present data would be consistent with a model for the ATPase molecule having two 100 kDa catalytic subunits with the catalytic sites facing the opposite direction (Fig. 5). 'Tightening' of the two subunits by Mg^{2+} (Schrijen *et al.*, 1983) may be visualized. Whereas

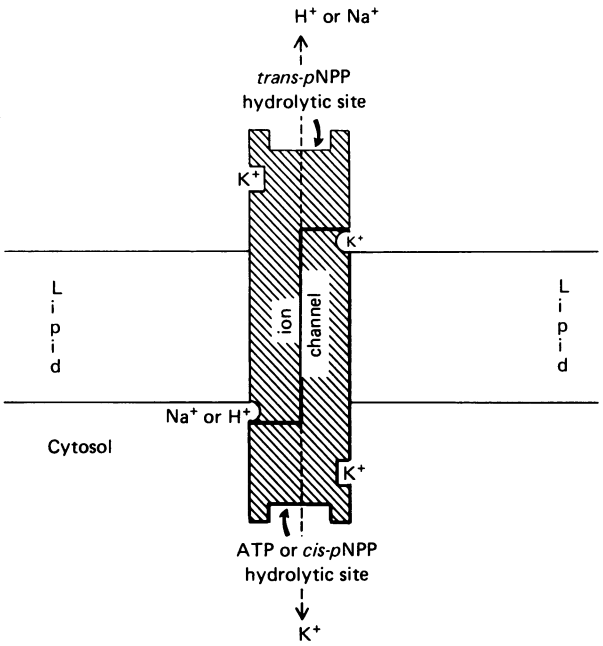

Fig. 5. Schematic view of possible mode of organization of catalytic subunits and cationic regulation in the H^+, K^+-transporting ATPase complex

Two identical catalytic subunits (about 100 kDa each) are arranged in close apposition and in opposite directions across the bilayer. The cytosolic ATP or *p*NPP (*cis*-*p*NPPase) hydrolytic site and transcytosolic *p*NPPase (*trans*-*p*NPPase) site are shown. The K^+ site responsible for the ATPase stimulation is located (designated by open half circle) across the bilayer in a partially hydrophobic environment, whereas for the *cis*-*p*NPPase the K^+ site (designated by open half rectangle) is on the same side in a completely hydrophilic environment as suggested from competition studies with highly charged spermine. Both K^+ sites for the ATPase and *cis*-*p*NPPase (shown by large and small-size K^+ to signify low and high affinities respectively) are located on the same catalytic subunit. Similar to *cis*-*p*NPPase, both the K^+ ligand site and catalytic site for the *trans*-*p*NPPase are located on the same catalytic subunit. The *trans*-*p*NPPase site can not hydrolyse ATP, but demonstrates an affinity for ATP which is about one-sixth that of the *cis*-*p*NPPase (or ATPase) site. Appropriate interaction between both the subunits is essential for the ATPase reaction, although each individual subunit can manifest the *p*NPPase activity. A cytosolic site for H^+ on the *trans*-*p*NPPase-containing peptide but oriented towards the *cis*-side has been visualized in accordance with our recent observation (Ray & Nandi, 1985*a*). The site showing Na^+ or H^+ implies that such model may also explain the behaviour of Na^+,K^+-ATPase if the specificity is changed from H^+ to Na^+ (Ray & Nandi, 1985*a*). The bilayer phase of the subunits is believed to have a channel or channels for the movement of the ions in both directions. On the basis of some limited evidence as mentioned in the text, it is postulated that the *trans*-*p*NPPase, together with its K^+ regulatory site, may function as an extension of the H^+ channel (beyond the bilayer phase). Similarly the *cis*-*p*NPPase or ATPase site may function as a part of the K^+ channel. It should be emphasized that main purpose of the model is to show fundamental differences in the H^+,K^+-ATPase and associated K^+-*p*NPPase reactions in terms of orientation of their catalytic and regulatory K^+ sites. Such differences clearly argue against *p*NPPase being a partial reaction of the H^+,K^+-ATPase.

an intimate association between these two subunits appears essential for the ATPase activity, only a single subunit seems to be sufficient for manifestation of the *p*NPPase reaction. There are reports in the literature which would be in accord with such an idea. Thus radiation-inactivation studies reported the M_r of the associated *p*NPPase to be half of the unit responsible for the Na^+,K^+-ATPase (Kepner & Macey, 1968; Ellory *et al.*, 1980; Richards *et al.*, 1981) and H^+,K^+-ATPase (Chang *et al.*, 1977) reactions. Also, solubilized ATPase complex subjected to column chromatography shows separation of low-M_r monomeric species having *p*NPPase reaction from the high-M_r ones containing H^+,K^+-ATPase activities (Soumarmon & Lewin, 1984). In addition, a recent observation with delipidated inactive Na^+,K^+-ATPase showing that the *p*NPPase is activated before the manifestation of the ATPase activity during relipidation (Ottolenghi, 1979) would be consistent with the above concept. In view of the observed antagonism between K^+ and spermine for both the *p*NPPase reaction (see above) and H^+ transport (Ray *et al.*, 1982) from the lumen side of the secretory membrane, the transcytosolic *p*NPPase (*trans-p*NPPase) site has been postulated to be acting as a part of the H^+ channel. Recent evidence suggests that cytosolic Na at high concentration may interfere with H^+ transport by interacting with a cytosolic H^+ site (Ray & Nandi, 1985*a*, *b*). The H^+ channel (carrying H^+ from the cell interior into the lumen) mentioned above will be analogous to the cytosolic ATPase (or *cis-p*NPPase) site, which is presumed to act as a part of the K^+ channel responsible for recycling of K^+ from the luminal environment into the cytosol (Ray & Nandi, 1985*b*). The latter assumption is based on the fact that interference with the transcytosolic (luminal) high-affinity K^+ site (Fig. 5) by 2-(3,4-dichlorphenylamino)quinolizium bromide inhibits both the K^+-dependent $E \sim P$ turnover (J. Nandi & J. K. Ray, unpublished work) and H^+ transport (Nandi *et al.*, 1983*a*).

Such a model (Fig. 5), although somewhat speculative, could explain many observations in the literature which hitherto remain unexplained or difficult to explain. Thus, in contrast with the highly competitive cationic inhibitors like spermine and Na^+ for the K^+-*p*NPPase reaction, there are studies (Saccomani *et al.*, 1980; Forte *et al.*, 1981; Jackson *et al.*, 1983; Nandi *et al.*, 1983*c*) with non-competitive inhibitors reporting complete inhibition of the H^+,K^+-ATPase and partial or no inhibition of the K^+-*p*NPPase activity. Demonstration of the protective effects of ATP against the inactivation of ATPase (Saccomani *et al.*, 1980; Forte *et al.*, 1981; Jackson *et al.*, 1983; Nandi *et al.*, 1983*c*) suggested the locus of such inhibitors to be at the catalytic domain or the *cis-p*NPPase site (Fig. 5). Under conditions of such target-specific blockage of the ATPase catalytic (*cis-p*NPPase) site, the *trans-p*NPPase site would be likely to remain unaffected and thus could explain the reported inhibition of only 50% of the total K^+-*p*NPPase activity (Jackson *et al.*, 1983; Nandi *et al.*, 1983*c*). In other cases where the *p*NPPase remains unaffected (Saccomani *et al.*, 1980) or transiently stimulated (Forte *et al.*, 1981), it appears likely that only *p*NPP (and not ATP) would have access to the modified catalytic site for subsequent hydrolysis, owing to the significantly smaller size of the *p*NPP molecule. In support of the latter view we have recently observed (see Table 3 and the Results section) that furosemide (which resembles somewhat the adenine moiety of ATP) competes with ATP for H^+,K^+-ATPase without inhibiting the K^+-*p*NPPase reaction. The model is also totally consistent with the report (Saccomani *et al.*, 1981) that only intravesicular (luminal) K^+ can fully protect the gastric microsomal H^+,K^+-ATPase from total inactivation by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a carboxy-group-specific agent, the extravesicular K^+ having no effect. The associated *p*NPPase (Saccomani *et al.*, 1981), on the other hand, is protected only 50% by K^+ on either side of the vesicle and complete protection was observed with K^+ on both sides. In addition, the reported (Sachs *et al.*, 1984) differences in the stoichiometric binding of various ligands to the H^+,K^+-ATPase could also be explained with the present scheme. Thus fluorescein isothiocyanate inhibition of the H^+,K^+-ATPase occurs with 1.5 nmol/mg of bound ligand, which is the same as the $E \sim P$ level. Azido-ATP, on the other hand, binds to a level of 3 nmol/mg, half of which appears as $E \sim P$ and the rest as bound nucleotide. Hence, with the exception of the half, or 1.5 nmol, of azido-ATP, which is likely to bind to the low-affinity ATP-binding site (*trans-p*NPP site), the rest of the observation clearly suggests binding at the active site of the enzyme. Also, consistent with our proposal (Fig. 5) there are reports (Askari, 1974; Ball, 1984) with antibodies specific to the catalytic subunit of the Na^+,K^+-ATPase, demonstrating that, under conditions of complete inhibition of the Na^+,K^+-ATPase activity, only 50% of the associated K^+-*p*NPPase was inhibited. There are numerous other reports in the literature involving differential effects on the Na^+,K^+-ATPase and associated K^+-*p*NPPase reactions which will be in accord with the proposed model. Thus the proposal (Fig. 5) appears to have the potential for providing a basic framework in the future development of a more detailed and unified mechanistic view for various univalent-cation-transporting ATPase systems.

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