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REVERSIBLE INACTIVATION OF PURIFIED ($Na^+ + K^+$)-ATPase FROM HUMAN RENAL TISSUE BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

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Summary

Human renal ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparations which exhibited a non-linear reaction rate, contained high levels of membrane-bound cyclic AMP-dependent protein kinase, while this latter activity was much less or absent in purified preparations. A non-linear reaction rate was observed in a purified preparation of ($Na^+ + K^+$)-ATPase by reconstituting the enzyme into lipid vesicles with cyclic AMP-dependent protein kinase. The addition of cyclic AMP to the ATPase assay of these lipid vesicles inactivated the ($Na^+ + K^+$)-ATPase. The cytoplasmic fraction of the cell contained a non-dialyzable factor, which prevented (or reversed) the cyclic AMP-mediated inactivation of the enzyme.

Some of the physical and kinetic properties of human kidney microsomal ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) were recently reported from this laboratory [1,2]. The ($Na^+ + K^+$)-ATPase reaction was non-linear with reaction time in the whole kidney homogenate and early stages of the treatment of the microsomes, but the rate was linear in the final preparation [2]. The appearance of linearity of the reaction was accompanied by loss of cyclic AMP-dependent protein kinase activity [1].

Non-linearity of the enzyme reaction with assay time was initially noted in our preliminary studies with unpurified microsomal ($Na^+ + K^+$)-ATPase (from rat kidney [3,4]), which was reversibly inactivated when pre-incubated with (Mg^{2+})-ATP. Other investigators have also noted apparent conversion of activities of ($Na^+ + K^+$)-ATPase of dog and lamb kidney when the enzyme was exposed to (Mg^{2+})-ATP under various conditions [5,6]. These studies led to the postulation that ($Na^+ + K^+$)-ATPase exists as an interconvertible active and inactive form [3—6]. This laboratory has obtained evidence to suggest that the

inactivation may be mediated by a cyclic AMP-dependent protein kinase and that reactivation is facilitated by a factor present in the soluble fraction of the cell [7]. The present report describes the restoration of cyclic AMP-dependent non-linearity to a purified ($Na^+ + K^+$)-ATPase (reacting in a linear manner) by reconstitution of the renal enzyme with cyclic AMP-dependent protein kinase. In addition, the cytoplasmic fraction of the cell has been found to contain some factor(s) that prevent or reverse the cyclic AMP-mediated non-linearity of the reconstituted ($Na^+ + K^+$)-ATPase reaction.

Methods

Source of materials. ATP (Tris salt), cyclic AMP, soluble bovine heart cyclic AMP-dependent protein kinase (2 pmol/mg), theophylline, EGTA histone (calf thymus), egg phosphatidylcholine and cholic acid were obtained from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]ATP was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were from Fisher Scientific Co., Pittsburgh, Pa.

Purification of ($Na^+ + K^+$)-ATPase from human renal tissue. The collection of human kidneys at autopsy and the purification of ($Na^+ + K^+$)-ATPase was described previously [2]. The microsomes treated only with 0.6 mg deoxycholate per ml had a non-linear reaction rate with time and are designated deoxycholate enzyme. This preparation was dialyzed for 4 h twice against 1000 vols. of 100 mM imidazole buffer (pH 7.0) to remove all deoxycholate. The next steps were NaI treatment, solubilization with deoxycholate, precipitation with glycerol, resolubilization with deoxycholate and $(NH_4)_2SO_4$ fractionation. The final ($Na^+ + K^+$)-ATPase preparation had a linear reaction rate and is designated purified enzyme.

Assays. ATPase was assayed as described previously [2] at 37°C in 100 mM imidazole (pH 7.0), 6 mM ATP, 6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 0.02% bovine serum albumin and 0.05—0.1 mg protein/ml. The reaction was started by addition of enzyme to the prewarmed reaction mixture. That activity referred to as a total ATPase was determined in the absence of ouabain; (Mg^{2+})-ATPase activity with 1 mM ouabain and ($Na^+ + K^+$)-ATPase as the difference between the total and (Mg^{2+})-ATPase. In the purified preparations exhibiting linear reaction rates (free of (Mg^{2+})-ATPase), ouabain was omitted from the assay. The activity of ($Na^+ + K^+$)-ATPase is expressed as mol liberated phosphate/kg protein per h.

The cyclic AMP-dependent protein kinase was assayed with endogenous protein or with exogenous histone as described previously [8]. The cyclic AMP-dependent activity was the difference between the activity in the presence and absence of 10 μM cyclic AMP. Cyclic AMP-dependent protein kinase activity is expressed as incorporated mol ^{32}P/kg protein per h.

Protein was determined by the Lowry procedure [9].

Preparation of lipid vesicles. Purified ($Na^+ + K^+$)-ATPase was incorporated into lipid vesicles by a procedure modified from that previously described [10]. A mixture containing 40 mg egg phosphatidylcholine and 20 mg cholic acid per ml in 25 mM imidazole (pH 7.0), 1 mM disodium EDTA, 10 mM $MgCl_2$, 400 mM NaCl, and 40 mM KCl was sonicated at 4°C for 2.5 h with an S75 sonifier

(Branson Instruments). This was then mixed with a suspension of purified $(Na^+ + K^+)$-ATPase in buffer and ions such that the final concentrations were 25 mM imidazole (pH 7.0), 1 mM disodium EDTA, 5 mM $MgCl_2$, 400 mM NaCl, 40 mM KCl, 20 mg phosphatidylcholine and 10 mg/ml cholic acid, and 1 mg/ml enzyme protein. When indicated, cyclic AMP-dependent protein kinase was also included in a concentration of 2 mg/ml. The entire suspension was then dialyzed with constant stirring for 2 days at 4°C against 1000 vols. of 25 mM imidazole (pH 7.0), 1 mM disodium EDTA, 400 mM NaCl, and 40 mM KCl. $(Na^+ + K^+)$-ATPase activity in vesicles was based upon the theoretical amount of ATPase protein in vesicles if all available ATPase protein had been incorporated. Protein kinase activity in vesicles was based upon a similar assumption for protein kinase protein. The kinase activity decayed at 4°C with a half-time of 4 days.

Electron microscopy. Purified $(Na^+ + K^+)$-ATPase and vesicles containing purified $(Na^+ + K^+)$-ATPase and cyclic AMP-dependent protein kinase were centrifuged at 100 000 × *g* for 1 h. The pellets were suspended in 3 vols. of 100 mM sodium cacodylic buffer (pH 7.1) and resedimented at 100 000 × *g* for 1 h. These pellets were then fixed in 1.5% glutaraldehyde and postfixed in 1% OsO_4. Sections were scanned in an Associated Electrical Industries AEI-EM-3G electron microscope.

$(NH_4)_2SO_4$ fractionation. The 100 000 × *g* cellular supernatant from which the microsomes were obtained was dialyzed at 4°C for 3 h in 25 mM imidazole (pH 7.0)/1 mM disodium EDTA (imidazole/EDTA). It was then brought to 30% $(NH_4)_2SO_4$ at 4°C by the addition of solid $(NH_4)_2SO_4$ crystals and centrifuged at 10 000 × *g* for 20 min. The resulting 30% pellet was resuspended in 4 vols. of imidazole/EDTA and dialyzed at 4°C for 14 h twice in 1000 vols. of imidazole/EDTA.

Results

The $(Na^+ + K^+)$-ATPase reaction in human kidney microsomes and the first several stages of purification was non-linear with assay time [2]. The activity was nearly zero by 8 min of assay, but the purified $(Na^+ + K^+)$-ATPase exhibited a linear reaction rate for at least 35 min.

The non-linearity of the $(Na^+ + K^+)$-ATPase reaction with time in the partially purified deoxycholate enzyme is shown in Fig. 1. This was not due to a limiting amount of substrate in that the addition of more enzyme to a complete assay system, which had been incubating for 15 min, caused the hydrolysis of ATP to resume.

It was apparent from these observations that the $(Na^+ + K^+)$-ATPase activity in partially purified preparations of the enzyme was decaying during the ATPase assay. This decay of activity was a reversible process. If the 100 000 × *g* supernatant from which the microsomes were obtained was dialyzed and added directly into the ATPase assay, after the $(Na^+ + K^+)$-ATPase reaction had slowed, the hydrolysis of ATP by the enzyme resumed and was maintained at a constant rate, Fig. 2. Furthermore, the non-linearity of the enzyme reaction could be almost totally prevented by the addition of the 100 000 × *g* supernatant at the beginning of the ATPase assay (Fig. 2). These findings suggested

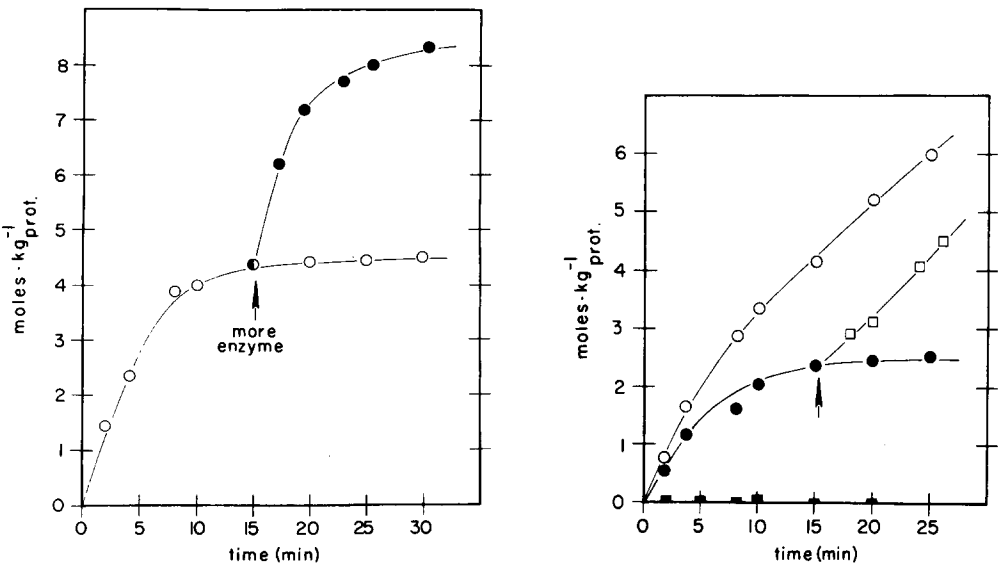


Fig. 1. The activity of $(Na^+ + K^+)$-ATPase (deoxycholate-treated) added to the reaction medium of an inactivated enzyme. The ATPase assay was run in duplicate at 0.06 mg protein per ml. At 15 min of assay as indicated by the arrow, additional enzyme (0.06 mg deoxycholate enzyme/ml) was added to one of the tubes (●———●).

Fig. 2. Reversal of $(Na^+ + K^+)$-ATPase non-linearity by the 100 000 × *g* supernatant. The control $(Na^+ + K^+)$-ATPase activity was determined on a deoxycholate-treated preparation (●———●). The 100 000 × *g* supernatant was dialyzed (at 15 mg/ml) against 25 mM imidazole, pH 7.0, and added in a concentration of 0.23 mg/ml either at the beginning of the ATPase assay (○———○), or as indicated by the arrow, after 15 min of assay (□———□). There was no ATPase activity in the 100 000 × *g* supernatant itself (■———■).

that the depression in $(Na^+ + K^+)$-ATPase activity which occurred in the assay represented a reversible inactivation of the enzyme.

Since the purified $(Na^+ + K^+)$-ATPase exhibited a linear reaction rate, it was felt that the cause of the non-linearity had been eliminated by the purification. Table I shows the endogenous cyclic AMP-dependent protein kinase activity at various stages of purification of the $(Na^+ + K^+)$-ATPase. The kinase activity in this Table is due to the phosphorylation of endogenous proteins in the ATPase preparations as no other substrate (such as histone) was added. Membrane preparations in which the $(Na^+ + K^+)$-ATPase reaction is non-linear, contain high cyclic AMP-dependent protein kinase activity. In contrast, more purified preparations of $(Na^+ + K^+)$-ATPase, which exhibit a linear reaction rate, contain little or no detectable cyclic AMP-dependent protein kinase.

During the course of the presence investigation it was observed that procedures other than enzyme purification would eliminate the non-linearity of the $(Na^+ + K^+)$-ATPase reaction. The effect of hypothermic storage on a deoxycholate treated enzyme prepration is shown in Fig. 3 and Table II. The deoxycholate enzyme had a non-linear reaction rate. If this preparation was kept at 2°C for 48 h, the $(Na^+ + K^+)$-ATPase exhibited a linear reaction rate. Identical results were obtained if the deoxycholate preparation was frozen (—90°C) and

TABLE I

THE ENDOGENOUS CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY AT VARIOUS STAGES OF PURIFICATION OF THE $(Na^+ + K^+)$-ATPase

$(Na^+ + K^+)$-ATPase was purified and assayed as described and shown previously [2]. The cyclicAMP-dependent protein kinase activity was measured at 0.1—0.5 mg protein per ml as described in Methods. The specific activity of the $(Na^+ + K^+)$-ATPase in the non-linear preparations was determined by the early part of the reaction curve which was the closest approximation of the initial enzymatic rate.

Purification step	Cyclic AMP-dependent protein kinase	$(Na^+ + K^+)$-ATPase	
	mol ^{32}P incorporated · kg^{-1} prot. · h^{-1}	mol · kg^{-1} prot. · h^{-1}	Reaction character
Microsomes	$0.276 \cdot 10^{-3}$	4	non-linear
Deoxycholate-treated enzyme	$0.208 \cdot 10^{-3}$	22	non-linear
NaI-treated enzyme	$0.110 \cdot 10^{-3}$	35	non-linear
Glycerol-precipitated enzyme	$0.0284 \cdot 10^{-3}$	70	linear
$(NH_4)_2SO_4$ enzyme	0	72	linear

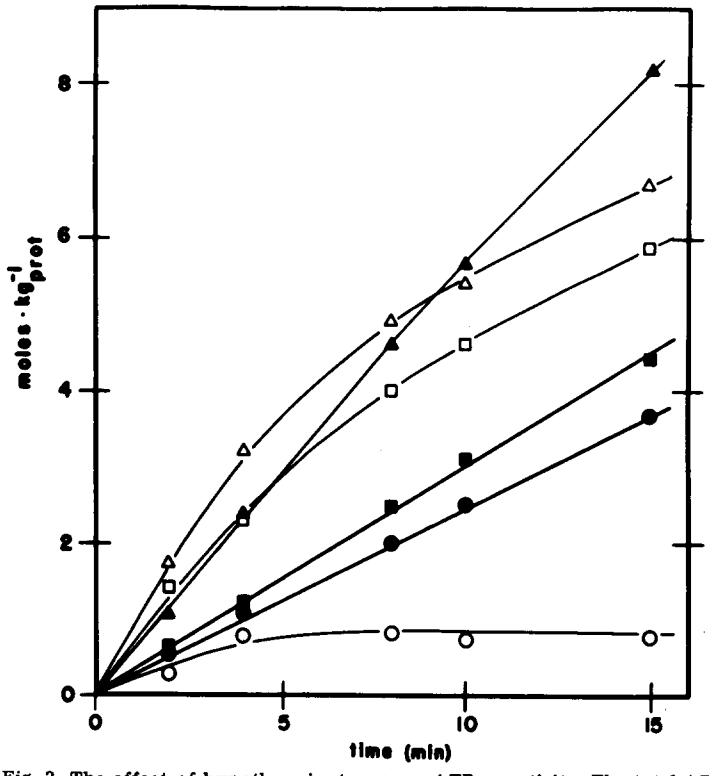


Fig. 3. The effect of hypothermic storage on ATPase activity. The total ATPase (triangles), the ATPase in the presence of ouabain designated (Mg^{2+})-ATPase (squares) and the difference designated $(Na^+ + K^+)$-ATPase (circles) activities were determined in a deoxycholate-treated preparation. The open symbols represent the activities at the end of dialysis (fresh preparation); see Table II. The filled symbols represent the activities in the same fraction after it had been stored at 2°C for 48 h, without further dialysis (hypothermic storage); Table II.

TABLE II

THE EFFECT OF HYPOTHERMIC STORAGE ON CYCLIC AMP-DEPENDENT PROTEIN KINASE IN $(Na^+ + K^+)$-ATPase AT THE DEOXYCHOLATE TREATMENT PURIFICATION STAGE

Condition	Cyclic AMP-dependent protein kinase	$(Na^+ + K^+)$-ATPase		
	mol ^{32}P incorporated · kg^{-1} prot. · h^{-1}	Reaction in character	mol · kg^{-1} prot. · h^{-1}	
			Early curve *	Late curve *
Control	$0.2010 \cdot 10^{-3}$	non-linear	10.8	0
Hypothermic	$0.0155 \cdot 10^{-3}$	linear	25.0	25.0
Freeze-thawing	$0.0151 \cdot 10^{-3}$	linear	25.0	25.0

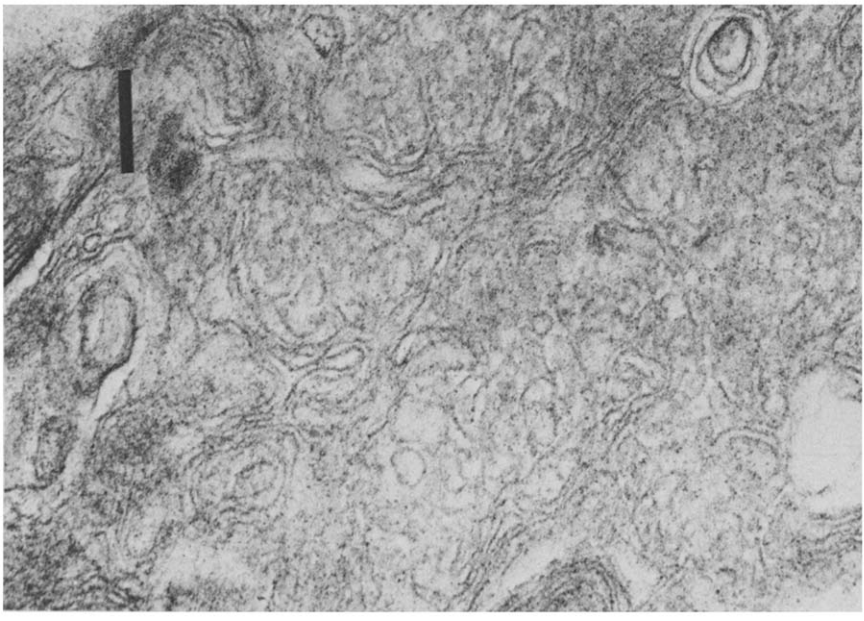
* The activities of $(Na^+ + K^+)$-ATPase were determined on a deoxycholate enzyme prepared similarly to that in Table I. The deoxycholate-treated preparation was dialyzed twice for 4 h at 2°C against 1000 vols. of 25 mM imidazole, pH 7.0, and assayed (designated control). The preparation was then stored 48 h at 2°C and reassayed (hypothermic). Also some of the control preparation was frozen at —90°C and thawed in a water bath at 4°C. The procedure was repeated six times and then assayed (freeze-thawing). The $(Na^+ + K^+)$-ATPase phosphate liberation curves for the control and hypothermic are shown in Fig. 3. Activities are represented based on the first 2—4 min of reaction (early) and latter 10—15 min of reaction (late). Protein kinase was measured as described in Table I.

thawed (4°C) repeatedly. These procedures also resulted in a loss of endogenous cyclic AMP-dependent protein kinase activity (Table II). Hypothermic storage has been previously noted by other authors to increase $(Na^+ + K^+)$-ATPase activities [11,12].

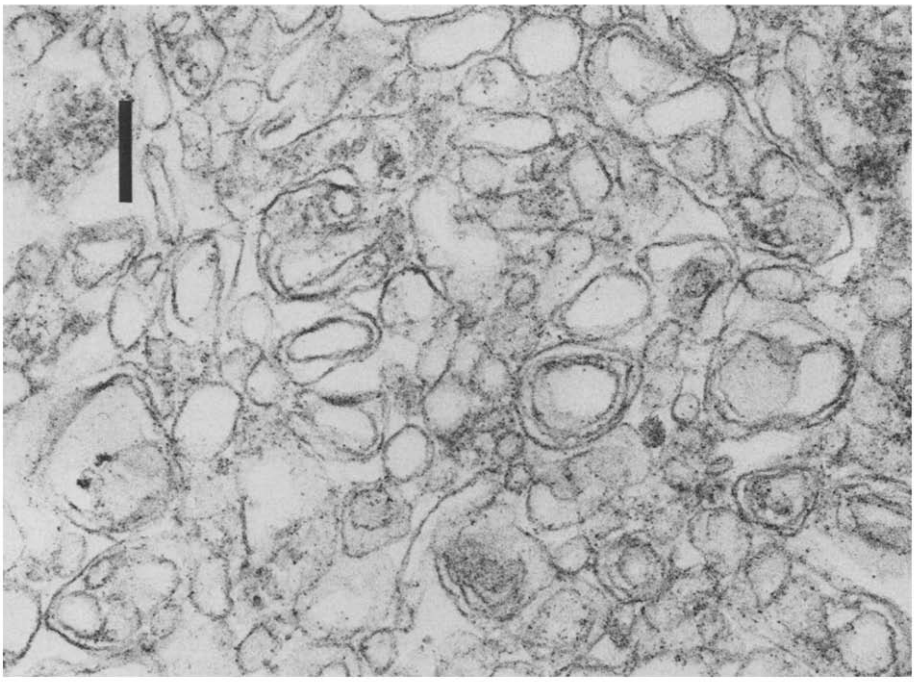
Several investigators have successfully incorporated purified $(Na^+ + K^+)$-ATPase into artificial lipid vesicles which are capable of actively transporting Na^+ and K^+ [10,13,14]. Purified human renal $(Na^+ + K^+)$-ATPase (linear reaction rate) was reconstituted into lipid vesicles. In some cases both $(Na^+ + K^+)$-ATPase and cyclic AMP-dependent protein kinase were reconstituted together into vesicles. It is not known what fraction of available protein is actually incorporated into lipid vesicles by this procedure [13]. In the present study, electron micrographs of the $(Na^+ + K^+)$-ATPase preparation were made before and after formation of the lipid vesicles (Fig. 4). The purified enzyme consists of strands of membrane material (Fig. 4A). In contrast, Fig. 4B shows a heterogeneous population of vesicles.

The $(Na^+ + K^+)$-ATPase activity in reconstituted vesicles was linear with assay time (Fig. 5A). The specific activity of the $(Na^+ + K^+)$-ATPase falls about 6—8-fold when the purified enzyme was incorporated into lipid vesicles. An 8—10-fold drop in specific activity has been reported by others for purified $(Na^+ + K^+)$-ATPase from *Squalus acanthias* incorporated in lipid vesicles [10].

The activity in vesicles which also contained protein kinase was less than in vesicles containing only $(Na^+ + K^+)$-ATPase (Fig. 5A). The addition of 100 μM cyclic AMP to the assay of vesicles containing both $(Na^+ + K^+)$-ATPase and protein kinase caused the $(Na^+ + K^+)$-ATPase to inactivate during the ATPase assay. A lower concentration of cyclic AMP (1 μM) was ineffective in causing inactivation, while 10 μM cyclic AMP (not shown) was only partially effective. 100 μM cyclic AMP had no effect on the activity in vesicles which contained only purified $(Na^+ + K^+)$-ATPase. Cyclic AMP-dependent protein kinase and cyclic



A



B

Fig. 4. Electron micrographs of lipid vesicles containing purified ($Na^+ + K^+$)-ATPase. Lipid vesicles were made and prepared for electron microscopy as described in Methods. These are micrographs (X 64750) of the glycerol precipitated enzyme before (A) and after (B) reconstitution into lipid vesicles. The solid lines represent 0.2 μm.

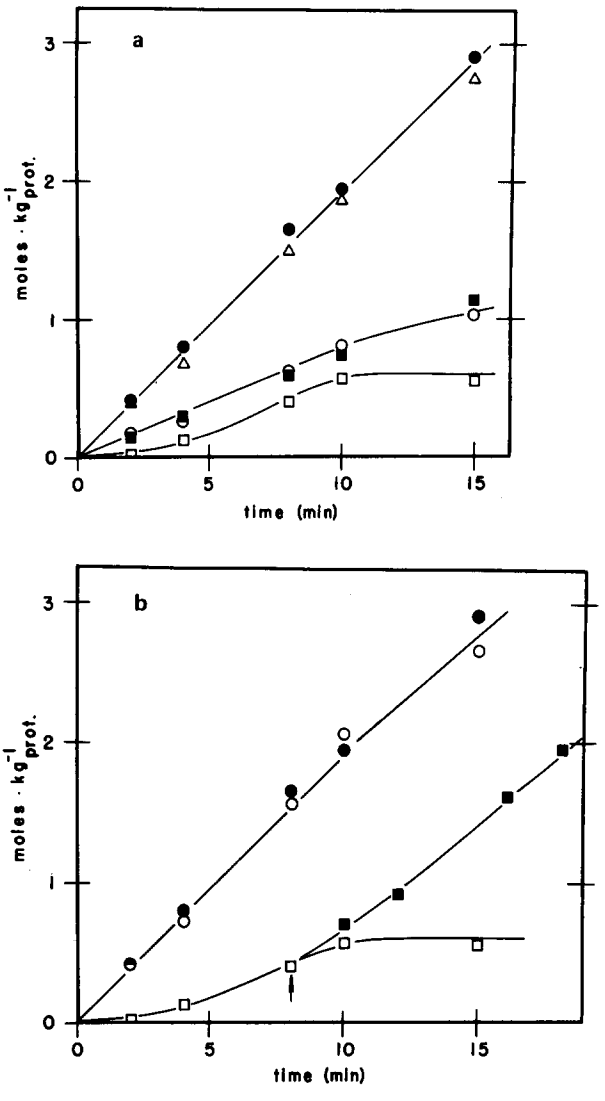


Fig. 5. $(Na^+ + K^+)$-ATPase reconstituted into lipid vesicles with and without cyclic AMP-dependent protein kinase. Lipid vesicles were prepared and $(Na^+ + K^+)$-ATPase activity in the vesicles was assayed at 0.15 mg protein per ml as described in Methods . (A), $(Na^+ + K^+)$-ATPase alone (●———●); $(Na^+ + K^+)$-ATPase with protein kinase (■———■); $(Na^+ + K^+)$-ATPase alone assayed with 100 μM cyclic AMP (△———△); $(Na^+ + K^+)$-ATPase with protein kinase assayed with 1 μM cyclic AMP (○———○) and with 100 μM cyclic AMP (□———□). (B), Lipid vesicles were the same as in Fig. 5A. $(Na^+ + K^+)$-ATPase alone (●———●) (same as in Fig. 5A); $(Na^+ + K^+)$-ATPase with protein kinase and 100 μM cyclic AMP (□———□) (same as in Fig. 5A). The 30% $(NH_4)_2SO_4$ fraction of the 100 000 × *g* cell supernatant (Methods) was added at the beginning of the assay (○———○) or added at the arrow after 8 min of incubation (■———■).

AMP which were simply added to the assay of a linear $(Na^+ + K^+)$-ATPase preparation were not effective in restoring non-linearity (not shown).

The 30% $(NH_4)_2SO_4$ fraction of the 100 000 × *g* supernatant added into the $(Na^+ + K^+)$-ATPase reaction medium would reverse and prevent the cyclic AMP-mediated inactivation of $(Na^+ + K^+)$-ATPase, which was reconstituted into lipid vesicles with cyclic AMP-dependent protein kinase, Fig. 5B. This was very

similar to the effects of the 100 000 × *g* supernatant on a deoxycholate preparation (Fig. 2).

The vesicles containing ($Na^+ + K^+$)-ATPase and protein kinase contained significant cyclic AMP-dependent protein kinase activity, $2.27 \cdot 10^{-3}$ mol $^{32}P \cdot kg^{-1} \cdot h^{-1}$. This activity was due purely to the phosphorylation of structures in the vesicles as no other substrate (such as histone) was added. The cyclic AMP-dependent protein kinase activity was much less, however, when the glycerol precipitated enzyme and protein kinase were not reconstituted together into vesicles but were simply added together into the kinase assay: $0.30 \cdot 10^{-3}$ mol $^{32}P \cdot kg^{-1} \cdot h^{-1}$.

Discussion

The findings presented in this study are compatible with the hypothesis that non-linearity of the ($Na^+ + K^+$)-ATPase reaction is mediated in part by a membrane bound cyclic AMP-dependent protein kinase. This hypothesis is based upon several findings: (1) loss of non-linearity from a preparation by purification is accompanied by loss of cyclic AMP-dependent protein kinase activity; (2) cyclic AMP-dependent protein kinase activity decays with repeated freezing and thawing or hypothermic storage of a non-linear membrane preparation and at the same time the reaction becomes more linear; (3) reconstitution of purified ($Na^+ + K^+$)-ATPase into lipid vesicles with heart cyclic AMP-dependent protein kinase, restores cyclic AMP-dependent non-linearity.

There is other evidence in the literature to show that membrane preparations which contain ($Na^+ + K^+$)-ATPase also contain adenylate cyclase and cyclic AMP-dependent protein kinase activities as shown for rat liver [15], erythrocytes [16,17], and kidney [18].

It seems likely, therefore, that during the assay of crude membrane preparations for ($Na^+ + K^+$)-ATPase activity, enough cyclic AMP might be produced to stimulate membrane protein kinase activity. Based upon those reports and the present findings, this may result in the inactivation of ($Na^+ + K^+$)-ATPase during the ATPase assay.

An interesting finding associated with non-linearity was that hypothermic storage or repeated freezing and thawing would cause a non-linear enzyme to become linear. This may explain the well known observation that aging of membrane preparations at 2—4°C will result in an increase in ($Na^+ + K^+$)-ATPase activity [11,12,19].

One would expect that phosphorylation of some membrane component of ($Na^+ + K^+$)-ATPase by protein kinase would be responsible for the non-linearity of the ATPase reaction. It is not clear if phosphorylation of a catalytic component of ($Na^+ + K^+$)-ATPase results in enzyme inactivation or whether the phosphorylation occurs on some other regulatory protein. It is known that the peptide of the human renal enzyme corresponding to 92 500 daltons undergoes Na^+-dependent phosphorylation during the ($Na^+ + K^+$)-ATPase reaction [2]. This laboratory thus far has been unsuccessful in identifying the receptor on ($Na^+ + K^+$)-ATPase for phosphorylation by the cyclic AMP-dependent protein kinase. Sodium dodecyl sulfate polyacrylamide electrophoresis, which nicely separates the subunits of ATPase, has not given sufficient resolution above

background to distinhuish from that occurring on the 92 500 dalton peptide. A recent report does show that one can effect cyclic AMP-dependent protein kinase phosphorylation of heart ($Na^+ + K^+$)-ATPase [20]. However, with the heart enzyme the site of phosphorylation was not ascertained.

The inactivation of ($Na^+ + K^+$)-ATPase which can be medidated by cyclic AMP and protein kinase is a reversable process. The 100 000 $\times g$ supernatant, which represents the cytoplasmic fraction of the cell, contains some factor(s) which will prevent and reverse enzyme inactivation.

The factor has not been characterized, except that it is non-dialyzable, heat labile and precipitable by ammonium sulfate. It is postulated that the factor(s) may be a protein phosphatase. A protein phosphatase has been identified in the 10 000 $\times g$ supernatant of renal medullary tissue homogenates which catalyzes the release of ^{32}P from renal plasma membrane phosphorylated by cyclic AMP-dependent protein kinase [21]. However, other possibilities entering into consideration include phosphodiesterases [22] and protein kinase inhibitors [23].

In 1969, Mozsik reported that agents which increased cyclic AMP levels also caused a depression of ($Na^+ + K^+$)-ATPase activity in rat heart and gastric tissues, including human gastric mucosa [24]. Mozsik suggested that a relationship existed between adenylate cyclase and ($Na^+ + K^+$)-ATPase such that stimulation of the former led to inhibition of the other. It has been shown that epinephrine and cyclic AMP inhibit ($Na^+ + K^+$)-ATPase activity in liver cell membranes [25,26].

The evidence showing inhibition of ($Na^+ + K^+$)-ATPase by catecholamines [24,26] is in contrast to other evidence showing an apparent stimulation of the enzyme by catecholamines [6,27]. In the latter instance [6], the effect was that relatively high levels of catecholamines prevented the inactivation of ($Na^+ + K^+$)-ATPase which could be carried out by preincubating the enzyme with K^+, Mg^{2+}, ATP. Thus, the apparent protective effect of relatively high levels of catecholamines may be unrelated to any effects relatively low levels of catecholamines might exert on stimulating the adenylcyclase cyclic AMP-dependent protein kinase system to cause inactivation of ($Na^+ + K^+$)-ATPase.

The studies here and others lend support to the idea that ($Na^+ + K^+$)-ATPase exists in interconvertible active and inactive molecular forms. This further emphasizes the extreme complexity which surrounds the regulation of the ctivity of the enzyme. The relative role of hormonal effects on the adenylcyclase-cyclic AMP-dependent protein kinase system versus other catalytic proteins and ionic constituents affecting activity of ATPase is unclear at this time.

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