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THE EFFECT OF HYDROXYLAMINE, MERCAPTANS, DIVALENT METALS AND CHELATORS ON ($Na^+ + K^+$)-ATPase

A POSSIBLE CONTROL MECHANISM

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SUMMARY

In the absence of Ca^{2+}, 20 mM hydroxylamine activates and in the presence of 20 μM Ca^{2+} it inhibits the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3). Mn^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, and Cu^{2+} can substitute for the Ca^{2+}-induced hydroxylamine inhibition at concentrations below 10 μM. Ba^{2+}, Sn^{2+}, Sr^{2+}, and Fe^{2+} have no Ca^{2+}-like effect.

This result is obtained in the presence of ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid, a chelator with a high stability constant for Ca^{2+}. If 8-hydroxyquinoline, a chelator with a low stability constant for Ca^{2+}, but a high stability constant for Cu^{2+}, is used instead of ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid, Ca^{2+} has no effect on the hydroxylamine–($Na^+ + K^+$)-ATPase. However, Cu^{2+} at concentrations of 1 μM will induce inhibition of the hydroxylamine–($Na^+ + K^+$)-ATPase in the presence of 8-hydroxyquinoline. Hydroxylamine in the presence of Ca^{2+} does not split the acylphospho intermediate of the ($Na^+ + K^+$)-ATPase, but rather prevents the Na^+-dependent phosphorylation of the enzyme. The mercaptans, 2,3-dimercaptopropanol and 2-mercaptoethanol have a biphasic effect on ($Na^+ + K^+$)-ATPase. They inhibit at low concentrations (10–100 μM) and activate at high concentrations (above 1 mM). The inhibition is increased in the presence of 30 μM Ca^{2+}. Both mercaptans reverse the Ca^{2+}-induced hydroxylamine inhibition at concentrations above 1 mM. *N*-ethylmaleimide reverses the Ca^{2+}-induced hydroxylamine inhibition between 10–100 μM, if it reaches the enzyme before hydroxylamine. The reactivation apparently is due to alkylation of a sulfhydryl group different from the sulfhydryl group by which *N*-ethylmaleimide inhibits the ($Na^+ + K^+$)-ATPase.

The results are interpreted by assuming a heavy metal intrinsic to the enzyme and bound to a storage site. Hydroxylamine may decrease the affinity of the storage site for this heavy metal so it can bind to an inhibitory site. The latter site has no function by itself, but when it forms a complex with the intrinsic heavy metal, it acts

Abbreviations: BAL, 2,3-dimercaptopropanol (British anti-Lewisite); EGTA, ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid; TES, *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid.

as an inhibitor, preventing Na^+-dependent phosphorylation. If a chelator like ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid is present, it will prevent the binding of the heavy metal to the inhibitory site and no inhibition occurs. However, if the chelator is bound by an excess of a divalent metal like Ca^{2+}, the chelator is not able to prevent hydroxylamine inhibition.

INTRODUCTION

Hydroxylamine at 20 mM concentration activates the ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in the absence of Ca^{2+} and inhibits this enzyme in the presence of 10 μM Ca^{2+} (refs. 1, 2). The inhibition is not due to the formation of an hydroxamate from the acylphospho intermediate of the ($Na^+ + K^+$)-ATPase[2,3]. Hydroxylamine apparently attacks the enzyme near the Na^+-site. It diminishes the competition between Mg^{2+} and Na^+ at this site. The most reactive hydroxylamine derivatives are those with a free hydroxyl group like *N*-methylhydroxylamine. Sulfite has a dual effect on ($Na^+ + K^+$)-ATPase similar to that of hydroxylamine. An attack on an unhindered carbonyl is unlikely due to the enhancing effect of a free hydroxyl group on hydroxylamine.[2] Mn^{2+} and Cd^{2+} (ref. 2), but not Ba^{2+} and Sr^{2+} (ref. 1) can replace Ca^{2+} for the hydroxylamine inhibition of ($Na^+ + K^+$)-ATPase. The hydroxylamine effect is specific for the ouabain-sensitive ($Na^+ + K^+$)-ATPase[1].

N-Ethylmaleimide inhibits the ($Na^+ + K^+$)-ATPase between 1–10 mM (ref. 4) by enhancing a Na^+-dependent ADP–ATP exchange reaction[5]. Equimolar 2,3-dimercaptopropanol (British anti-Lewisite, BAL)–arsenite at 0.1–1 mM has a similar effect[6]. In a preliminary note, we have shown that mercaptans by themselves have a dual effect on ($Na^+ + K^+$)-ATPase[7]. They inhibit the enzyme at about 10 μM and stimulate it above 1 mM. Ca^{2+} (30 μM) increases the inhibitory effect of mercaptans, but has no effect on their stimulatory action. Mercaptans between 0.1 and 1 mM overcome the Ca^{2+}-induced inhibition of the hydroxylamine–enzyme establishing full activity above 1 mM mercaptan. This paper is an attempt to explain the mechanism of the Ca^{2+}-induced inhibition on the ($Na^+ + K^+$)-ATPase by hydroxylamine.

METHODS

($Na^+ + K^+$)-ATPase was prepared according to the method described by POST AND SEN[8]. Unless otherwise stated, the activity of the enzyme was determined by incubating an aliquot (10–20 μg protein) at 37° for 20 min with 150 mM Na^+, 10 mM K^+, 6 mM Mg^{2+}, 4 mM Tris–ATP, 20 mM Tris–*N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) (pH 7.5) and 0.1 mM ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid (EGTA). When Ca^{2+} was present, 0.13 mM Ca^{2+} was added to give a free Ca^{2+} concentration of 30 μM. All cations were added in the chloride form. The activity in the presence of 1 mM ouabain instead of Na^+ and K^+ was subtracted from the total activity except in the experiment of Fig. 1. The activity of the ($Na^+ + K^+$)-ATPase is expressed in units/mg protein, one unit being 1 μmole P_i released from ATP in 1 min at 37°. Hydroxylamine·HCl was adjusted to pH 7.5 with Tris immediately before use to minimize the formation of NH_4^+.

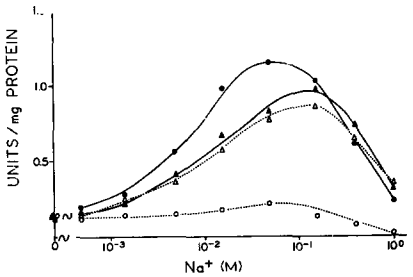


Fig. 1. Effect of hydroxylamine on brain cortex $(Na^+ + K^+)$-ATPase. The enzyme was incubated as described in METHODS at increasing Na^+ concentrations. The ouabain-insensitive ATPase is not subtracted. ▲—▲, control, 0 mM Ca^{2+}; △— — —△, control, 30 μM free Ca^{2+}; ●—●, 20 mM hydroxylamine, 0 mM Ca^{2+}; ○— — —○, 20 mM hydroxylamine; 30 μM free Ca^{2+}.

Preparation of [γ-^{32}P]ATP and phosphorylation of the $(Na^+ + K^+)$-ATPase was carried out according to POST AND SEN[9].

RESULTS

Hydroxylamine probably increases the Na^+ affinity of the $(Na^+ + K^+)$-ATPase of rabbit kidney cortex by diminishing the competition between Mg^{2+} and Na^+ at the Na^+-site[2]. Ca^{2+} at 10 μM inhibits the hydroxylamine–enzyme. To see if this is a general property of $(Na^+ + K^+)$-ATPase, we tested the effect of hydroxylamine and Ca^{2+} on the $(Na^+ + K^+)$-ATPase of rabbit brain cortex and guinea pig kidney cortex at different Na^+ concentrations. The results obtained on the $(Na^+ + K^+)$-ATPase of rabbit brain cortex are shown in Fig. 1. As already reported for the rabbit kidney cortex, hydroxylamine shifted the half maximal Na^+ activation from 12 to 6 mM and increased the activity of the Ca^{2+}-free enzyme over most of the Na^+ concen-

TABLE I

EFFECT OF DIVALENT METALS ON $(Na^+ + K^+)$-ATPase ACTIVITY

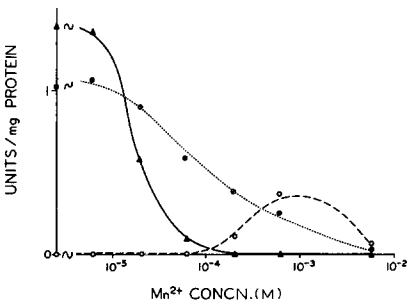
The incubation medium contained: 6 mM $MgCl_2$, 150 mM NaCl, 10 mM KCl, 4 mM Tris–ATP, 0.05 mM Tris–EGTA and 0.06 mM divalent metal with or without 20 mM hydroxylamine. The reaction was carried out at 37° for 20 min.

Divalent metal	*Activity (%)*	
	0 mM hydroxylamine	*20 mM hydroxylamine*
———	100	126
Ba^{2+}	105	124
Sn^{2+}	89	135
Sr^{2+}	104	94
Fe^{2+}	61	61
Ca^{2+}	79	18
Mn^{2+}	62	10
Cd^{2+}	46	6
Zn^{2+}	40	0
Pb^{2+}	31	0
Cu^{2+}	24	0

trations tested. Addition of 30 μM free Ca^{2+} to the hydroxylamine–enzyme caused inhibition at all Na^+ concentrations tested. A similar result was obtained with (Na^+ + K^+)-ATPase from guinea pig kidney cortex.

We have previously shown that Mn^{2+} and Cd^{2+} show an inhibitory effect on the hydroxylamine–enzyme similar to that of Ca^{2+} (ref. 2). Ba^{2+} and Sr^{2+} could not replace Ca^{2+} (ref. 1). Table I shows an extended study on the effects of divalent cations on (Na^+ + K^+)-ATPase. At 60 μM in the presence of 50 μM EGTA, Ba^{2+}, Sn^{2+} and Sr^{2+} had negligible effects on the activity of the enzyme with or without hydroxylamine. At the same concentration, Fe^{2+}, Ca^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, and Cu^{2+} inhibited the hydroxylamine-free enzyme to different degrees. Fe^{2+} did not inhibit further in the presence of hydroxylamine. Mn^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, and Cu^{2+} showed an increased inhibitory effect in the presence of 20 mM hydroxylamine similar to that already observed with Ca^{2+}.

Mn^{2+} not only had a Ca^{2+}-like inhibitory effect on the (Na^+ + K^+)-ATPase,



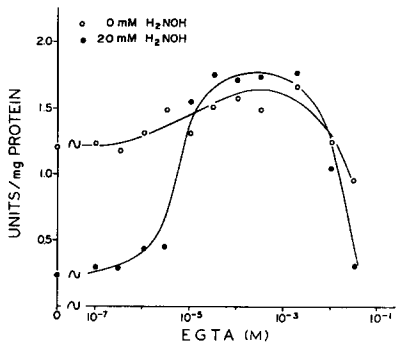


Fig. 2. Effect of Mn^{2+} on kidney cortex (Na^+ + K^+)-ATPase. ●— — —●, 6 mM $MgCl_2$; ○— —○, no $MgCl_2$; ▲—▲, 6 mM $MgCl_2$, 20 mM hydroxylamine.

Fig. 3. Effect of EGTA on kidney cortex (Na^+ + K^+)-ATPase. The only divalent metal added was 6 mM Mg^{2+}. ○—○, 0 mM hydroxylamine; ●—●, 20 mM hydroxylamine.

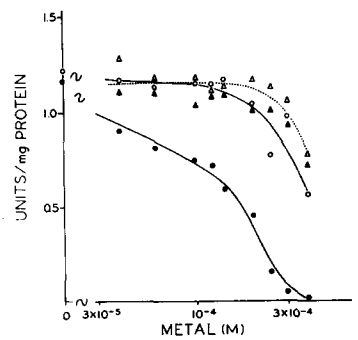
but could also replace Mg^{2+} for activation (Fig. 2). In the absence of Mg^{2+}, Mn^{2+} activated the enzyme between 0.1 and 1 mM. However, the Mn^{2+} activation of the (Na^+ + K^+)-ATPase was only one-third of the Mg^{2+} activation. This was probably due to the overlapping inhibitory effect of Mn^{2+} with half maximal inhibition at about 0.1 mM. Hydroxylamine caused a shift of inhibition to lower Mn^{2+} concentrations, similar to that observed with Ca^{2+} (ref. 1). The exact half maximal inhibitory Mn^{2+} concentration in the presence of hydroxylamine could not be determined since 50 μM EGTA was present in the incubation medium. Ca^{2+} or any other divalent cation (besides Mn^{2+}) shown in Table I could not replace Mg^{2+} for activation.

EGTA itself increased the activity of the (Na^+ + K^+)-ATPase slightly between 1–100 μM (Fig. 3). Above 1 mM, EGTA inhibited the activity of the enzyme probably by chelating Mg^{2+}. Hydroxylamine added to the (Na^+ + K^+)-ATPase in the absence of EGTA had no consistent effect on the enzyme activity. Some enzyme preparations tested over the last 3 years were not inhibited at all, others were inhibited to different degrees. If EGTA was added in increasing concentrations to enzymes inhibited by hydroxylamine (Fig. 3), the enzyme was activated between 1–100 μM EGTA. The

highest activity of the hydroxylamine–enzyme was greater than the highest activity of the hydroxylamine-free enzyme. The EGTA concentration necessary for activation of the hydroxylamine-inhibited enzyme varied from enzyme to enzyme between 1–100 μM EGTA.

The same result as shown in Fig. 3 was obtained when 8-hydroxyquinoline, diethyldithiocarbamate or EDTA was used instead of EGTA. However, in the presence of 0.3 mM 8-hydroxyquinoline (Fig. 4), Ca^{2+} was not able to inhibit the hydroxylamine–enzyme more than the hydroxylamine-free enzyme. Cu^{2+}, however, caused a similar inhibition as shown earlier with Ca^{2+} in the presence of EGTA[1]. Half maximal inhibition of the hydroxylamine–enzyme was obtained at a ratio of Cu^{2+} to 8-hydroxyquinoline of 1:2. The inhibition of the hydroxylamine-free enzyme by Cu^{2+} is similar to that by Ca^{2+}. The same difference between Ca^{2+} and Cu^{2+} as shown with 8-hydroxyquinoline was obtained when 0.3 mM EDTA was used as chelating agent. The effect of Cd^{2+} and Mn^{2+} was similar to that of Ca^{2+}, inhibition of the hydroxylamine–enzyme in the presence of EGTA, but no inhibition in the presence of 8-hydroxyquinoline or EDTA.

The optimal concentration of hydroxylamine for activation of the Ca^{2+}-free enzyme and inhibition of the Ca^{2+}-containing enzyme is 20 mM (ref. 1). Since hydroxylamine splits the acylphospho intermediate of the acid-denatured $(Na^+ + K^+)$-ATPase instantaneously[10], we first thought hydroxylamine inhibited the enzyme in the presence of Ca^{2+} by splitting the acylphospho intermediate forming the hydroxamate[1]. However, as Fig. 5 shows, this is not true. If 20 mM hydroxylamine was added to the enzyme 10 sec after it was phosphorylated from [γ-^{32}P]ATP in the presence of Mg^{2+} and Na^+, no dephosphorylation took place within 60 sec. Dephosphorylation occurred only when the hydroxylamine concentration was increased to 200 or 600 mM. This dephosphorylation was dependent on hydroxylamine concentration, but was time



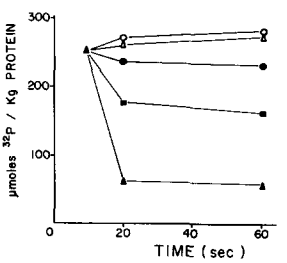


Fig. 4. Effect of Ca^{2+} and Cu^{2+} on kidney cortex $(Na^+ + K^+)$-ATPase in the presence of 0.3 mM 8-hydroxyquinoline with or without *N*-methylhydroxylamine. △— — —△, 0 mM *N*-methylhydroxylamine, Ca^{2+}; ▲— — —▲, 20 mM *N*-methylhydroxylamine, Ca^{2+}; ○—○, 0 mM *N*-methylhydroxylamine, Cu^{2+}; ●—●, 20 mM *N*-methylhydroxylamine, Cu^{2+}.

Fig. 5. Effect of hydroxylamine on the phosphorylated kidney cortex $(Na^+ + K^+)$-ATPase. The enzyme was phosphorylated from [γ-^{32}P]ATP in the presence of 20 mM Na^+, 1 mM Mg^{2+}, 0.1 mM EGTA, 0.13 mM Ca^{2+} and 20 mM Tris–TES, pH 7.5. The reaction was started at 0 sec by addition of 40 μM [γ-^{32}P]ATP. At 10 sec, hydroxylamine or K^+ was added. The reaction was terminated at the time indicated by addition of a 20-fold volume of 300 mM trichloracetic acid containing 0.5 mM [γ-^{32}P]ATP. ○—○, control; △—△, 20 mM hydroxylamine; ●—●, 200 mM hydroxylamine; ■—■, 600 mM hydroxylamine; ▲—▲, 20 mM K^+.

TABLE II

EFFECT OF HYDROXYLAMINE ON PHOSPHORYLATION OF $(Na^+ + K^+)$-ATPase
The results are expressed as μmoles ^{32}P/kg protein.

	*Preincubated**			*Not preincubated***		
	Na^+	K^+	$Na^+ - K^+$	Na^+	K^+	$Na^+ - K^+$
Control						
No Ca^{2+}	259	15	244	250	15	235
0.13 mM Ca^{2+}	255	19	236	244	10	234
20 mM hydroxylamine						
No Ca^{2+}	242	16	226	249	21	228
0.13 mM Ca^{2+}	90	19	71	247	13	234

* Preincubated: the enzyme was preincubated with or without 20 mM hydroxylamine for 10 min at 37° in a medium containing 20 mM Tris–TES (pH 7.5) 2 mM $MgCl_2$, 0.1 mM Tris–EGTA with and without 0.13 mM $CaCl_2$ and with either 20 mM NaCl or 20 mM KCl. The enzyme was then phosphorylated for 10 sec at 0° by adding 40 μM [γ-^{32}P]ATP.

** Not preincubated: the enzyme was phosphorylated for 10 sec at 0° in the same medium, but without preincubation. At 10 sec, either 20 mM hydroxylamine or water was added and the reaction was stopped at 20 sec.

independent. The same result was obtained if Ca^{2+} was added as in Fig. 5, or if Ca^{2+} was deleted. Addition of 20 mM K^+ resulted in 80% dephosphorylation.

If the $(Na^+ + K^+)$-ATPase was incubated with 20 mM hydroxylamine prior to the addition of [γ-^{32}P]ATP, a Ca^{2+}-dependent hydroxylamine effect could be observed (Table II). In the absence of Ca^{2+}, the hydroxylamine-incubated enzyme showed the same Na^+-dependent phosphorylation as the control. In the presence of 30 μM free Ca^{2+}, the Na^+-dependent phosphorylation of the hydroxylamine-incubated enzyme was only 30% of the control. The Na^+-independent phosphorylation (in the presence of K^+) was not affected by hydroxylamine with or without Ca^{2+}. *N*-Ethylmaleimide at 10 mM inhibits the $(Na^+ + K^+)$-ATPase[4] and increases a Na^+-dependent ADP-ATP exchange reaction of the enzyme[5]. At 100 times lower concentrations, *N*-ethylmaleimide overcame the Ca^{2+}-induced inhibition of the hydroxylamine–enzyme (Fig. 6). Half maximal activation of the Ca^{2+}-inhibited hydroxylamine–enzyme was achieved at 70 μM *N*-ethylmaleimide. The Ca^{2+}-free hydroxylamine–enzyme showed a small additional activation at the same low *N*-ethylmaleimide concentration. Ethacrynic acid is an α,β-unsaturated ketone and a sulfhydryl-alkylating agent like *N*-ethylmaleimide. It inhibits the $(Na^+ + K^+)$-ATPase at around 1 mM (ref. 11). However, ethacrynic acid did not reverse the Ca^{2+}-induced inhibition of the hydroxylamine–enzyme at any concentration.

If the $(Na^+ + K^+)$-ATPase was preincubated with *N*-ethylmaleimide or hydroxylamine prior to the activity test (Fig. 7), the Ca^{2+}-induced hydroxylamine inhibition was reversed only when the enzyme was preincubated with *N*-ethylmaleimide, but not when it was preincubated with hydroxylamine. The effect was the same whether Ca^{2+} was present during hydroxylamine preincubation or if it was added after preincubation.

An equimolar BAL–arsenite complex at 0.1–1 mM has an effect similar to that of *N*-ethylmaleimide on $(Na^+ + K^+)$-ATPase. It inhibits the activity of the enzyme

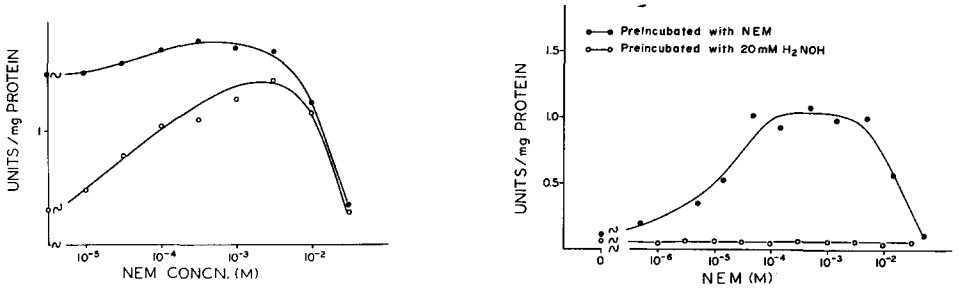


Fig. 6. Effect of *N*-ethylmaleimide (NEM) on kidney cortex $(Na^+ + K^+)$-ATPase. *N*-Ethylmaleimide was added at different concentrations to the incubation medium together with 20 mM hydroxylamine. ●—●, 20 mM hydroxylamine, 0 mM $CaCl_2$; ○—○, 20 mM hydroxylamine, 50 μM $CaCl_2$.

Fig. 7. Effect of *N*-ethylmaleimide (NEM) on the preincubated kidney cortex $(Na^+ + K^+)$-ATPase. The enzyme was preincubated either with *N*-ethylmaleimide at the concentration stated (●—●) or with 20 mM hydroxylamine (○—○) in the presence of 0.1 mM EGTA, 0.13 mM Ca^{2+} and 20 mM Tris–TES (pH 7.5) for 20 min at 23°. At the end of the preincubation time, the rest of the medium was added and the activity of the enzyme determined.

and enhances a Na^+-dependent ADP–ATP exchange reaction[6]. BAL alone at 10 μM inhibited the activity of the $(Na^+ + K^+)$-ATPase (Fig. 8). Above 0.1 mM, BAL alone stimulated the enzyme. Addition of 30 μM Ca^{2+} increased the inhibitory effect of BAL. In the presence of 20 mM hydroxylamine, BAL also inhibited the $(Na^+ + K^+)$-ATPase at 10 μM. Increasing BAL concentrations returned the activity to that observed with hydroxylamine in the absence of BAL. Between 10 μM and 1 mM, BAL reversed the inhibition of the enzyme due to Ca^{2+} and hydroxylamine. A similar effect was seen with 2-mercaptoethanol, but 10 times higher concentrations were necessary (Fig. 9). The biphasic mercaptan effect was specific for the ouabain-sensitive $(Na^+ + K^+)$-ATPase.

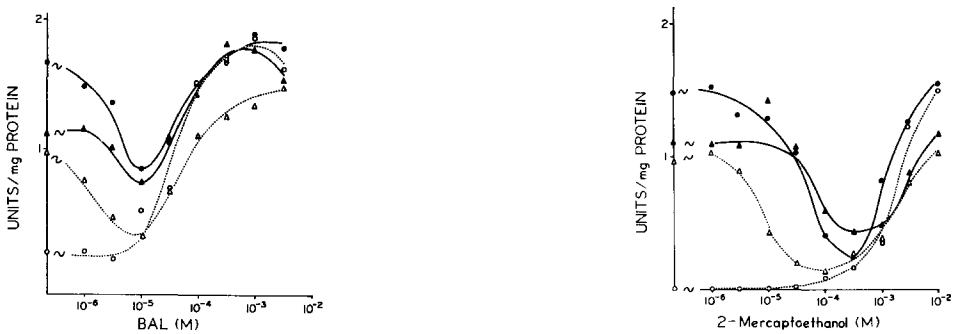


Fig. 8. Effect of BAL on kidney cortex $(Na^+ + K^+)$-ATPase. The enzyme was incubated with different concentrations of BAL with or without 20 mM hydroxylamine and with or without 30 μM free Ca^{2+}. ▲—▲, control, 0 mM Ca^{2+}; △— — —△, control, 30 μM Ca^{2+}; ●—●, 20 mM hydroxylamine, 0 mM Ca^{2+}; ○— — —○, 20 mM hydroxylamine, 30 μM Ca^{2+}.

Fig. 9. Effect of 2-mercaptoethanol on kidney cortex $(Na^+ + K^+)$-ATPase. Conditions and curves as in Fig. 8.

DISCUSSION

The typical biphasic effect of hydroxylamine on the $(Na^+ + K^+)$-ATPase described earlier for the rabbit kidney cortex[1,2] appears to be a general property of $(Na^+ + K^+)$-ATPases. It can be obtained on other tissues (brain) and in other species (guinea pig). This result shows again the close similarity of the $(Na^+ + K^+)$-ATPase of different animal species and tissues[12].

An intrinsic heavy metal

When EGTA is added to the incubation medium of the $(Na^+ + K^+)$-ATPase, no hydroxylamine inhibition occurs unless Ca^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, or Cu^{2+} is added (Table I). When no EGTA is added, hydroxylamine inhibits the enzyme in the absence of Ca^{2+} (Fig. 3). This indicates the presence of minute amounts of an intrinsic divalent metal in the enzyme. The inconsistent inhibition by hydroxylamine in the absence of chelating agents and divalent metals may be due to different removal of an intrinsic metal during preparation and purification of the enzyme.

When EGTA is replaced by 8-hydroxyquinoline or EDTA, Ca^{2+}, Cd^{2+} or Mn^{2+} are not able to induce an inhibition of the hydroxylamine–enzyme. In contrast, Cu^{2+}, induces similar inhibition with all chelating agents tested. EGTA is a specific chelator for Ca^{2+} with a high stability constant of 11.0 for Ca^{2+}, whereas, 8-hydroxyquinoline has a very low stability constant of about 3.3 for Ca^{2+}, but a high stability constant for heavy metals (about 12.6 for Cu^{2+})[13]. This is a strong indication that the intrinsic metal is a heavy metal. The effect of Ca^{2+} is apparently secondary. In the presence of EGTA, Ca^{2+} will compete with the intrinsic metal for chelation with EGTA and release the intrinsic metal for inhibition of the hydroxylamine–enzyme. In the presence of 8-hydroxyquinoline, Ca^{2+} cannot compete and the intrinsic metal will not be able to inhibit the hydroxylamine–enzyme.

Unique role of Mn^{2+}

Mn^{2+} inhibits the native $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} with a half maximal concentration of 0.1 mM and the hydroxylamine-$(Na^+ + K^+)$-ATPase in the presence of EGTA with a half maximal concentration of less than 1 μM. In addition, Mn^{2+} is able to replace Mg^{2+} for activation with a half maximal concentration of about 0.3 mM. The inhibitory effect of Mn^{2+} appears to be due to competition with Na^+ at the Na^+-site[14]. Since the inhibition of the hydroxylamine–enzyme by divalent metals is not due to competition with Na^+ or Mg^{2+} on the Na^+- or Mg^{2+}-site[2], Mn^{2+} seems to be able to affect the $(Na^+ + K^+)$-ATPase on at least three different sites; Mg^{2+}-site (activation), Na^+-site (inhibition) and hydroxylamine-sensitive site (competing with a heavy metal for chelation).

Concentration quenching

Equimolar concentrations of BAL and arsenite at about 1 mM inhibit the $(Na^+ + K^+)$-ATPase by preventing the K^+-dependent dephosphorylation after Na^+-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase[6]. An excess of BAL over arsenite reverses this inhibition. Figs. 8 and 9 show that BAL and 2-mercaptoethanol inhibit the enzyme by themselves when added at concentrations between 10 and 100 μM. They activate the enzyme above 0.1–1 mM. A similar type of concentration

quenching, whereby the inhibitory effect decreases while the concentration of the inhibitor increases, occurs with 8-hydroxyquinoline for the inhibition of growth of *Staphylococcus aureus*[15] and with dimethyldithiocarbamic acid, a fungicide, for the inhibition of growth of *Aspergillus niger*[16]. Inhibition occurs when at equimolar concentrations a 1:1 complex between Cu^{2+} and the chelating agent is formed. When the chelating agent is in excess, a 1:2 or 1:3 complex will be formed. Cu^{2+} is not able to exert its inhibitory effect under these circumstances[17]. The observed concentration quenching of mercaptans on the $(Na^+ + K^+)$-ATPase (Figs. 8 and 9) could be similarly explained by the presence of an intrinsic heavy metal in the enzyme preparation, as discussed above.

Mercaptans inhibit the Ca^{2+}-free hydroxylamine–enzyme in the same way as they inhibit the native enzyme. This indicates mercaptans and hydroxylamine attack the $(Na^+ + K^+)$-ATPase at different sites, although Ca^{2+} increases the inhibition by both agents. The activation of the Ca^{2+}–hydroxylamine-inhibited enzyme by mercaptan above 0.1 mM may be due to concentration quenching of the intrinsic metal.

A sulfhydryl group

N-Ethylmaleimide at 10–100 μM prevents Ca^{2+}–hydroxylamine inhibition of the $(Na^+ + K^+)$-ATPase, if it reaches the enzyme first. If hydroxylamine reaches the enzyme first, *N*-ethylmaleimide has no effect (Fig. 7). This indicates that a sulfhydryl group is involved in the interaction of Ca^{2+} and hydroxylamine with the enzyme. This sulfhydryl group should be different from the sulfhydryl group by which *N*-ethylmaleimide (10 mM) inhibits the enzyme (Fig. 7). Ethacrynic acid inhibits the $(Na^+ + K^+)$-ATPase at 1 mM (ref. 11), but it does not reverse the Ca^{2+}–hydroxylamine inhibition of the enzyme. This is in agreement with the findings of BANERJEE AND SEN[18] that ethacrynic acid and *N*-ethylmaleimide, both α,β-unsatured ketones, attack at different sites of the enzyme.

Hydroxylamine prevents Na^+-dependent phosphorylation

Hydroxylamine splits the acylphospho intermediate of the $(Na^+ + K^+)$-ATPase in the acid-denatured enzyme[10]. In the native enzyme, the acylphospho intermediate is protected from attack by hydroxylamine, as seen in Fig. 5 and Table II. The addition of 20 mM hydroxylamine to the phosphorylated enzyme did not cause dephosphorylation, with or without Ca^{2+}. The decrease in phosphorylation at higher hydroxylamine concentrations (200–600 mM) as seen in Fig. 5 is probably due to the presence of NH_4^+, a degradation product of hydroxylamine. NH_4^+ is able to replace K^+ for dephosphorylation[19]. This is in agreement with CHIGNELL AND TITUS[3], who obtained a similar result to that shown in Fig. 5 with beef brain $(Na^+ + K^+)$-ATPase. Addition of *N*-methylhydroxylamine, which has a higher potency of Ca^{2+}-dependent inhibition than hydroxylamine (ref. 2), but which is free of NH_4^+ contamination, does not dephosphorylate the phosphorylated enzyme even at 800 mM (ref. 3). If hydroxylamine together with Ca^{2+} is added before the addition of ATP (Table II), no Na^+-dependent phosphorylation takes place. This shows that Ca^{2+} and hydroxylamine together inhibit the $(Na^+ + K^+)$-ATPase by inhibition of Na^+-dependent phosphorylation.

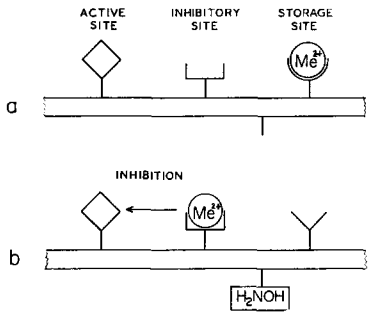


Fig. 10. Proposed control mechanism of $(Na^+ + K^+)$-ATPase. a. Without hydroxylamine attack. b. With hydroxylamine attack. H_2NOH = hydroxylamine.

A possible control mechanism

The described phenomena can be explained with the following model (Fig. 10); an intrinsic heavy metal is bound to a storage site, which has a high affinity for this metal. Hydroxylamine is known to increase or decrease the affinity of the $(Na^+ + K^+)$-ATPase for different essential cations[2]. Hydroxylamine may attack at or near the proposed storage site decreasing its affinity for the intrinsic metal. If a chelating agent like EGTA is present, the intrinsic heavy metal will be chelated by it and will therefore not be available for inhibition. However, if the chelating agent is absent or bound by excess Ca^{2+}, the intrinsic metal will attach to an inhibitory site. This inhibitory site would be without significance for the activity of the $(Na^+ + K^+)$-ATPase unless it forms a complex with the intrinsic metal and thereby prevents the Na^+-dependent phosphorylation. It could be a sulfhydryl group. If this sulfhydryl group is not available, for instance due to protection by excess mercaptan or due to alkylation by *N*-ethylmaleimide, no inhibition occurs.

A mechanism as described in the above model could be utilized *in vivo* to control the transport of Na^+ and K^+ through the cell membrane by turning different numbers of $(Na^+ + K^+)$-ATPase sites off or on. The intrinsic heavy metal would be the switch. It could be regulated by some kind of transmitter substance which acts like hydroxylamine, for instance sulfite[2].

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