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An Na^+/K^+-ATPase inhibitor protein from rat brain cytosol

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A protein isolated from rat brain cytosol is found to inhibit Na^+/K^+-ATPase in rat brain and kidney and H^+/K^+-ATPase from toad gastric mucosa, but has no effect on Ca^{2+},Mg^{2+}-ATPase and Ca^{2+}-ATPase isolated either from rat testis or goat spermatozoa. The inhibitor has been partially purified by ammonium sulphate precipitation followed by gel-filtration through Sephadex G-100. The inhibitor seems to bind at or close to the ATP binding site of Na^+/K^+-ATPase, such that the binding of the inhibitor to ATPase is reversible and competitive in nature with respect to the substrate. Optimum inhibition is observed at around the phase transition temperature of brain Na^+/K^+-ATPase and the inhibitory activity is only partially dependent on -SH or $-NH_2$ group(s) of the inhibitor protein.

Introduction

Na^+/K^+-ATPase (the Na-pump, as it is commonly called) is responsible for the transport of Na^+ and K^+ ions across the cell membranes at the expense of the hydrolysis of ATP. The pump is specifically inhibited by cardiac glycoside ouabain [1]. The enzyme is also inhibited by endogenous glycosides [2–8] and some endogenous peptides of varying molecular weights [9–11]. The inhibition is found to be very specific for Na^+/K^+-ATPase. Recently, Arnaiz et al. reported that brain extract from rat either stimulates or inhibits Na^+/K^+-ATPase [12]. In the present communication we describe an inhibitor isolated from rat brain cytosol that inhibits Na^+/K^+-ATPase from rat brain and kidney and H^+/K^+-ATPase from toad gastric mucosa.

Materials and Methods

Materials

Imidazole hydrochloride, ATP disodium salt, Sephadex G-100, phenylmethylsulphonyl fluoride were purchased from Sigma, USA; histidine hydrochloride, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, β-mercaptoethanol, EDTA, trichloroacetic acid, ammonium molybdate, ascorbic acid were from Sisco Research Laboratory (India) and all other chemicals used were of analytical grade available in the market.

Methods

Preparation of microsomal membranes. Male albino rats of the Charles foster strain weighing about 100 g were killed after anesthetization with chloroform and the brain, testis and kidney were collected. The microsomal membranes were isolated from the above organs as described before [13,14]. The supernatant from post $100\,000 \times g$ centrifugation of brain was collected and is termed cytosolic or soluble fraction. The membranes from goat spermatozoa were collected as described by Sikdar et al. [15]. The gastric microsomal membranes were isolated from toad gastric mucosa as described [16]. All the fractions were stored at −20°C until use.

Enzyme activity assay. Na^+/K^+-ATPase activity from rat brain and kidney microsomal membranes was determined colorimetrically as described [17,18]. Briefly, the reaction mixture in a volume of 1 ml containing 30 mM histidine in 25 mM sucrose and 1 mM EDTA (pH 7.5), 2 mM $MgCl_2$, 15–25 μg microsomal membrane proteins, without and with 130 mM NaCl and 20 mM KCl was preincubated for 5 min at 37°C. The reaction was initiated with the addition of 3 mM ATP and incubated for 30 min more. The reaction was stopped by the addition of 0.2 ml of 30% ice-cold TCA and the liberated inorganic phosphate was estimated colorimetrically as described by Sen et al. [18]. Na^+/K^+-ATPase activity was measured as the difference in

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Abbreviations: PMSF, phenylmethylsulphonyl fluoride; βME, β-mercaptoethanol; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; DNFB, 2,4-dinitrofluorobenzene.

activity between Mg^{2+}, Na^+, K^+ and Mg^{+2} alone and was found to be 95% sensitive to ouabain. The Mg^{2+}-ATPase activity was found to be about 25–30% of Mg^{2+},Na^+/K^+-ATPase activity.

Ca^{2+}-ATPase and Ca^{2+},Mg^{2+},-ATPase activities were determined according to the established procedures published from our laboratory [14–15]. For Ca^{2+},Mg^{2+}-ATPase, the assay mixture contained in a final volume of 1 ml, 50 mM imidazole in 25 mM sucrose, 0.5 mM EDTA, 1 mM βME (pH 7.5), 1 mM $MgCl_2$, 10–15 μg of either rat testicular or goat spermatozoa membranes, without and with 3 mM $CaCl_2$. For Ca^{2+}-ATPase activity assay, Mg^{2+} was excluded from the reaction mixture and the pH was maintained at 8.5. After preincubation for 5 min at 37°C, the reaction was initiated with the addition of 3 mM ATP and incubated further for 30 min. The inorganic phosphate was estimated colorimetrically as described above. Mg^{2+}-dependent Ca^{2+}-ATPase activity was calculated as the difference in activity between Mg^{2+}+ Ca^{2+} and Mg^{2+} alone. Ca^{2+}-ATPase activity was determined against a blank that contained all the ingredients except calcium.

K^+-ion stimulated ATPase (H^+/K^+-ATPase) in gastric microsomal membranes was determined as described [19–20]. Briefly, the incubation mixture contained in a total volume of 1 ml 100 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 3 mM ATP, 50 μg membrane protein with and without 10 mM KCl. After 30 min incubation at 30°C, the reaction was stopped with TCA and the liberated P_i was measured colorimetrically as described above. The ATPase was found to be insensitive to ouabain.

Protein was estimated following the method of Lowry et al. [21] using bovine serum albumin as standard.

Partial purification of the inhibitor. The cytosolic fraction from rat brain was sequentially precipitated with ammonium sulphate to a saturation of 35%, then to 65%. Each time, the fraction was centrifuged at $100\,000 \times g$ for 1 h, pellet resuspended in 25 mM imidazole buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM βME (pH 7.5) and dialysed against 50 vols. of the same buffer for 24 h with two changes. After estimation of protein, each fraction was tested for its inhibitory activity. The protein precipitated with 35% ammonium sulphate was found to be more active and was then subjected to gel-filtration in a column of Sephadex G-100. For gel equilibration and elution the above buffer was used. Fractions of 2 ml each at a flow rate of 12 ml/h were collected and inhibitory activity of each fraction was assayed.

Effect of ATP, -SH or -NH_2 reactive probes on the inhibitory activity of the inhibitor. The inhibitor protein (approx. 20 μg) from gel-filtration column was added either to Na^+/K^+-ATPase enriched membranes which was preincubated alone or in the presence of 3 mM ATP for 5 min at 37°C. The enzyme activity was measured as described above.

In other experiments, the inhibitor was incubated either with 5 mM *N*-ethyl maleimide or 5 mM 2,4-dinitrofluorobenzene at 37°C for 1 h in 25 mM imidazole buffer (pH 7.5). Each was then dialysed for 24 h against the same buffer with two changes and then tested for the inhibitory activity. Concentrated solution of NEM and DNFB were prepared in dimethyl formamide.

Temperature effect. To study the effect of temperature on enzyme activity in the presence of inhibitor, the enzyme activity was measured at different temperatures and the liberated inorganic phosphate was estimated colorimetrically as described before.

For studying the effect of temperature on inhibitor, the inhibitor was preincubated at different temperatures for 1 h and its effect was tested on Na^+/K^+-ATPase activity.

Nature of inhibition. The enzyme enriched membrane fraction from rat brain was assayed in the presence of a fixed concentration of the inhibitor at different concentrations of ATP and the nature of inhibition was calculated from the double-reciprocal plot.

In order to determine whether the inhibition is reversible or irreversible, in our first approach, Na^+/K^+-ATPase was incubated with the inhibitor for 30 min at 37°C, spun at $100\,000 \times g$ for 1 h, the pellet resuspended in 25 mM imidazole buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM βME (pH 7.5) and assayed for activity. The process was repeated one more time. In another approach, a fixed amount of inhibitor was added to different concentrations of Na^+/K^+-ATPase.

To examine whether the inhibitor requires any monovalent ion, e.g., Na^+ or K^+ for its functioning, we preincubated Ca^{2+},Mg^{2+}-ATPase and Ca^{2+}-ATPase enriched rat testicular membranes in the presence of the above ions; this being followed by an activity assay in presence and absence of the inhibitor.

Results

The effect of different concentrations of crude inhibitor on transport enzyme activities isolated from different sources (Table I) showed that Na^+/K^+-ATPase either from rat brain or kidney and H^+/K^+-ATPase from toad gastric mucosa were inhibited by the inhibitor in a concentration dependent manner. However, the inhibitor did not affect Ca^{2+},Mg^{2+}-ATPase or Ca^{2+}-ATPase isolated either from rat testis or goat spermatozoa. A summary of the purification scheme as shown in Table II indicated that the inhibitory activity has been increased to 3-fold when the cytosolic fraction was fractionated with ammonium sulphate followed by gel filtration. The fraction 9 was

TABLE I

Effect of different concentrations of rat brain crude cytosolic protein on transport enzyme activities

The transport enzyme activities in different membranes were measured in the absence and presence of different concentrations of crude cytosolic protein and the liberated P_i was estimated colorimetrically as described in Materials and Methods. The results presented were the mean of six determinations obtained as duplicate assay on three different preparations.

Enzym	Spec. act. (μmol/mg protein per h)			
	control	inhibitor (μg)		
		27	55	110
Na^+/K^+-ATPase				
Rat brain	11.90	9.49	9.00	8.00
Rat kidney	38.80	34.50	24.29	16.96
H^+/K^+-ATPase				
Toad gastric mucosa	2.28	1.58	1.14	0.98
Ca^{2+},Mg^{2+}-ATPase				
Rat testis	2.57	2.52	2.47	2.69
Goat spermatozoa	29.23	26.79	29.02	30.44
Ca^{2+}-ATPase				
Rat testis	11.93	12.50	11.23	11.09
Goat spermatozoa	86.40	93.80	89.33	81.09

found to be enriched with the inhibitory activity after gel-filtration (Fig. 1) and the molecular mass of the protein was found to be about 80 kDa (data not shown).

The ATPase activity of the control as well as in presence of the inhibitor was found to be linear over a 30 min period (Fig. 2).

The inhibitory activity on Na^+/K^+-ATPase either when preincubated with ATP followed by the addition of the inhibitor protein or when ATP was added to the reaction mixture already preincubated with the inhibitor (Table III) revealed that preincubation of the

TABLE II

Summary of purification of inhibitor protein from rat brain cytosol

The unit of inhibitory activity was defined as the extent of inhibition of Na^+/K^+-ATPase activity by a fixed amount of the inhibitor which was then normalized to milligram of the inhibitor protein. The percent inhibition of Na^+/K^+-ATPase activity was calculated taking the activity of the control membrane without any inhibitor as 100. 20 μg of inhibitor protein was used in each case. The fraction obtained from 0–35% ammonium sulphate precipitation was fractioned on a Sephadex G-100 gel-filtration column as described in Materials and Methods. The fraction '9' was tested for the inhibitory activity.

Fraction	Protein (mg)	Inhibitory activity (units/mg protein)	% inhibition
Cytosol	28.7	71	20
$(NH_4)SO_4$			
0–35%	4.2	116	30
35–65%	3.9	27	7
Sephadex G-100	0.9	231	60

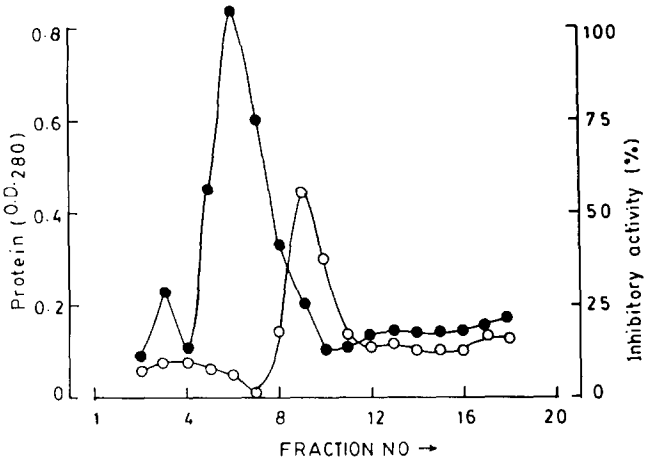

Fig. 1. Sephadex G-100 gel-filtration for the purification of inhibitor protein. About 4.2 mg of protein obtained after 35% ammonium sulphate precipitation of post 100000× *g* supernatant from rat brain was loaded on a column of Sephadex G-100 (2×30 cm) preequilibrated with 25 mM imidazole buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 1 mM ME. The protein was then eluted with the above buffer at a flow rate of 12 ml/h, the void volume was discarded and fractions of 2 ml each were collected. The fractions were read at 280 nm and then assayed for inhibitory activity on Na^+/K^+-ATPase as described in Materials and Methods. •, protein; ○, inhibitory activity.

ATPase with ATP protected its activity partially from inhibition.

To examine the role of -SH or $-NH_2$ group(s) of the inhibitor in inhibiting Na^+/K^+-ATPase activity, the inhibitor was incubated with NEM or DNFB, respectively, and dialysed for 24 h. The inhibitory activity was then tested when only partial dependency on the above

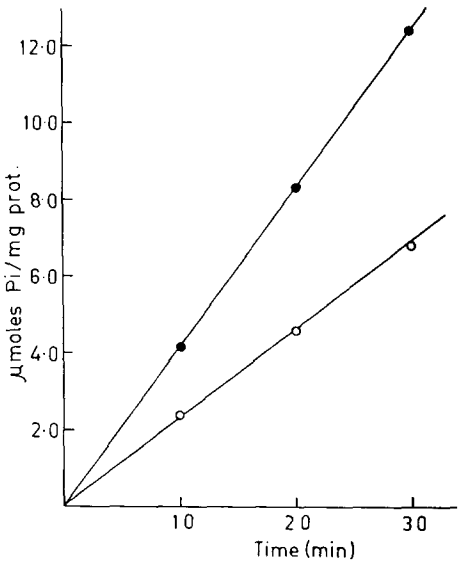

Fig. 2. Time-course of hydrolysis of Na^+/K^+-ATPase from microsomal membranes of rat brain in absence and presence of the inhibitor. Details were described in Materials and Methods. •, control ATPase; ○, in presence of the inhibitor ($n = 4$).

TABLE III

Effect of the inhibitor on Na^+/K^+-ATPase activity which was preincubated in the presence and absence of ATP

The preincubation of Na^+/K^+-ATPase enriched membranes from rat brain with or without ATP was done at 37°C. The activity was assayed and the liberated P_i was measured colorimetrically as described in Materials and Methods. 20 μg protein of fraction '9' from gel-filtration column was used in each case ($n = 3$).

	Spec. act. (μmol P_i / mg protein per h)	% inhibition
Na^+/K^+-ATPase (control)	15.25	–
Na^+/K^+-ATPase + inhibitor → incubated for 5 min → ATP → assay for activity	8.42	44.8
Na^+/K^+-ATPase + ATP → incubated for 5 min → inhibitor → assay for activity	12.62	17.3

TABLE IV

Effect of -SH and -NH_2 reactive probe on inhibitory activity of the inhibitor

The partially purified inhibitor (120 μg of fraction 9 from Sephadex G-100 column) in a volume of 1 ml was incubated either with 5 mM *N*-ethylmaleimide or 2,4-dinitrofluorobenzene at 37°C for 1 h, dialysed against 25 mM imidazole buffer (pH 7.5) for 24 h, the inhibitory activity was tested with 20 μg of inhibitor protein on Na^+/K^+-ATPase enriched membranes from rat brain. Control enzyme with the inhibitor which was not incubated with the probes were assayed simultaneously ($n = 3$).

	Spec. act. (μmol P_i / mg protein per h)	% inhibition
Na^+/K^+-ATPase (Control)	14.83	–
Na^+/K^+-ATPase + inhibitor	7.41	50
Na^+/K^+-ATPase + inhibitor treated with NEM	9.94	33
Na^+/K^+-ATPase + inhibitor treated with DNFB	10.86	27

TABLE V

Effect of inhibitor on Na^+/K^+-ATPase activity when assayed at different temperatures

Na^+/K^+-ATPase activity of rat brain microsomal membranes was assayed in presence and absence of 20 μg of the inhibitor protein (fraction 9 from Sephadex G-100) and liberated P_i was determined colorimetrically as described in Materials and Methods ($n = 3$).

Temperature (°C)	Spec. act. (μ mol P_i / mg protein per h)		% inhibition
	control	+ inhibitor	
30	9.06	6.74	25.5
33	16.68	11.68	30.0
37	24.82	12.98	47.7
40	26.09	19.43	25.5
45	33.40	30.20	9.6

TABLE VI

Na^+, K^+-ATPase activity of the microsomal membranes when assayed in the presence of inhibitor which was preincubated for 1 h at different temperatures

The inhibitor was preincubated at 35, 45, 60 and 90°C for 1 h, and an aliquot (20 μg) was used to check its inhibitory activity on Na^+/K^+-ATPase as described in Materials and Methods.

		Na^+/K^+-ATPase (μmol P_i / mg protein per h)
Control membranes		17.54
+ Inhibitor (no preincubation)		10.00
+ Inhibitor which was preincubated at:	35°C	10.50
	45°C	13.90
	60°C	16.10
	90°C	16.20

TABLE VII

Titration of inhibitor with different amounts of Na^+/K^+-ATPase enriched membranes

The enzyme activity was measured with different concentrations of enzyme at a fixed concentration (20 μg) of the partially purified inhibitor from gel-filtration column (fraction 9) and the liberated P_i was measured as described in Materials and Methods ($n = 4$).

Enzyme added (μg)	Spec. act. (μmol P_i /30 min)		% inhibition
	control	+ inhibitor	
31	0.429	0.261	39.0
62	0.859	0.625	27.1
156	1.949	1.566	19.7
187	2.286	2.140	6.4

TABLE VIII

Na^+/K^+-ATPase activity of the mirosomal membranes of rat brain after treatment with the inhibitor followed by washing, dilution and centrifugation

1 mg of mirosomal protein was incubated with 1.7 mg of the inhibitor protein for 30 min at 37°C in 1 ml of 25 mM imidazole buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 1 mM βME. The volume was made up to 10 ml and spun at 100000 × *g* for 1 h. The pellet was washed with the above buffer, suspended in 1 ml of the same buffer, 250 μl was kept aside and remaining amount was diluted to 10 ml, and centrifuged again at 100000 × *g* for 1 h. The pellet was suspended in 500 μl of the buffer. A control without the inhibitor was incubated and centrifuged in a similar way. Each fraction was assayed for protein and ATPase activity.

	Spec. act. (μmol P_i / mg protein per h)		% inhibition
	control	+ inhibitor	
	10.99	7.20	34.4
1 × centrifugation	11.88	9.58	19.4
2 × centrifugation	11.47	11.30	1.4

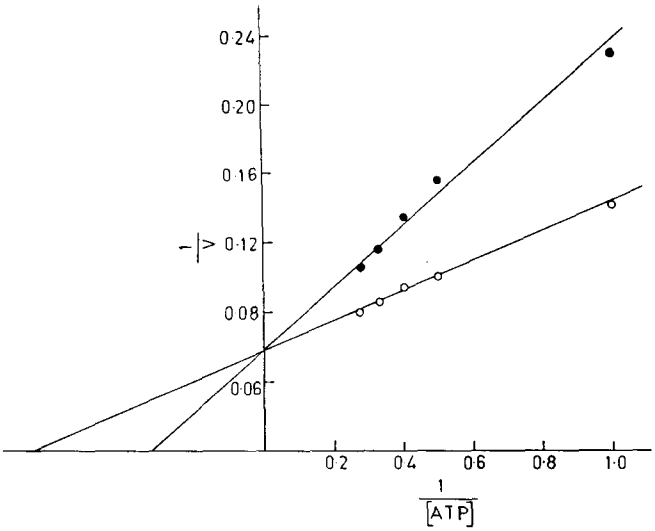

Fig. 3. Lineweaver-Burk plot of Na^+/K^+-ATPase when activity was assayed in absence or presence of the inhibitor protein (20 μg) with different concentrations of ATP. Details were described in Materials and Methods. ○, control ATPase; ●, in presence of the inhibitor ($n = 4$).

group(s) was observed (Table IV). When Na^+/K^+-ATPase activity in presence of the inhibitor was tested at different temperatures, maximum inhibition was obtained at 37°C (Table V). The inhibitor was found to lose its activity gradually at higher temperatures (Table VI).

The inhibitory activity was tested with varying concentration of the enzyme when the inhibition was found to be decreased with increasing concentration of the enzyme (Table VII), indicating that the inhibition may be reversible. Repeated dilution, washing and centrifugation led to a decrease in the inhibitory activity (Table VIII), suggesting that the inhibition was reversible. The inhibition was found to be competitive in nature with respect to the substrate as evident from Fig. 3, where the K_m of Na^+/K^+-ATPase for ATP

TABLE IX

Effect of inhibitor on Ca^{2+},Mg^{2+}-ATPase and Ca^{2+}-ATPase in absence and presence of Na^+ or K^+ or $Na^+ + K^+$

The rat testicular membranes enriched with Ca^{2+}, Mg^{2+} and Ca^{2+}-ATPase was assayed in absence and presence of the inhibitor (20 μg) in absence and presence of Na^+ (130 mM), K^+ (20 mM) or $Na^+ + K^+$ and the liberated P_i was estimated as described in Materials and Methods.

Ions added	Spec. act. (μmol P_i/mg protein per h)			
	control		+ inhibitor	
	Ca^{2+}, Mg^{2+}-ATPase	Ca^{2+}-ATPase	Ca^{2+}, Mg^{2+}-ATPase	Ca^{2+}-ATPase
None	4.51	7.15	5.29	7.20
$+Na^+$	4.17	6.16	4.85	6.23
$+K^+$	3.44	9.00	4.28	9.24
$+Na^+ + K^+$	2.65	7.89	3.04	7.78

assayed in the presence and absence of the inhibitor with different concentrations of the substrate was found to be different. The preincubation of Ca^{2+},Mg^{2+}-ATPase or Ca^{2+}-ATPase with monovalent ions, e.g., Na^+ or K^+ or $Na^+ + K^+$ did not help the inhibitor to inhibit these divalent-ion-dependent ATPases (Table IX), suggesting the inhibitor to be specific for monovalent-ion-dependent ATPase.

Discussion

Several endogenous substances are known to regulate Na^+/K^+-ATPase and Ca^{2+}-ATPase activities isolated from different sources. Thus catecholamine [22–24], thyroid hormones [25], vanadium [26], endogenous ouabain like glycosides [2–8], endogenous peptides [9–11], and brain extract [12] have been reported either to inhibit or stimulate Na^+/K^+-ATPase. A protein inhibitor of Ca^{2+}-ATPase from cytosol of sarcoplasmic reticulum has been reported by Narayanan et al. [27]. In the present study we have found an inhibitor protein in rat brain which inhibits Na^+K^+-ATPase isolated either from rat brain or kidney and H^+/K^+-ATPase from toad gastric mucosa. The inhibitor does not affect the activity of either Ca^{2+},Mg^{2+}-ATPase or Ca^{2+}-ATPase from rat testis or goat spermatozoa microsomal membranes (Table I) or rat skeletal muscle sarcoplasmic reticulum (data not shown). Thus the inhibitor could be specific for monovalent cation transporting ATPase. A monovalent cation transporting ATPase inhibitor has recently been reported by Araki et al. in porcine duodenum and intestine [9,10]. The inhibitor could be precipitated with ammonium sulphate to a saturation of 35% (Table II) which could further be purified by gel-filtration on Sephadex G-100 (Table II and Fig. 1). In gel-filtration, fraction 9 is enriched with the inhibitor protein, though the concentration of protein is not so significant in that particular fraction (Fig. 1). The inhibition is found to be competitive in nature (vide infra) thus it is likely that the inhibitor binds at or close to the ATP binding site, since preincubation of the enzyme enriched membranes with ATP protects the enzyme partially from inhibition (Table III). The other possibility of protection by ATP could be due to the conformational change of the enzyme when ATP binds to the enzyme making it resistant to the inhibitor. It has been reported previously that transport enzymes, e.g., Na^+/K^+-ATPase and H^+/K^+-ATPase could be protected from exogenous inhibitors by ATP [18,28–29].

When the activity of the inhibitor was tested in the presence of different group specific probes, it has been found that only part of the inhibitory activity depends on either -SH or $-NH_2$ group(s) (Table IV).

The phase transition temperature of the microsomal membranes of rat brain is found to be about 36.5°C

[30]. The maximum inhibition is obtained at a temperature around the phase transition temperature of the enzyme, i.e., at 37°C (Table V). Above this temperature less inhibition is observed. It is revealed from the data that the inhibitor can retain its full activity upto 35°C and starts losing the activity at and above 45°C (Table VI).

The inhibition seems to be reversible, since increasing concentration of the enzyme leads to a decrease in inhibition at a fixed concentration of the inhibitor (Table VII). The fact that the inhibition is reversible is strengthened from the finding that the activity of the inhibitor-treated membranes was reversed upon repeated dilution, centrifugation and resuspension (Table VIII). This type of reversible inhibition of Na^+/K^+-ATPase by an endogenous inhibitor has been reported recently [11]. The inhibitor is competitive in nature with respect to the substrate, since K_m is found to be affected, but V_{max} remained unchanged when the enzyme activity was measured at different concentrations of the substrate (Fig. 3). The inhibitor is found to be ineffective on Ca^{2+},Mg^{2+}-ATPase isolated from rat skeletal muscle (results not shown). The inhibitor seems to be specific for monovalent-ion-dependent ATPase and the inhibition is not due to any ionic effect, since preincubation of Ca^{2+},Mg^{2+}-ATPase or Ca^{2+}-ATPase with monovalent ions does not help the inhibitor in inhibiting these enzyme activities. In conclusion, though an inhibitor of Na^+/K^+-ATPase from rat duodenum [9–11] and Ca^{2+}-ATPase from sarcoplasmic reticulum [27] have recently been reported, no report of a Na^+/K^+-ATPase inhibitor protein from rat brain cytosol is available. The foregoing results indicate that the inhibitor is protein in nature and different from the endogenous glycoside-like inhibitor reported before [2–8]. The work on further purification, characterization and its effect on partial reactions of Na^+/K^+-ATPase, i.e., phosphorylation, ouabain binding, cation activation, etc., is in progress in our laboratory and will be published elsewhere.

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