Purification and functional characterization of a low-molecular-mass Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase modulator protein from rat brain cytosol

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A low-molecular-mass modulator protein having a molecular mass of about 12 kDa has been purified from rat brain cytosol following gel filtration and FPLC/Mono Q anion-exchange chromatographic separation. A number of protein fractions were obtained from an FPLC column when eluted with a 0.1 M NaCl hold gradient. One fraction (peak no. 5) was found to stimulate Ca^{2+} , Mg^{2+} -ATPase but inhibit Ca^{2+} -ATPase isolated from goat spermatozoa. The S_{50} (concentration producing 50 % stimulation) and I_{50} were found to be in the nanomolar range. The modulator seems to bind to Ca^{2+} , Mg^{2+} - or Ca^{2+} -ATPase at a site distal from the ATP binding site. The binding to both the ATPases is reversible and non-competitive in nature. The inhibitory activity is found to depend significantly on -SH or -NH₂ group(s) of the modulator, whereas no appreciable dependency

of the stimulatory effect was apparent. The study indicates that the modulator is not a glycoprotein. CD analysis suggests that the protein exists as an unordered secondary structure. An immuno-cross-reactivity study with specific antibody and inhibition by thapsigargin suggests that the Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPases from goat testes microsomal membranes are two isoforms of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) family. The modulator does not contain any Trp molecules, as evident from Trp fluorescence analysis. Amino acid analysis shows that glycine, serine, derivatives of tyrosine and phenylalanine are the predominant amino acids. The data suggest that the modulator is a negatively charged protein and is a good tool for distinguishing the regulation of Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase activities.

INTRODUCTION

The control of intracellular free calcium concentration is crucial for the maintenance of normal cell function and is regulated through the operation of several mechanisms, including the ATP-driven calcium pump [1,2]. Changes in free cytosolic calcium concentration play a vital role in the action of certain hormones and other stimuli (like calmodulin) on cell metabolism [1,3,4]. Ca^{2+} -ATPase in different organs generally requires Mg^{2+} for its activation [1,5–7], however Ca^{2+} -ATPase without a requirement for Mg^{2+} has also been reported [8–11]. Recently it has been reported from our laboratory that a Ca^{2+} -ATPase in rat testicular membranes [12] and goat spermatozoa membranes [13] could be activated by Ca^{2+} alone without the requirement for Mg^{2+} . We proposed that this Ca^{2+} -ATPase may be involved in fertility regulation [14] like Ca^{2+} , Mg^{2+} -ATPase. However, all available known inhibitors and/or stimulators failed to distinguish, in their effect, between Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase. It has been reported recently that a peptide of approximately 5 kDa from the venom of scorpion *pandinus* affects Ca^{2+} -release channels of the sarcoplasmic reticulum [15] and the regulation of an endogenous regulator protein of the sarcoplasmic reticulum calcium pump in heart muscle [16]. In the present report, we describe the purification and functional characterization of a modulator (regulator) protein from rat brain cytosol which can stimulate Ca^{2+} , Mg^{2+} -ATPase but inhibits Ca^{2+} -ATPase activities. Our findings suggest that these enzymes are two isoforms of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) family.

MATERIALS AND METHODS

Materials

ATP disodium salt, 2-mercaptoethanol, EDTA, EGTA, imi-

dazole hydrochloride, PMSF, benzamidine, Sephadex G-100, 5,5'-dithio(bis)-2-(nitrobenzoic acid) (Nbs_2), 2,4-dinitrofluorobenzene (DNFB), verapamil, trifluoperazine (TFP), thapsigargin, goat anti-mouse IgG, Nitro Blue Tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and dialysis tubings were purchased from Sigma Chemical Company. Mono Q HR 5/5 anion exchanger was from Pharmacia Biotech., histidine hydrochloride, sucrose, Tris/HCl, calcium chloride, magnesium chloride, SDS, acrylamide, bisacrylamide, tricine, glycine and Tween-20 were obtained from SISCO Research Laboratories, Bombay, India. Millipore filter (0.45 and 0.22 μm) from Millipore Corporation, dimethyl formamide and dimethyl sulphoxide were from Glaxo Chemicals, India, and octaethylene glycol mono-n-dodecyl ether (C_{12}E_8) was from Nikko Chemicals, Tokyo, Japan. Monoclonal (mouse) anti-SERCA2a antibody was from Affinity Bioreagents, Inc., U.S.A. Other reagents used were of analytical grade. Doubly distilled water was used throughout the study.

Isolation and purification of Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase enriched membranes from goat spermatozoa

Goat testes were collected from the local slaughter house just after the animals had been killed and were brought to the laboratory on ice. The caudal region of the testes was minced in 25 mM imidazole buffer containing 0.25 M sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol (pH 7.5). The suspension was passed through a nylon bag (mesh size 37 μm) to separate sperm from tissues, and the filtrate was homogenized gently in a glass homogenizer and centrifuged for 10 min at 600 g at 4 °C. The pellet was resuspended in half the volume of the same buffer and centrifuged again at 600 g for 10 min. The process was repeated once more and the supernatants were pooled together.

Abbreviations used: S_{50} , concentration producing 50 % stimulation; I_{50} , concentration causing 50 % inhibition; Nbs_2 , 5,5'-dithio(bis)-(2-nitrobenzoic acid); DNFB, 2,4-dinitrofluorobenzene; TFP, trifluoperazine; C_{12}E_8 , octaethylene glycol mono-n-dodecyl ether; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; NBT, Nitro Blue Tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

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About 95 % of the sperm found in the supernatants were motile. Microsomal membranes were prepared from the sperm as described [12,13]. Membranes enriched in Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPases were prepared by treatment with C_{12}E_8 followed by sucrose-density-gradient centrifugation as described previously [13]. The partially purified membrane fraction contains two major proteins with molecular masses of around 110 and 97 kDa. Protein was estimated by following the method of Lowry et al. [17] using BSA as standard.

Assay of ATPase activities

The Ca^{2+} , Mg^{2+} -ATPase activity was assayed as described previously [2,13,18]. The assay mixture contained, in a final volume of 0.25 ml (pH 7.5), 50 mM histidine, 25 mM sucrose, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM MgCl_2 , 3 mM ATP and 20 μg of membrane protein, with and without 3 mM CaCl_2 . The reaction mixture was preincubated for 5 min at 37 °C, followed by the addition of ATP and incubation for a further 30 min. The liberated P_i was estimated colorimetrically as described [13]. The values were expressed as the difference in activity in the presence of Mg^{2+} and Ca^{2+} and with Mg^{2+} alone.

For Ca^{2+} -ATPase, the assay mixture contained, in a final volume of 0.25 ml (pH 8.5), 50 mM imidazole in 25 mM sucrose, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 3 mM CaCl_2 , 3 mM ATP and 20 μg of membrane protein [13]. The reaction mixture was incubated and the reaction subsequently terminated as described above. The Ca^{2+} -ATPase activity was determined against a blank that contained all the ingredients except membrane protein. The liberated P_i was estimated as described [13].

Isolation of crude modulator protein from rat brain cytosol

Eight to ten albino rats of Charles Foster strain were killed after anaesthetization in chloroform. The brain was isolated by opening the skull and was homogenized in ice-cold buffer (pH 7.5) containing 25 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 1 mM 2-mercaptoethanol. The tissue was spun at 15000 g for 20 min in a cold centrifuge and the supernatant was collected and centrifuged at 100000 g for 1 h at 4 °C in an ultracentrifuge. The post-100000 g supernatant, termed the cytosol, was collected and stored at -20 °C for further use. This cytosolic fraction was found to be enriched with the modulator protein.

Purification of modulator protein

The cytosolic fraction from rat brain was loaded on to a Sephadex G-100 gel-filtration column pre-equilibrated with 10 mM Tris/HCl containing 1 mM benzamidine, 1 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.5). Fractions (3 ml) were collected in tubes at a flow rate of 12 ml/h, and the absorbance was recorded at 280 nm. The two major peaks obtained were tested against Ca^{2+} , Mg^{2+} -ATPase and Ca^{2+} -ATase from goat spermatozoa. The first peak contained the high-molecular-mass inhibitor protein of Na^+ , K^+ -ATPase while the second one contained the low-molecular-mass-modulator proteins of Na^+ , K^+ -ATPase, Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPases. Fractions 22–24 from the second peak were subjected to FPLC anion-exchange (Mono Q) column chromatographic separation. The column was washed with the buffer described above for 1 h, then eluted with NaCl gradient solution in 10 mM Tris/HCl buffer containing 0.1 mM benzamidine, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol (pH 7.5). The gradient was held at 0.1 M NaCl for 20 min. Altogether ten separate peaks were observed, of which eight were eluted with

0.1 M NaCl. Each fraction was collected, dialysed against buffer containing zero concentration of NaCl with four changes. The fractions were tested for modulatory (regulatory) activities against Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase from goat spermatozoa. Of the eight peaks from the 0.1 M NaCl hold eluent, peak no. 5 was found to stimulate Ca^{2+} , Mg^{2+} -ATPase but inhibit Ca^{2+} -ATPase activities at a significant level. For our further studies we used this fraction unless otherwise specified.

SDS/PAGE

Resolution of low-molecular-mass proteins/peptides requires the use of a gel system utilizing slower moving trailing ions [20] than the glycine of the Laemmli discontinuous gel-electrophoresis system [21]. The tricine gel system we used to resolve small proteins involves the use of a 4 % stacking gel at pH 6.8 followed by electrophoresis on a 16.5 % resolving gel at pH 8.8.

Concentrations of the modulator producing 50% stimulation (S_{50}) and 50% inhibition (I_{50})

To determine S_{50} and I_{50} values of the modulator, Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase activities were measured using different concentrations of the modulator at a fixed concentration of each enzyme separately, and the liberated P_i was estimated colorimetrically as described above.

Verapamil and TFP are two known inhibitors of Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPases. The effect of the modulator was tested on these enzymes in the absence and presence of verapamil and TFP.

Thapsigargin, a specific inhibitor of microsomal Ca^{2+} -ATPase, was also tested against the ATPases.

Temperature stability study

Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase activities were measured as described above in the presence of the modulator which was preincubated at different temperatures for 30 min.

Nature of modulation

Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase activities were measured in the presence of different concentrations of ATP at a fixed concentration of the modulator. The nature of modulation was determined from a Lineweaver–Burk plot of the velocity of the reaction versus substrate concentration. In order to determine whether the modulation is reversible or irreversible, the enzyme-enriched membranes (250 μg) were incubated with the regulator (50 μg) in a volume of 1 ml for 30 min at 37 °C. Keeping an aliquot aside, the rest was then diluted to 10 ml with the same buffer containing 1 mg/ml BSA and centrifuged for 1 h. The pellet was resuspended in 25 mM imidazole buffer containing 0.25 M sucrose, 1 mM 2-mercaptoethanol, 1 mM EDTA at pH 7.5. An aliquot was kept aside and the remaining mixture, after dilution with the same buffer in the presence of BSA was centrifuged as above. Each suspended pellet was assayed for protein and Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase activities.

Effect of -SH, and -NH₂ groups on the modulatory activity of the regulator

To examine the role of the -SH group(s) of the modulator in its activity, it was incubated with 50 μM Nbs₂ for 30 min at 37 °C. To study the role of -NH₂ group(s), the regulator protein was incubated with 50 μM DNFB for 30 min at 37 °C. In all cases

10 mM Tris/HCl buffer, pH 7.5, was used. Each test solution was dialysed for 24 h against the same buffer with two changes and tested for its activity, i.e. its stimulatory or inhibitory effect on Ca²⁺,Mg²⁺-ATPase or Ca²⁺-ATPase respectively.

Electrophoresis and immunoblotting

Goat testes microsomal membranes (100 µg), enriched with Ca²⁺,Mg²⁺- and Ca²⁺-ATPase, were separated on 7.5% gel under reducing conditions [21] and transferred to nitrocellulose paper [22]. The nitrocellulose paper was blocked overnight with 3% BSA in Tris/saline buffer, pH 7.5, at 4 °C. The nitrocellulose blot was incubated for 2 h at room temperature [23] with a 1:1000 dilution of monoclonal (mouse) anti-SERCA2a antibody (IgG2a) in Tris/saline buffer containing 0.5% BSA followed by extensive washing four times, 3 min each, with 0.05% Tween-20 in Tris/saline buffer. Similar washing was done thrice with only Tris/saline buffer, pH 7.5. The blot was incubated for 1 h with goat anti-mouse IgG as a conjugate (1:5000 dilution) followed by similar washing as described above. Immunoreactive bands were revealed with NBT-BCIP reagents as a chromogenic substrate for goat antimouse IgG.

Tryptophan fluorescence study

The emission spectra of ATPases (100 µg) (alone and in the presence of Mg²⁺ + Ca²⁺ or Ca²⁺) were recorded in the absence and presence of 50 µg of the modulator protein in a buffer system of 10 mM Tris/HCl/0.1 mM EDTA/0.1 mM 2-mercaptoethanol (pH 7.5) from 300 to 370 nm at an excitation wavelength of 280 nm using a Perkin-Elmer spectrofluorometer. A blank was run with the same buffer simultaneously.

CD study

The CD spectra of the ATPases (100 µg) (alone and in the presence of Mg²⁺ + Ca²⁺ or Ca²⁺) were taken in the absence and presence of the modulator protein (50 µg) in the same buffer system as in the fluorescence study. The spectra were measured in a Jasco spectropolarimeter (model 600) at between 200 and 250 nm.

Amino acid analysis

About 10 µg of protein free from all ions was digested with 6 M HCl in a nitrogen atmosphere followed by derivatization with phenylisothiocyanate and run through a Pico.Tag HPLC column (amino acid analysis system, Millipore corporation, Waters Chromatographic division). From the chromatogram, peaks were identified by comparing the retention time with known standards obtained from Sigma Chemicals.

RESULTS

A summary of the purification of low-molecular-mass proteins and their effect on Ca²⁺,Mg²⁺- and Ca²⁺-ATPase is shown in Table 1. It is evident from the Table that the crude cytosolic protein fraction does not show a significant effect on these ATPases at the concentration of the regulator used. A significant effect was observed with the crude regulator at a higher concentration (results not shown). When separated on a Sephadex G-100 column, the second peak was found to stimulate Ca²⁺,Mg²⁺-ATPase and inhibit Ca²⁺-ATPase. When the second

Table 1 Summary of purification of low-molecular-mass regulator protein from rat brain cytosol

Cytosolic protein (40 mg) from rat brain was loaded on to a Sephadex G-100 gel filtration column which was pre-equilibrated with 10 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA/0.1 mM benzamide/0.1 mM 2-mercaptoethanol. Two peaks were obtained. The second (later) peak (fraction nos. 22–24 showing Ca²⁺,Mg²⁺- and Ca²⁺-ATPase regulatory and Na⁺,K⁺-ATPase inhibitory activities were pooled together) were subjected to FPLC fractionation on an anion-exchange column (Mono Q). The column was washed thoroughly with the above buffer and eluted with different concentrations of NaCl. A number of fractions were collected from 0.1 M NaCl hold eluent. Each fraction was dialysed against the same buffer without NaCl for 48 h with four changes, and the regulatory activity of each was tested with 5 µg of regulator on Ca²⁺,Mg²⁺-ATPase and Ca²⁺-ATPase activities respectively. Results are means ± S.D. of three experiments. ns, not significant. + values, stimulation; — values, inhibition.

Fraction	Protein (mg)	Activity change (%)	
		Ca ²⁺ ,Mg ²⁺ -ATPase	Ca ²⁺ -ATPase
Cytosol	40.0	ns	ns
Pooled second peak from Sephadex G-100 (fraction nos. 22–24)	0.600 ± 0.10	+ 20.0 ± 2.1	— 8.5 ± 1.0
Eluent from FPLC column:			
Peak no.			
1	0.040 ± 0.012	+ 34.0 ± 3.2	+ 3.0 ± 0.8
2	0.012 ± 0.003	+ 28.0 ± 2.6	— 2.0 ± 0.5
3	0.022 ± 0.006	+ 35.0 ± 2.5	+ 9.0 ± 1.2
4	0.028 ± 0.007	+ 35.0 ± 3.0	nd
5	0.044 ± 0.010	+ 30.0 ± 3.5	— 70.0 ± 4.6
6	0.008 ± 0.004	+ 33.5 ± 2.2	+ 10.0 ± 1.6
7	0.013 ± 0.005	+ 27.0 ± 2.0	+ 14.0 ± 1.8
8	0.020 ± 0.007	+ 42.0 ± 2.8	+ 6.5 ± 0.6

Figure 1 SDS/PAGE of the regulator protein at different stages of purification

Protein was resolved on 16.5% acrylamide according to the method of Schagger and Von Jagow [20]. Lane 1, molecular-mass markers (values given on the left); lane 2, crude cytosolic protein; lane 3, pooled fractions 22–24 from Sephadex G-100 second peak; and lane 4, peak fraction 5 from the Mono Q/FPLC column.

peak from Sephadex was fractionated on an FPLC anion-exchange column (Mono Q), eight fractions could be obtained using a 0.1 M NaCl hold eluent. Though all the fractions showed a stimulatory effect on Ca²⁺,Mg²⁺-ATPase and a different level of stimulatory/inhibitory effect on Ca²⁺-ATPase, fraction number 5 was found to significantly stimulate Ca²⁺,Mg²⁺-ATPase and inhibit Ca²⁺-ATPase. We used this fraction for all our future studies.

The molecular mass of the protein in fraction 5 was found to

Figure 2 Effect of different concentrations of the modulator on $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase activities

(a) $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase and (b) Ca^{2+} -ATPase activities were assayed in the presence of different concentrations of regulator as described in the text. Each plot is representative of the mean $\pm$ S.D. of three similar experiments.

be around 12 kDa when analysed on SDS/16.5%-PAGE (Figure 1).

The effect of different concentrations of the modulator protein on $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase and Ca^{2+} -ATPase shows that the S_{50} of the modulator for $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase is 0.63 nM (Figure 2a), while the I_{50} against Ca^{2+} -ATPase is 0.35 nM (Figure 2b).

The modulator was found to show an additive effect on Ca^{2+} -ATPase, whereas a counteractive effect was observed on $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase when tested in the presence of verapamil or TFP (Table 2). Thapsigargin, a potent inhibitor of SERCA-type ATPases, was found to inhibit $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase activities in an identical manner (results not shown).

The effect of the modulator on ATPases was found to be reduced (both stimulation and inhibition) on dilution, repeated centrifugation and washing, suggesting that the binding of the regulator is reversible (results not shown).

The nature of the regulation of ATPases by the regulator was evaluated from a Lineweaver–Burk plot of the enzyme activities at different substrate concentrations, and the modulation was found to be non-competitive in nature (results not shown).

Table 3 shows the role of -SH and - NH_2 groups of the regulator in affecting its modulatory activity. The results indicate that the inhibitory activity is dependent more on these groups, than the stimulatory activity.

An immuno-cross-reactivity study with SERCA2a ATPase

Table 3 Effect of -SH or - NH_2 group-reacting probes on the regulatory activity of the regulator

The regulator was incubated either with Nbs₂ (50 μM) or DNFB (50 μM) for 30 min at 37 °C, each in 10 mM Tris buffer containing 0.25 M sucrose and 1 mM EDTA (pH 7.5). The reaction mixtures were dialysed overnight against the above buffer to remove excess probe. A sample containing the same concentration of protein in buffer without any probe was run simultaneously. $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase and Ca^{2+} -ATPase activities were checked in the presence of 5 μg and 3 μg of each of the probe-treated regulators respectively, as described in the Materials and methods section. Results are means $\pm$ S.D. of three independent experiments.

Condition	Activity (%)	
	$\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase	Ca^{2+} -ATPase
Control	100	100
+ Regulator	134 $\pm$ 3.6	57 $\pm$ 4.2
+ Regulator + DNFB	130 $\pm$ 2.5	81 $\pm$ 6.3
+ Regulator + Nbs ₂	121 $\pm$ 2.2	76 $\pm$ 5.0

Figure 3 Immunoblot of $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase

A 100 μg portion of $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase enriched membranes from goat testes spermatozoa was run on SDS/7.5%-PAGE, transferred to nitrocellulose paper and blotted with a monoclonal (mouse) anti-SERCA2a antibody as described in the Materials and methods section. The cross-reactive proteins of molecular mass 110 and 97 kDa are indicated. The data represents one of the three experiments.

antibody shows two cross-reacted bands with molecular masses of approximately 110 and 97 kDa (Figure 3).

Tryptophan fluorescence of $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase enriched membranes in the presence and absence of different ions and with and without the modulator protein are shown in Figures 4a and 4b. The results indicate that the regulator itself does not show any Trp fluorescence; however, the intrinsic Trp fluorescence of ATPases was affected in the presence of the modulator.

Table 2 Effect of different concentrations of verapamil and TFP either alone or in combination with a fixed concentration of regulator on $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase activities

$\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase and Ca^{2+} -ATPase activities were assayed at different concentrations of verapamil and TFP in the presence and absence of a fixed concentration of regulator (5 and 3 μg respectively) as described in the text. Results are means $\pm$ S.D. of three similar experiments. 1, 3, 5: Enzyme + 0.1, 0.2 and 0.3 mM verapamil respectively. 2, 4, 6: Enzyme + 0.1, 0.2 and 0.3 mM verapamil respectively + regulator (5 μg). 7, 9, 11: Enzyme + 10, 25, 50 μM trifluoperazine respectively. 8, 10, 12: Enzyme + 10, 25, 50 μM trifluoperazine respectively + regulator (3 μg).

		Inhibition of $\text{Ca}^{2+}, \text{Mg}^{2+}$ - and Ca^{2+} -ATPase activities (%)											
	Control	1	2	3	4	5	6	7	8	9	10	11	12
$\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase	0	50.0 $\pm$ 5.5	36.3 $\pm$ 6.0	66.1 $\pm$ 6.2	50.0 $\pm$ 6.4	74.2 $\pm$ 6.8	59.7 $\pm$ 6.1	48.0 $\pm$ 5.2	30.3 $\pm$ 4.8	75.2 $\pm$ 8.3	52.3 $\pm$ 6.4	90.0 $\pm$ 7.5	70.0 $\pm$ 7.8
Ca^{2+} -ATPase	0	20.0 $\pm$ 5.1	29.0 $\pm$ 5.3	32.0 $\pm$ 5.6	42.0 $\pm$ 7.2	40.0 $\pm$ 4.2	50.1 $\pm$ 6.8	6.8 $\pm$ 3.0	20.0 $\pm$ 4.1	20.4 $\pm$ 4.4	35.5 $\pm$ 5.3	26.0 $\pm$ 6.1	40.0 $\pm$ 6.0

Figure 4 Tryptophan fluorescence of (a) Ca²⁺,Mg²⁺-ATPase at different concentrations of the modulator and (b) Ca²⁺-ATPase at different concentrations of the regulator

(a) Fluorescence change was measured at two different concentrations of modulator: 1, buffer; 2, modulator (50 μ g); 3, purified goat testicular membranes (100 μ g); 4, purified goat testicular membranes (100 μ g) with 1 mM MgCl₂ and 3 mM CaCl₂; 5, with 25 μ g of modulator; and 6, with 50 μ g of modulator. The emission spectrum was recorded as described in the text. The results represent one of the three similar experiments. (b) Fluorescence quenching was measured with two different concentrations of regulator: 1, buffer; 2, regulator (50 μ g); 3, purified goat testicular membranes (100 μ g); 4, purified membranes (100 μ g) with 3 mM CaCl₂; 5, with 25 μ g of regulator; and 6, with 50 μ g of regulator. The emission spectrum was recorded as described in the text. The spectrum represents one of the three similar experiments.

The CD spectra of the modulator protein alone and/or ATPase-enriched membranes are shown in the Figures 5a and 5b in the presence of different ions. The results suggest an unordered conformation of the modulator protein, which, when it reacted with the ATPase, led to a change in the conformation of the latter.

Amino acid analysis of the modulator protein is shown in Table 4. The data show that derivatives of tyrosine (X) and phenylalanine (Y) together contribute about 31 %, aspartic acid and glutamate combined 14 %, and a 20 % contribution is from hydrophobic amino acids like glycine (Gly) and alanine (Ala). Only 3 % of basic amino acids like histidine, arginine, proline and lysine are present. This suggests that the modulator is a negatively charged hydrophobic molecule.

Table 4 Amino acid composition of the regulator protein

About 10 μ g of regulator protein free from all ions was digested with 6 M HCl in a nitrogen atmosphere, followed by derivatization with PITC, and run through a Pico.Tag HPLC column. The experiment was repeated twice. X and Y represent derivatives of tyrosine (Tyr) and phenylalanine (Phe) respectively.

Amino acid	Residue (moles of protein)	Composition (%)
Asp	6	5.2
Glu	11	9.0
Ser	15	12.8
Gly	17	14.2
His	1	0.5
Arg	1	0.5
Thr	2	1.3
Ala	7	6.1
Pro	1	1.2
X	16	13.0
Val	1	0.8
Met	2	1.7
Cys	9	7.2
Ile	8	6.4
Leu	2	1.6
Y	22	18.0
Lys	1	0.8
Total no. of residues 122		

DISCUSSION

Sperm microsomes enriched in Ca²⁺-ATPase catalyse ATP hydrolysis [12,13] and are involved in Ca²⁺ transport with properties similar to those from the microsomes of rat myometrium [24] and human liver [25]. In bull spermatozoa, calcium transport activity is regulated by Ca²⁺,Mg²⁺-ATPase located in the outer acrosomal membrane [26]. The ATPases play a vital role in regulating sperm motility through the maintenance of the intracellular concentration of calcium [27]. We have reported recently that Ca²⁺-ATPase activity is inhibited similarly to Ca²⁺,Mg²⁺-ATPase activity by two contraceptive drugs, chlorpromazine [14,28] and gossypol [14,29]. These findings suggest a possible role of Ca²⁺-ATPase in fertility regulation, similar to that of Ca²⁺,Mg²⁺-ATPase. The ATPase is found to be involved in Ca²⁺-uptake in an ATP-dependent manner similar to Ca²⁺,Mg²⁺-ATPase [30].

Figure 5 CD spectra of the Ca²⁺,Mg²⁺- and Ca²⁺-ATPase alone or in the presence of the regulator protein

The spectra were recorded between 200 and 250 nm in 10 mM Tris/HCl buffer (pH 7.5) containing 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol in a Jasco spectropolarimeter (model no. 600). Buffer CD was subtracted from each spectrum. The spectra are representative of more than three similar results. (a) $\cdots$, regulator (50 μ g); $—$, purified goat testicular membranes (100 μ g); $---$, purified membranes (100 μ g) with 1 mM MgCl₂ and 3 mM CaCl₂; and $-.-.-$, with 50 μ g of regulator. (b) $\cdots$, regulator (50 μ g); $—$, purified goat testicular membranes (100 μ g); $---$, purified membranes (100 μ g) with 3 mM CaCl₂ and $-.-.-$, with 50 μ g regulator.

Recently, a 5 kDa peptide from the scorpion *pandinus*, which affects Ca^{2+} -release channels of the sarcoplasmic reticulum, has been reported [15]. The presence of an endogenous inhibitor protein of the sarcoplasmic reticulum calcium pump in heart muscle has been reported by Narayanan et al. [16]. We demonstrated that a low-molecular-mass protein isolated from rat brain stimulates Ca^{2+} , Mg^{2+} -ATPase but inhibits Ca^{2+} -ATPase (Table 1). When pooled fractions from the Sephadex G-100 second peak (fraction nos. 22–24 in Table 1) were loaded on to a Mono Q column in FPLC, eight fractions were identified and each was collected from the 0.1 M hold NaCl eluent (Table 1). They were found to be of almost similar molecular masses (12–13 kDa) [31]. From the Table, it is obvious that peak no. 5 is the most effective fraction for stimulation of Ca^{2+} , Mg^{2+} -ATPase and inhibition of Ca^{2+} -ATPase. Fraction 3 is found to be a potent inhibitor of Na^+ , K^+ -ATPase [31]. This finding is very interesting, in that it could be used to distinguish the activities of these two ATPases, which has not been possible to date. The purity and molecular mass of peak fraction 5 was checked on SDS/16.5 %-PAGE (Figure 1), and was further confirmed by capillary-gel electrophoresis (results not shown).

During isolation and purification, different protease inhibitors were used in the isolation medium to avoid any possible effect of endogenous protease. This procedure also ruled out the possibility that the modulator is a protease-like substance (results not shown). The modulator is found to stimulate Ca^{2+} , Mg^{2+} -ATPase with an S_{50} of 0.63 nM (Figure 2a) and inhibit Ca^{2+} -ATPase with an I_{50} value of 0.35 nM (Figure 2b).

Verapamil and TFP are two inhibitors of Ca^{2+} - and Ca^{2+} , Mg^{2+} -ATPases. We have tested the effects of the modulator protein in the presence and absence of verapamil and TFP. It can be seen from Table 2 that the inhibitory effect of the modulator on Ca^{2+} -ATPase is enhanced by the addition of either of these agents, whereas the stimulatory effect on Ca^{2+} , Mg^{2+} -ATPase is reduced by them (Table 2). The findings suggest that in one case an additive effect is observed (Ca^{2+} -ATPase), whereas in other case a counteractive effect is obtained (Ca^{2+} , Mg^{2+} -ATPase). The findings also suggest that the binding sites of the modulator and these agents are different.

Inhibition by thapsigargin [32,33] (results not shown) along with immuno-cross-reactivity studies (Figure 3) suggest that these are isoforms of microsomal SERCA-type ATPases [34] having different sensitivities to Mg^{2+} .

The modulator is found to be quite stable up to 60 °C, above which the activity begins to be lost (results not shown). The stability of the modulator at high temperature is not unusual; we have reported an inhibitor protein of Na^+ , K^+ -ATPase from rat brain cytosol which is found to be stable up to 60 °C [31].

The binding of the modulator to ATPases is reversible, as revealed from a dilution and centrifugation study (results not shown). The effect was found to be non-competitive in nature in both cases with respect to the substrate (results not shown). In other words, it can be said that the binding sites of the modulator and ATP on the enzyme molecule are different.

To study the role of different functional group(s), i.e. $-\text{NH}_2$ or $-\text{SH}$ of the modulator, the groups were allowed to react with reactive probes, such as DNFB and Nbs₂ respectively. The data show that the stimulatory activity of Ca^{2+} , Mg^{2+} -ATPase has not changed much, whereas the inhibitory activity of Ca^{2+} -ATPase was reduced significantly on reaction with these probes (Table 3), suggesting that these groups are involved in modulating the inhibitory activity of the modulator more than the stimulatory effect. The role of reactive functional groups in modulating the activities of different enzymes have been reported previously [11,19,35–39].

The fluorescence data (Figures 4a and 4b) suggest that the modulator protein does not show any Trp fluorescence. The Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase enriched membranes are found to contain intrinsic Trp, which, when excited at 280 nm, shows an emission absorption at 335 nm that undergoes a change in the presence of the regulator protein. The Trp fluorescence enhancement with respect to Ca^{2+} , Mg^{2+} -ATPase and quenching in the case of Ca^{2+} -ATPase is in conformity with the stimulatory effect on Ca^{2+} , Mg^{2+} -ATPase and the inhibitory effect on Ca^{2+} -ATPase. Since the microsomal membranes constitute about 75–80 % of the ATPase proteins, it is suggested that changes observed are mostly due to these proteins. The quenching in the present study is comparable with that of sarcoplasmic reticulum Ca^{2+} -ATPase [40].

The CD spectra (Figures 5a and 5b) indicate that no well-known structural elements exists; however, an unordered structure [41] may exist, possibly containing a high content of β -turn [42] helix and an anti-parallel β sheet, causing the thermostability of the regulator up to 60 °C [43].

Amino acid analysis data shown in Table 4 suggest that the modulator is a hydrophobic negatively charged protein. No tryptophan residue was detected from the fluorescence data. A calcium-binding protein of comparable molecular mass (12 kDa) has recently been reported [44] which was found to be hydrophobic, negatively charged and to have no tryptophan residue. Calcium is believed to bind to a calcium-binding protein in the hydrophobic loop of the molecule. Whether our modulator protein behaves similarly remains to be established.

In an attempt to partially sequence the modulator protein, preliminary analysis of nine residues from the N-terminal end shows about 62 % sequence identity with fatty acid binding protein (results not shown).

In conclusion, though we have isolated and purified the modulator from brain, we tested the effect on Ca^{2+} , Mg^{2+} - and Ca^{2+} -ATPase from spermatozoa of rat and goat, since the ATPase activities are largely enriched in spermatozoa rather than in brain. Brain contains very low levels of activity which is also affected similarly (results not shown). Furthermore, there are reports showing that the brain controls spermatogenesis through these ATPases by the regulator from brain cannot be ruled out.

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