Phosphorylation and dephosphorylation of Mg^{2+}-independent Ca^{2+}-ATPase from goat spermatozoa

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We reported previously that a Ca^{2+}-ATPase in rat testes and goat spermatozoa could be activated by Ca^{2+} alone without Mg^{2+}, though it has a lot of similarities with the well known Ca^{2+}, Mg^{2+}-ATPase. Recently, we were successful in isolating the phosphorylated intermediate of the former enzyme under control conditions i.e., in the presence of low concentration of Ca^{2+} and at low temperature. Increase of the concentration of Ca^{2+} and/or temperature lead to dephosphorylation. Based on our observations, we proposed a reaction scheme comparable to that of Ca^{2+}, Mg^{2+}-ATPase. The findings strengthened our previous report that Mg^{2+}-independent Ca^{2+}-ATPase is involved in Ca^{2+} transport and Ca^{2+} uptake like Ca^{2+}, Mg^{2+}-ATPase.

1. Introduction

The control of intracellular free calcium concentration is crucial for the maintenance of normal cell functions and is regulated through the operation of several mechanisms including ATP driven calcium pump (Carafoli and Crompton 1978; Lynch and Cheung 1979). Changes in free cytosolic calcium concentration play a vital role in the action of certain hormones and other stimuli on cell metabolism (Carafoli and Crompton 1978; Charest *et al* 1983; Joseph and Williams 1983), cell growth (Moolenaar *et al* 1986), muscle contraction (Potter and Johnson 1982), fertility regulation (Garbers and Kopf 1980) and signal transduction (Nishizuka 1992) etc. Ca^{2+}-ATPase in different organs require Mg^{2+} for its activation (Carafoli and Crompton 1978; Niggli *et al* 1979; Moore *et al* 1975; Robinson 1976). However, Ca^{2+}-ATPase without the requirement of Mg^{2+} has also been reported (Vijaysarathi *et al* 1980; Gupta and Venkitasubramanian 1983; Jackowaski *et al* 1979; Shami and Radde 1971). It has been reported from our laboratory that a Ca^{2+}-ATPase in rat testicular membranes (Nagdas *et al* 1988) and goat spermatozoa (Sikdar *et al* 1991) could be activated by Ca^{2+} alone. We proposed that this Ca^{2+}-ATPase may be involved in fertility regulation (Mazumder *et al* 1991) like Ca^{2+}, Mg^{2+}-ATPase. In the present report, we describe the phosphorylation and dephosphorylation phenomena of the Mg^{2+}-independent Ca^{2+}-ATPase under various conditions and based on the observation propose a reaction scheme for the overall reaction.

2. Materials and methods

2.1 *Chemicals and radiochemicals*

ATP disodium salt, 2-mercaptoethanol (2-ME), EDTA, ethylene glycol-bis (2-amino ethylether) N,N,N′,N′ tetra-acetic acid (EGTA), imidazole, 1,2-cyclohexanediamine tetraacetic acid (CDTA), phenylmethylsulfonyl fluoride (PMSF) and Na-vanadate were purchased from Sigma Chemicals Co., USA. Sucrose, calcium chloride were from SISCO Research Laboratories, Mumbai. [γ-^{32}P]ATP (3000 Ci/mmol) from Bhabha Atomic Research Centre, Mumbai. Membrane filters (0·45 μm) from Millipore Corporation. All other reagents used were of analytical grade obtained from local market. Double distilled water was used throughout the study.

2.2 *Collection of goat testes, isolation and purification of ATPase enriched membranes*

Goat testes were collected from the local slaughter house just after sacrifice of the animal and brought to the

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laboratory on ice. The caudal region of the testes were minced in 25 mM imidazole buffer containing 0·25 M sucrose, 1 mM EDTA and 1 mM 2-ME (pH 7·5) (buffer A). Membranes enriched with Ca^{2+}, Mg^{2+}- and Ca^{2+}-ATPase were prepared as described earlier (Sikdar *et al* 1991). Briefly, the sperms were homogenized in a glass homogenizer and spun for 10 min at 600 *g* in cold. The pellet was resuspended in the buffer, centrifuged again at 600 *g*. The process was repeated once more. The pooled supernatant was spun at 12,000 *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 100,000 *g* for 1 h. The pellet (microsomes) was resuspended in the above buffer and assayed for protein. It was then treated with 0·05% octaethyl glucoside ($C_{12}E_8$) containing 0·1 mM PMSF and 1 mM ATP to a final concentration of 1 mg protein per ml. After stirring for 10 min at 4°C, the mixture was layered on the top of a gradient consisting 5 ml each of 20, 25, 30, 34 and 37% sucrose and spun at 50,000 *g* for 4·5 h in a SW27 swinging bucket rotor. The band which appeared between 34 and 30% sucrose was collected, diluted three times with 25 mM imidazole buffer (pH 7·5) containing 1 mM 2-ME and spun at 100,000 *g* for 1 h. The pellet was suspended in buffer A and assayed for protein and ATPase activities. The purified fraction shows two protein bands of molecular mass 110 and 97 kDa on 7·5% SDS-PAGE (Bhattacharyya and Sen 1998). Proteins were estimated following the method of Lowry *et al* (1951) using bovine serum albumin as standard. The enzyme activities were assayed as described below. However for calcium uptake study non-detergent treated microsomal membranes were used (Sikdar *et al* 1991).

2.3 *Enzyme activity assay*

The Mg^{2+}-dependent Ca^{2+}-ATPase activity was assayed according to previously established procedures (Sikdar *et al* 1991; Nandi *et al* 1981). The values were expressed as the difference in activities between Mg^{2+}, Ca^{2+} and Mg^{2+} alone. The Mg^{2+}-independent Ca^{2+}-ATPase was assayed as described previously (Sikdar *et al* 1991). The assay medium (1 ml) contained 25 mM imidazole, 25 mM sucrose, 0·5 mM EDTA, 1 mM 2-ME (pH 8·5), 4 mM $CaCl_2$ and 3 mM ATP. The mixture was preincubated for 5 min at 37°C and the reaction was initiated with the addition of 10–15 µg detergent treated membrane protein and incubated for 30 min. It was terminated with the addition of 0·2 ml of 30% ice-cold TCA. The liberated inorganic phosphate was estimated colorimetrically following the method of Sen *et al* (1981). A tube containing all the ingredients except membrane protein was run simultaneously as blank. The free concentration of calcium in the reaction mixture was adjusted by the addition of EGTA (Sillen and Martell 1971). After chelating endogenous Mg^{2+} (which was found to be very low when measured by atomic absorption spectrophotometer) with CDTA, the ATPase activity obtained was solely due to free Ca^{2+} alone.

2.4 *^{45}Ca uptake study with microsomal membrane vesicles*

Calcium uptake by the Mg^{2+}-independent Ca^{2+}-ATPase was measured in a 1 ml reaction mixture containing 25 mM imidazole buffer (pH 8·5), 10 mM KCl, 0·5 mM $CaCl_2$ containing ^{45}Ca (sp. activity 2,000 cpm/pmol) and 4 mM ATP. The reaction was started by the addition of non-detergent treated microsomal vesicles (100 µg protein) and the mixture incubated at 37°C. At a different time an aliquot of 20 µl was removed and diluted to 1 ml with ice-cold buffer containing 0·5 mM $CaCl_2$ and 2 mM EGTA. The suspension was rapidly filtered through a 0·45 µm Millipore filter, which was then washed with 20 ml cold $CaCl_2$-EGTA (1 mM). The filters were taken in scintillation fluid and the radioactivity counted in a liquid scintillation counter.

Table 1. Mg^{2+}-independent and Mg^{2+}-dependent Ca^{2+}-ATPase activities in different fractions isolated from goat spermatozoa.

	µmol P_i/mg/h		
	Mg^{2+}-dependent		Mg^{2+}-independent
Fraction	Mg^{2+} alone	Mg^{2+} + Ca^{2+}	Ca^{2+}
Microsomal membranes	165 ± 15	309 ± 18	360 ± 20
Detergent treated density gradient fraction	336 ± 13	676 ± 21	718 ± 23

The post 10,000 *g* fraction from goat spermatozoa homogenate was centrifuged at 100,000 *g* for 1 h and the resultant pellet (microsomal membranes) was treated with $C_{12}E_8$ followed by density gradient centrifugation. The layer enriched in enzyme activities was collected and assayed for protein and enzyme activities as described in the text (n = 4).

To examine if the uptake is energy dependent, we have carried out the uptake experiment in the absence of ATP To study the effect of A23187 on Ca^{2+} uptake by the ATPase, we added the ionophore at a final concentration of 1 μM after 10 min; at a different time after addition, an aliquot of 20 μl was withdrawn and radioactivity measured as described above. The ionophore was prepared in dimethyl sulphoxide. The solvent alone had no effect on Ca^{2+} uptake at the concentration used.

2.5 *Phosphorylated intermediate study with the Mg^{2+}-independent Ca^{2+}-ATPase enriched membranes*

This has been done under different conditions to standardize the optimum conditions for the formation of phosphorylated intermediate (E ~ P).

2.5a *Time course of phosphorylation*: Rate of phosphorylation was measured at a different time of incubation at 4°C in the presence of 1·7 μM free Ca^{2+} under above optimum conditions with 50 μg membrane protein. The level of E ~ P formed was measured as described above.

2.6 *Effect of different concentrations of Ca^{2+} on phosphorylated intermediate (E ~ P) formation*

ATPase enriched membranes were phosphorylated with [γ-^{32}P]ATP in different concentrations of free Ca^{2+} (50 nM–250 μM) at 4°C for 3 s in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 80 mM KCl, 0·5 mM 2-ME (pH 6·8) in a total reaction volume of 1 ml. The free calcium concentration was calculated according to the method of Sillen and Martell (1971). The reaction was stopped with 0·2 ml of ice-cold 30% TCA and filtered through 0·45 μm membrane filter and washed thoroughly with 10 ml cold 10 mM Na_2HPO_4 containing 1 mM cold ATP. The radioactivity on the membrane filters was counted in a scintillation counter.

2.6a *Incorporation of ^{32}P to membrane ATPase at different concentrations of ATP*: The incorporation was carried out at different concentrations of [γ-^{32}P]ATP under above conditions at 1·7 μM free Ca^{2+}.

2.6b *Dephosphorylation of phosphorylated intermediates*: After the intermediate was allowed to form for 3 s in the presence of 1·7 μM free Ca^{2+}, protein and 5 μM [^{32}P]ATP, calcium concentration in the reaction mixture was increased and incubated for 30 s more. The reaction was terminated with TCA and the radioactivity was measured as described before.

2.7 *Effect of vanadate on phosphorylation and dephosphorylation*

To examine which reaction step phosphorylation or dephosphorylation is affected by vanadate (an inhibitor of Ca^{2+}-ATPase), we have performed the phosphorylation and dephosphorylation experiments in the presence of

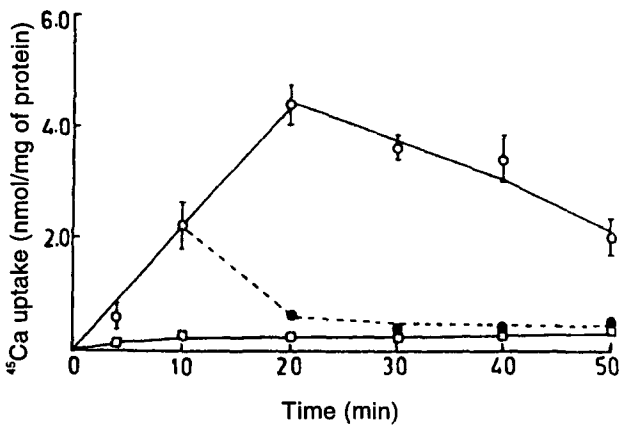

Figure 1. Time course of Ca^{2+} uptake by Mg^{2+}-independent Ca^{2+}-ATPase enriched detergent untreated microsomal membrane vesicles. (○), Control in complete medium; (□), in the absence of ATP; (●), in the presence of A23187 (n = 4).

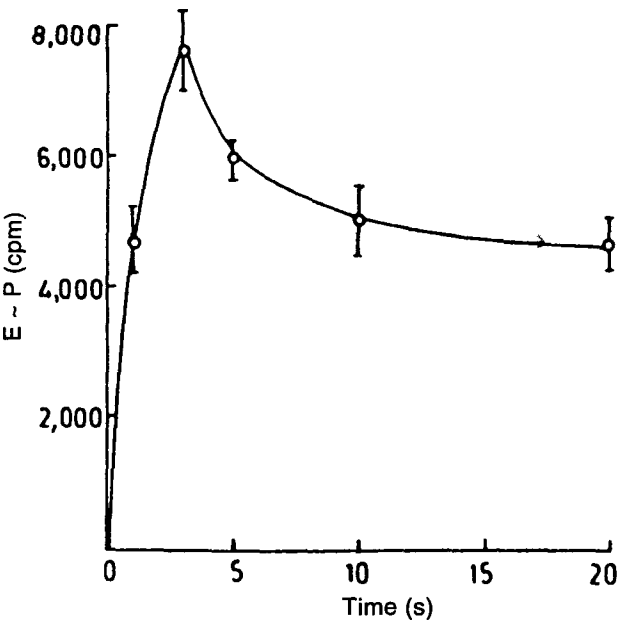

Figure 2. Time course of phosphorylation of Mg^{2+}-independent Ca^{2+}-ATPase from goat spermatozoa. Phosphorylation of ATPase enriched membranes was done in a volume of 1 ml reaction mixture of 50 mM Tris-HCl buffer (pH 6.8) containing 1 mM EDTA, 80 mM KCl, 0.5 mM 2-ME in the presence of 5 μM [γ-^{32}P]ATP, 1 μM $CaCl_2$ and 50 μg membrane protein at 4°C. At different intervals of time an aliquot of 0.2 ml was taken out and the radioactivity was measured as described in the text (n = 3).

50 μM vanadate under optimum conditions. After termination of the reaction with ice-cold TCA, the radioactive counts were determined in each case as described above.

3. Results and discussion

Mg^{2+}-dependent and independent Ca^{2+}-ATPase activities are shown in table 1. Though the activities of both Mg^{2+}-dependent and independent enzymes are predominant in this fraction, the activity of the latter is higher than the former. The high level of Mg^{2+}-independent Ca^{2+}-ATPase activity found in goat spermatozoa is comparable with that of rat testicular membranes (Nagdas *et al* 1988). The treatment with octyl glucoside enhances the enzyme activity under control conditions.

Figure 1 shows the rate of Ca^{2+} uptake at points of different time under various conditions. It is evident from the figure that uptake reaches a maximum level at 20 min. The lowering of uptake beyond 20 min may be due to the leakiness of the membranes thereby loosing the vesicular structure. It can also be seen that in the absence of ATP, no Ca^{2+}-uptake takes place suggesting that Ca^{2+}-uptake by Ca^{2+}-ATPase is energy dependent. Accumulated Ca^{2+} is rapidly and completely released by the calcium ionophore A 23187. The fact that the uptake is absolutely ATP dependent suggests that a significant proportion of the prepared vesicles are oriented inside out (Enyedi *et al* 1988; Sumida *et al* 1988).

Figure 2 shows time course of phosphorylation of Mg^{2+}-independent Ca^{2+}-ATPase. It is seen from the figure that maximum phosphorylation of the ATPase is obtained at 3 s, beyond which it decreases. It could so happen that the phosphorylated intermediate ($E_1 \sim P$) which forms at 3 s may undergo dephosphorylation at longer time of incubation in the presence even of low concentration of Ca^{2+}.

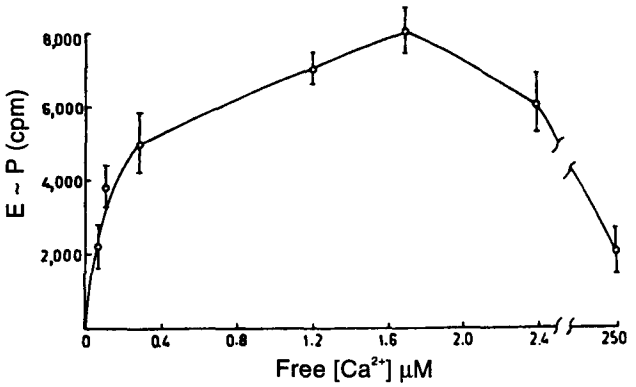

Figure 3. Effect of different concentrations of free Ca^{2+} on the formation of phosphorylated intermediates. Free calcium concentration was calculated as described in the text. The reaction was continued for 3 s with 5 μm [γ-^{32}P]ATP. The other conditions were same as in figure 2 ($n = 3$).

Figure 3 shows the rate of formation of phosphorylated intermediate of ATPase at different concentrations of Ca^{2+}. It is evident from the figure that phosphorylation increases up to 1·7 μM Ca^{2+} concentration above which dephosphorylation takes place. The finding suggests that higher concentration of Ca^{2+} is responsible for dephosphorylation of the intermediate. This step of the reaction is comparable to the dephosphorylation of the Ca^{2+}, Mg^{2+}-ATPase in the presence of Ca^{2+} (Schurmans-Stekhoven and Bonting 1981). The concentration of free Ca^{2+} required for optimum phosphorylation is relatively high but interesting, since the finding is reproducible (Nagdas *et al* 1988; Sikdar *et al* 1991; Bhattacharyya and Sen 1998). Possibly the transport of calcium takes place through the binding of low affinity site. It has been shown by Quist and Roufogalis (1975) that Ca^{2+}-ATPase with high affinity for Ca^{2+} can easily be removed from membranes and this suggests that low affinity site plays basic role in active calcium transport.

The study of the phosphorylation of ATPase at different concentrations of ATP shows that optimum concentration of substrate (ATP) to obtain maximum phosphorylation is 5 μM (data not shown) which is comparable to Ca^{2+}, Mg^{2+}-ATPase reported in other sources (Miessner 1973).

Vanadate is an inhibitor of Mg^{2+}-independent Ca^{2+}-ATPase (Sikdar *et al* 1991) and also an inhibitor of P-type ATPases (Stryer 1988). To examine which step of the overall reaction sequence (phosphorylation or dephosphorylation) is inhibited, we have performed an experiment in the presence of vanadate and the finding is shown in table 2. From the table it may be suggested that vanadate inhibits the phosphorylation step of the overall reaction sequence. The inhibition by vanadate suggests that Mg^{2+}-independent Ca^{2+}-ATPase is a P-type ATPase. Since in all P-type ATPases phosphorylation takes place on Asp residue (Stryer 1988), it is therefore logical to conclude that in Mg^{2+}-independent Ca^{2+}-ATPase in the present study, phosphorylation takes place on Asp residue.

Table 2. Phosphorylaton and dephosphorylation of the Mg^{2+}-independent Ca^{2+}-ATPase and the effect of vanadate.

	Phosphorylated product (cpm)	Dephosphorylated product (cpm)
Control	15698 ± 550	6802 ± 400
+ vanadate	5634 ± 250	4298 ± 325

Phosphorylation of the ATPase was done at 4°C for 3 s in the presence of 1.7 μM Ca^{2+} and 5 μM [^{32}P]ATP. After 3 s 250 μM Ca^{2+} was added and incubated for 30 s more. In another set of experiment 50 μM vanadate was added in the reaction medium either during phosphorylation or during dephosphorylation. The radioactive counts in each case were measured as described in the text ($n = 3$).

It is pertinent to mention here that recently we have reported a Ca^{2+}, Mg^{2+}- and a Ca^{2+}-ATPase from goat testes microsomal membranes which belong to two isoforms of SERCA family having different sensitivity to Mg^{2+} (Bhattacharyya and Sen 1998). From the ongoing findings we propose a scheme for the overall reaction of this Ca^{2+}-ATPase comparable with the Mg^{2+}, Ca^{2+}-ATPase (Ikemoto 1975; Schatzmann 1975) except that phosphorylation and dephosphorylation is regulated by different concentrations of calcium ion i.e., phosphorylation of the enzyme takes place at lower concentration of calcium and dephosphorylation at higher concentration:

$$E \xleftarrow{\text{Ca(low)} + \text{ATP}} E.\text{Ca.ATP} \xleftarrow{\text{Ca, ADP}} E_1 \sim P \xleftarrow{\text{Ca(high)}} E + P_i.$$

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