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Calcium uptake and Ca^{2+}-ATPase activity in goat spermatozoa membrane vesicles do not require Mg^{2+}

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Abstract. Microsomal membrane vesicles isolated from goat spermatozoa contain Ca^{2+}-ATPase, and exhibit Ca^{2+} transport activities that do not require exogenous Mg^{2+} .The enzyme activity is inhibited by calcium-channel inhibitors, *e.g.* verapamil and diltiazem, like the well known Ca^{2+} , Mg^{2+}-ATPase. The uptake of calcium is ATP (energy)-dependent and the accumulated Ca^{2+} can be completely released by the Ca^{2+} ionophore A23187, suggesting that a significant fraction of the vesicles are oriented inside out.

Keywords. Ca^{2+}-ATPase; Ca^{2+} uptake; microsomal membranes; spermatozoa (goat).

1. Introduction

The optimum amount of extracellular calcium essential for sperm motility varies from species to species. There is a steep concentration gradient of Ca^{2+} between the inside of the sperm and the outside, with the cytoplasmic level being about 10^3–10^4-fold lower than the extracellular level. Changes in free cytosolic calcium concentration play a vital role in the action of certain hormones and other internal stimuli (like calmodulin) on cell metabolism (Carafoli and Crompton 1978; Charest *et al* 1983; Joseph and Williamson 1983). The coordinated and forward motility of mature mammalian spermatozoa is regulated by the intracellular level of free calcium (Brokaw *et al* 1974), so that its presence is essential for fertilization (Yanagimachi 1988). The control of intracellular free Ca^{2+} concentration, which is crucial for the maintenance of normal cell function, is regulated through the operation of several mechanisms, including ATP-driven calcium pumps and Mg^{2+}, Ca^{2+}-ATPases (Carafoli and Crompton 1978; Lynch and Cheung 1979).

Ca^{2+}-ATPases in different organs generally require Mg^{2+} for their activation (Carafoli and Crompton 1978; Niggli *et al* 1979; Moore *et al* 1975; Robinson 1976). However, a Ca^{2+}-ATPase without the requirement of Mg^{2+} has also been reported (Vijaysarathi *et al* 1980; Gupta and Venkitasubramanian 1983; Jackowski *et al* 1979; Shami and Radde 1971; Zhao and Dhalla 1988). We have previously reported a Ca^{2+}-ATPase in rat testicular sperm (Nagdas *et al* 1988) and goat sperm (Sikdar *et al* 1991) membranes that could be activated by Ca^{2+} on its own, that is, without any Mg^{2+}. We have also suggested that the enzyme could have a role in fertility regulation (Mazumder *et al* 1991). In the present study we report that the Ca^{2+}-ATPase of goat spermatozoa is involved in calcium transport. We examine the physiological significance of the Ca^{2+}-ATPase and compare its properties with those of the well-known Mg^{2+}, Ca^{2+}-ATPase.

2. Materials and methods

2.1 *Materials*

ATP, *β*-mercaptoethanol *(β*-ME*),* EDTA, EGTA, imidazole, 1,2-cyclohexanediamine-tetraacetic acid (CDTA), verapamil, diltiazem and A23187 were purchased from Sigma Chemical Co., USA; sucrose, calcium chloride, magnesium chloride were from Sisco Research Laboratories, Bombay; $^{45}CaCl_2$ from Bhabha Atomic Research Centre, Bombay; nylon bags (mesh size 37 μm) from Small Parts Incorporation, USA; Millipore filters (0.45 μm) from Millipore Corporation; dimethylformamide (DMF) and dimethyl sulphoxide (DMSO) were from Glaxo Chemicals (India); octaethylene glycol mono-*n*-dodecyl ether (C_{12} E_8) from NIKKO Chemicals, Tokyo, Japan. All other reagents used were of analytical grade. Double-distilled water was used throughout the study.

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

Goat testes were collected from the local slaughter house just after sacrifice and brought to the laboratory on ice. The caudal regions of the testes were minced in 25 mM imidazole buffer containing 0·25 mM sucrose, 1 mM EDTA and 1 mM *β*-ME (buffer A. pH 7·5). The suspension was passed through a nylon bag (mesh size 37μm) to separate sperm from tissues, and the filtrate 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. About 95% of the sperm found in the supernatants were motile. Microsomal membranes were prepared from the sperm as described (NagDas *et al* 1988; Sikdar *et al* 1991). Membranes enriched in ATPase were prepared by treatment with C_{12} E_8 followed by sucrose density gradient centrifugation as described earlier (Sikdar *et al* 1991; Sikdar 1991). The partially purified membrane fraction contained two proteins of molecular weight around 93 and 102 kDa (Sikdar *et al* 1991; Adhikary 1992). Protein was estimated following the method of Lowry *et al* (1951) using bovine serum albumin as standard, and assayed for enzyme activity as described below.

2.3 *Enzyme assay*

The Mg^{2+}, Ca^{2+}-ATPase in the above membrane fraction was assayed as previously described (NagDas *et al* 1988; Nandi *et al* 1981). The values were expressed as the difference between ATPase activity in presence of Mg^{2+} + Ca^{2+} and that in presence of Mg^{2+} alone. For Ca^{2+} -ATPase, the assay mixture contained, in a final volume of 1 m (pH 8·5); 25 mM imidazole in 25 mM sucrose, 0·5 mM EDTA, 1 mM *β*-ME, 3 mM $CaCl_2$, 4mM ATP and 10–15 μg of membrane protein (Sikdar *et al* 1991). The Ca^{2+} -ATPase activity was determined against a blank that contained all the ingredients except membrane protein. After a 30 min incubation at 37°C, the reaction was terminated by addition of 0·2 ml of

30% ice-cold TCA. and free inorganic phosphate was determined following the method of Sen *et al* (1981). The low-affinity Ca^{2+} -ATPase activity was determined as the difference between the rate of ATP hydrolysis when membranes were incubated in the standard asay medium containing 200 μM CDTA without Ca^{2+} (basal activity) and the rate in presence of 200 μM CDTA with different concentrations of Ca^{2+}. After chelation of endogenous Mg^{2+} (which was found to be very low when measured by atomic absorption spectroscopy) with CDTA, the ATPase activity obtained was due to the free Ca^{2+} alone (Sillen and Martell 1971).

2.4 *Inhibition of ATPase activities by calcium-channel blockers*

Membranes were incubated with different concentrations of inhibitors in the ATPase assay buffer for 20 mm at 37°C. The reactions were then initiated with the addition of ATP and either Mg^{2+} + Ca^{2+} or Ca^{2+} alone, and incubation was continued for 30 min in a shaker bath at 37°C. The liberated P_i was estimated as described above. Verapamil and diltiazem solutions were prepared in double-distilled water.

2.5 *^{45}Ca uptake study with microsomal membrane vesicles*

Calcium uptake by the Ca^{2+}-ATPase was measured in a 1 ml reaction mixture containing 25 mM imidazole (pH 8·5), 10 mM KCl, 0·5 mM $CaCl_2$, 0·5 mM $^{45}CaCl_2$, (sp. act. 2,000 cpm/pmol) and 4mM ATP. For the Mg^{2+} , Ca^{2+} -ATPase, 1 mM $MgCl_2$ was added in 50 mM histidine buffer, pH 7·5. The reaction was started by the addition of intact microsomal vesicles (100 μg protein) and the mixture incubated at 37° C. At different times 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. The filters were taken up in scintillation fluid and the radioactivity counted in a liquid scintillation counter.

To study the effect of external calcium concentration on Ca^{2+} uptake, we measured uptake over a fixed time (20 min) with different concentrations of calcium chloride.

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 different times 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.

3. Results

The effect of different concentrations of Ca^{2+} in the presence of CDTA is shown in figure 1. The ATPase activity obtained is due to Ca^{2+} since endogenous Mg^{2+} was chelated by CDTA. The K_m for Ca_{2+} calculated from the double reciprocal plot (inset) is 375 μM.

The calcium antagonists verapamil and diltiazem inhibited both Ca^{2+}-ATPase

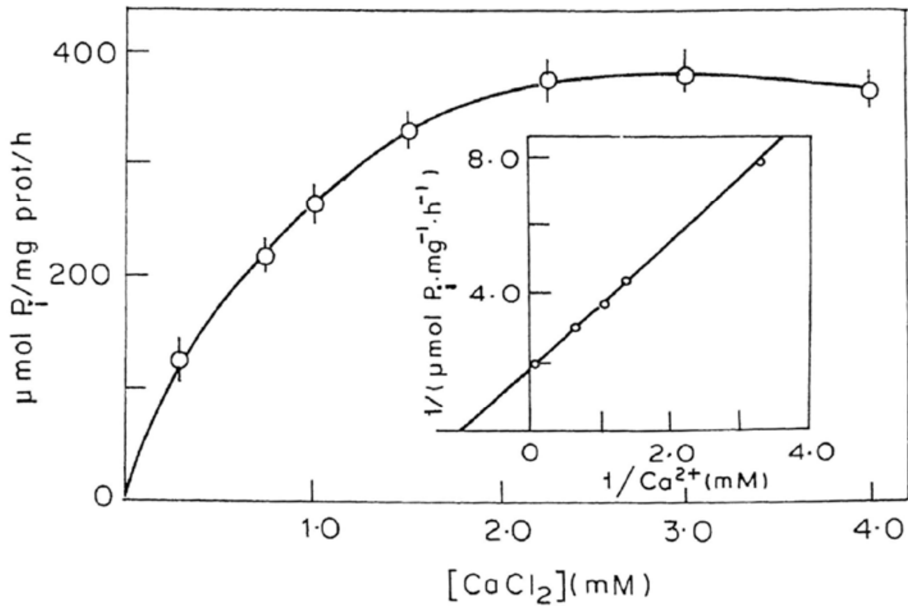

Figure 1. Effect of different concentrations of CaCl $_2$ on Ca^{2+}-ATPase in detergent-treated purified goat sperm membranes in the presence of 200 μM 1, 2-cyclohexanediamine tetraacetic acid (CDTA). The activity in the absence of $CaCl_2$ but in the presence of CDTA is negligible. The details are given in §2. The results shown are the means with standard errors, from four separate experiments.

and Mg^{2+}, Ca^{2+}-ATPase activities in a concentration-dependent manner. For 50% inhibition the concentrations of verapamil were 160 and 46 μM for the two ATPases respectively (figure 2), and with diltiazem, 220 and 100 μM respectively (figure 3).

The rate of $^{45}Ca^{2+}$ uptake was measured in the presence of ATP with microsomal membrane vesicles enriched in the enzyme activities. The uptake was linear for 10 min in absence of Mg^{2+} and for 20 min in the presence of Mg^{2+} (figure 4). The lower rate in the presence of Mg^2 + is consistent with the observation that the Ca^2-ATPase (no Mg^{2+}) is more active than the Mg^{2+}, Ca^{2+}-ATPase (Sikdar *et al* 1991). As shown in figure 5 the uptake reached a plateau at 3 mM $CaCl_2$ with both enzymes. For the calculation of uptake a tube containing all the ingredients except membrane protein was run as a blank; the count here was subtracted from that in the sample tube. The filtering and washing were done as described in the methods.

The experiment on the effect of the calcium ionophore A23187 and ATP on Ca^{2+} uptake by Ca^{2+}-ATPase shows that addition of the ionophore after 10 min of uptake caused immediate release of accumulated calcium (figure 6).

4. Discussion

Sperm microsomes enriched in Ca^{2+}-ATPase catalyse ATP but Mg^{2+}-independent Ca^{2+} transport with properties similar to those of microsomes of rat

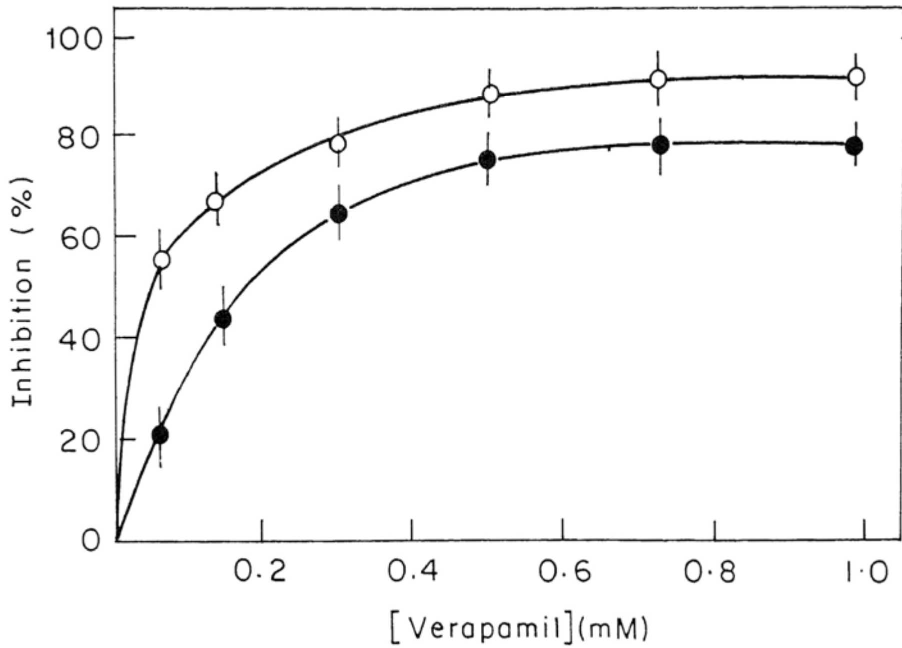

Figure 2. Effect of verapamil on Mg^{2+}, Ca^{2+}-ATPase (○) and Ca^{2+}-ATPase (●) activities. The detergent-treated purified membrane fraction was incubated with different concentrations of verapamil for 20 min at 37° C, and enzyme activities were measured as described in §2 ($n = 4$).

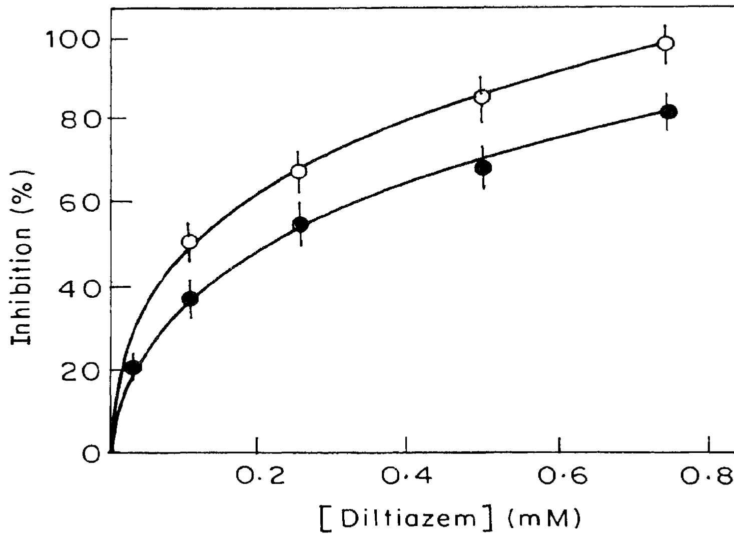

Figure 3. Effect of different concentrations of diltiazem on Mg^{2+} Ca^{2+}-ATPase (O) and Ca2+-ATPase (●) activities in purified membranes. The other conditions were as in figure 2 ($n = 5$).

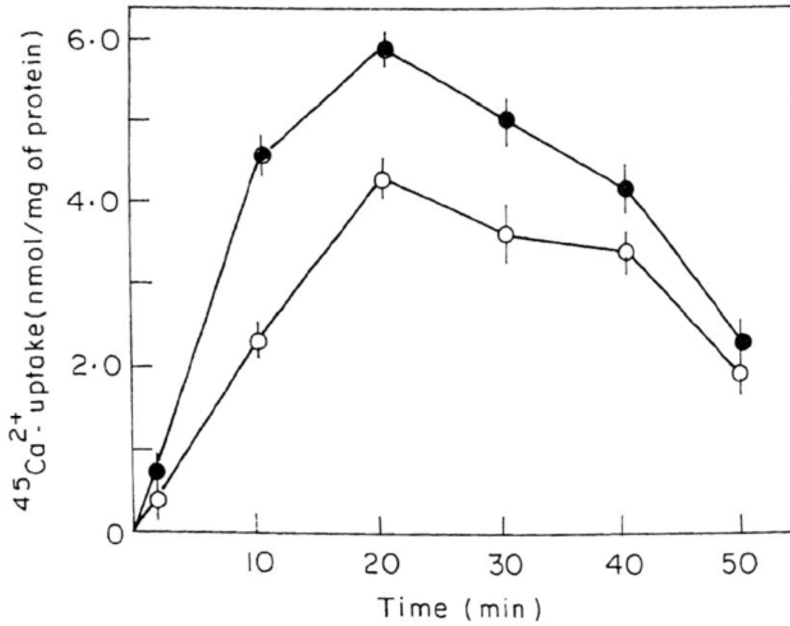

Figure 4. Time course of $^{45}Ca^{2+}$ uptake by -ATPase enriched detergent-untreated microsomal membrane vesicles. Mg^{2+} , Ca^{2+} (○) and Ca $^{2+}$ -ATPase (●). The details are given in §2 (*n* = 4).

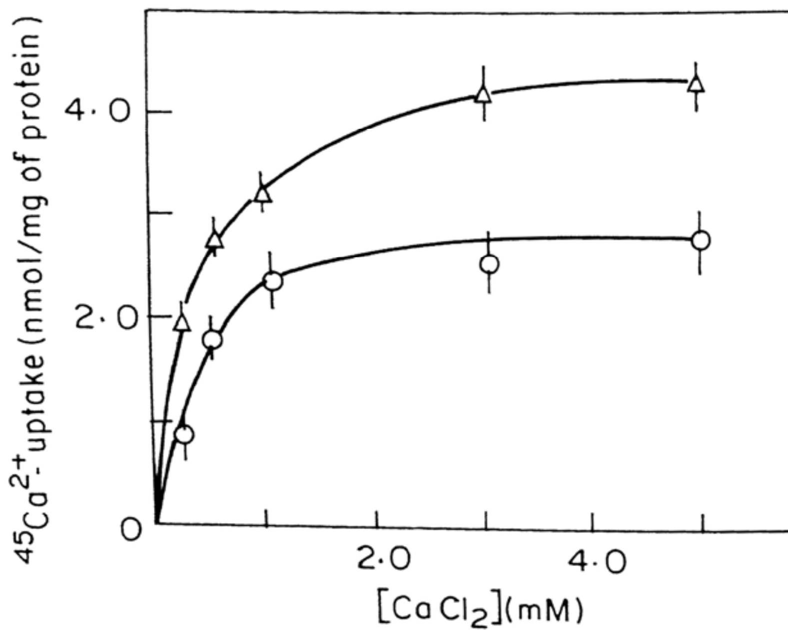

Figure 5. $^{45}Ca^{2+}$-uptake by Mg^{2+} , Ca^{2+} (O) and Ca^{2+} (Δ) ATPase in microsomal membrane vesicles at different calcium concentrations. The uptake was measured at 20 min as described in §2 (n=4).

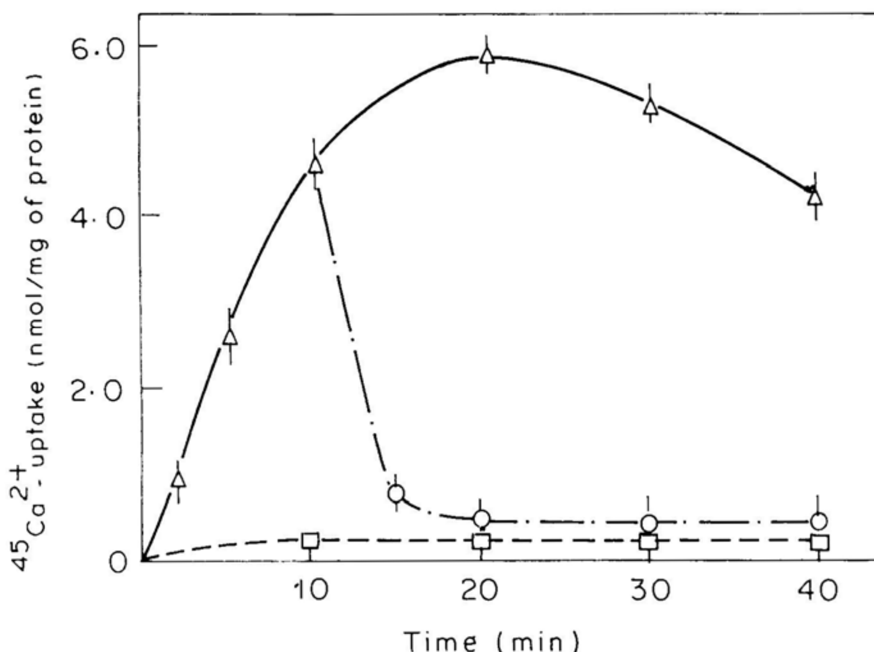

Figure 6. $^{45}Ca^{2+}$ uptake by Ca^{2+}-ATPase in the microsomal membranes of goat spermatozoa in complete (Δ) and ATP-depleted (□)media, and the effect of A23187(○). A23187 (1 μM) was added after 10 min of Ca^{2+} uptake in the complete medium and uptake was monitored in the presence and absence of ionophore for 40 min (n = 3).

myometrium (Enyedi *et al* 19881 and human liver (Spamer *et al* 1987). In bull spermatozoa calcium transport activity is regulated by the Mg^{2+}, Ca^{2+}-ATPase located in the outer acrosomal membrane (Breitbart and Rubinstein 1983). The ATPases-play a vital role in regulation of sperm motility through the maintenance of intracellular concentration of calcium (Breitbart *et al* 1985). In our previous studies we have found that the Ca^{2+} -ATPase activity is inhibited, like the Mg^{2+} , Ca^{2+}-ATPase. by two contraceptive drugs, chlorpromazine (Mazumder *et al* 1990. 1991) and gossypol (Mazumder *et al* 1991). This suggests a possible role for Ca 2+-ATPase in fertility regulation.

The Ca^{2+}-ATPase is found to have a low affinity for Ca^{2+} (figure 1). Quist and Roufogalis (1975) showed that Ca^{2+}-ATPase with high affinity for Ca^{2+} can easily be removed from membranes and suggested that the low-affinity site plays the basic role in active calcium transport. However, how the enzyme under study acts through the low-affinity site remains to be investigated.

Calcium-channel inhibitors have been reported to inhibit Mg^{2+} ' Ca^{2+} -ATPase (Kim and Raess 1988: Atlas and Adler 1981: Lee and Tsien 1983). In the present study we have shown that they inhibit both Ca^{2+}-ATPase and Mg^{2+}, Ca^{2+}-ATPase activities in a concentration-dependent manner (figures 2,3). Since calcium has been shown to compete with these inhibitors (Lee and Tsien 1983), we incubated membranes with the inhibitors in the absence of calcium and then

initiated the ATPase reaction with ATP and Ca^{2+} or Mg^{2+} +Ca^{2+} The Mg^{2+}, Ca^{2+}-ATPase is more sensitive to verapamil (figure 2) and diltiazem (figure 3). The inhibition of Ca^{2+} -ATPase activity by verapamil and diltiazem leads us to believe that the enzyme is involved in Ca^{2+} transport, like Mg^{2+} , Ca^{2+}-ATPase (Atlas and Adler 1981). Higher I_{50} values of calcium antagonists for Mg^{2+} , Ca^{2+}-ATPase have been reported previously by Atlas and Adler (1981) also. Concentrations of the inhibitors beyond the highest shown here could not be used because of the appearance of a precipitate.

The $^{45}Ca^{2+}$ -uptake study reveals that uptake is higher with Ca^{2+}-ATPase than with Mg^{2+}, Ca^{2+} -ATPase (figure 4). This is also consistent with our earlier data that the ATPase activity of Ca^{2+}-ATPase is higher (Sikdar *et al* 1991; Sikdar 1991). Mg^{2+}-independent Ca^{2+} uptake and Ca^{2+} -ATPase activities have been reported in smooth-muscle membrane vesicles (Thorens 1979).

In the absence of ATP no calcium uptake takes place (figure 6), suggesting that Ca^{2+} uptake by Ca^{2+} -ATPase is energy-dependent. Accumulated Ca^{2+} is rapidly and completely released by the calcium ionophore A23187. 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). The apparent reduction of Ca^{2+} uptake seen in figures 5 and 6 over longer periods could be due to loss of vesicular structure of the membranes.

In conclusion we suggest that both Ca^{2+}-ATPase and Mg^{2+},Ca^{2+}-ATPase have calcium transport activity in goat spermatozoa. This activity is essential to maintain the required levels of intracellular calcium and to regulate sperm motility and the fertilization process. However, we still do not know whether these are two separate enzymes or only one with different catalytic sites. Since there are similarities in properties between the two activities (Sikdar *et al* 1991), it is conceivable that they may be. the same enzyme but with distinct, different catalytic sites. Further work with reconstituted enzyme is in progress in our laboratory to explore this question.

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