Biochimica et Biophysica Acta, 728 (1983) 349–355
Elsevier Biomedical Press

BBA 71548

CALCIUM TRANSPORT AND Ca^{2+}-ATPase ACTIVITY IN RAM SPERMATOZOA PLASMA MEMBRANE VESICLES

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(Received August 30th, 1982)

Key words: $(Ca^{2+} + Mg^{2+})$-ATPase; Ca^{2+} transport; (Ram spermatozoa)

Plasma membrane vesicles, isolated from ejaculated ram sperm, were found to contain Ca^{2+}-activated Mg^{2+}-ATPase and Ca^{2+} transport activities. Membrane vesicles that were exposed to oxalate as a Ca^{2+}-trapping agent accumulated Ca^{2+} in the presence of Mg^{2+} and ATP. The V_{max} for Ca^{2+} uptake was 33 nmol/mg protein per h, and the K_m values for Ca^{2+} and ATP were 2.5 μM and 45 μM, respectively. 1 μM of the Ca^{2+} ionophore A23187, added initially, completely inhibited net Ca^{2+} uptake and, if added later, caused the release of Ca^{2+} previously accumulated. A Ca^{2+}-activated ATPase was present in the same membrane vesicles which had a V_{max} of 1.5 μmol/mg protein per h at free Ca^{2+} concentration of 10 μM. This Ca^{2+}-ATPase had K_m values of 4.5 μM and 110 μM for Ca^{2+} and ATP, respectively. This kinetic parameter was similar to that observed for uptake of Ca^{2+} by the vesicles. The Ca^{2+}-ATPase activity was insensitive to ouabain. Both Ca^{2+} transport and Ca^{2+}-ATPase activity were inhibited by the flavonoid quercetin. Thus, ram spermatozoa plasma membranes have both a Ca^{2+} transport activity and a Ca^{2+}-stimulated ATPase activity with similar substrate affinities and specificities and similar sensitivity to quercetin.

Introduction

The optimum amount of extracellular calcium essential for motile function in spermatozoa may vary from species to species. In the 1–10 mM range prevailing both in sea water and in mammalian tissue fluids, a steep, inward concentration gradient is established, since the intracellular free Ca^{2+} content is about 0.1 to 1 μmol/liter cell water.

Maintenance of a concentration difference of this magnitude would seem to operate through several possible mechanisms. One of the most known mechanisms includes the calcium pump or the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane [1,2]. An alternative mechanism, based upon coupling between Ca^{2+} efflux and Na^+ influx, has been reported in various tissues including brain and heart [3,4]. However, the most extensively studied Ca^{2+}-pump has been that present in red blood cell plasma membranes [2,5]. In mammalian spermatozoa, the mechanism regulating intracellular Ca^{2+} concentration has not been fully characterized. Bradley and Forrestor have shown the presence of ATP-requiring Ca^{2+} pump [6] and Na^+/Ca^{2+} antiporter [7] in plasma vesicles isolated from ram spermatozoa. In order to gain more specific information concerning the basic properties of the $(Ca^{2+} + Mg^{2+})$-ATPase, we have studied purified ram sperm plasma membranes. The kinetic parameters and substrate requirements of Ca^{2+} transport and Ca^{2+}-stimulated ATPase activities of the plasma membranes are described.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid.

Materials and Methods

Preparation of sperm plasma membranes. Semen was collected from rams by electric induction. The fresh semen was immediately transferred to ice. The sperm cells were pelleted by centrifugation at $1500 \times g$ for 10 min; then the cells were washed four times in buffer comprising 0.25 M sucrose/10 mM histidine (pH 7.4). The washed cells were resuspended in hypotonic medium (10 mM histidine (pH 7.4)/0.5 mM EDTA) and disrupted by ultraturrax using the Janke and Kunkel K6 IKA WERK Typ. TP18-10, in the following way: 10 s low rate, 3 s high, 7 s low, 3 s high and 7 s low rate. Low and high rates represent 3000 and 14000 rpm, respectively. The suspension was centrifuged at $3000 \times g$ for 10 min, and the supernatant was removed and centrifuged at $6000 \times g$ for 10 min. The supernatant was removed and centrifuged at $35000 \times g$ for 30 min; then the pellet was resuspended in the hypotonic medium. The suspension was layered on a discontinuous sucrose gradient composed of 0.5, 1.0 and 1.5 M sucrose solutions prepared in 10 mM histidine (pH 7.4). The gradient was centrifuged at 30000 rpm for 18 h using a SW 41 rotor, in Spinco (Beckman) ultracentrifuge. The membrane fraction located just above the 1.5 M sucrose layer was removed and diluted with 10 mM histidine (pH 7.4)/0.1 mM EDTA at 4°C. The protein concentration was estimated by the method of Lowry et al. [8] using bovine serum albumin as standard of reference. The membranes were stored at −20°C for up to 2 weeks prior to analysis. These membranes showed an 15-fold enrichment of the plasma membrane marker ($Na^+ + K^+$)ATPase and less than 4% of the cytochrome *c* oxidase specific activity found in whole cell homogenates. When examined by transmission electron microscopy, the membranes were vesicular and mitochondria were not identified.

ATPase activity. The ATPase activity was measured in a 1 ml medium containing 18 mM histidine/18 mM imidazole buffer (pH 6.8), 0.1 M KCl, 3 mM $MgCl_2$, 5 mM sodium-oxalate, 0.18 mM $CaCl_2$ (10 μM free Ca), 0.2 mM EGTA, 0.1 mM ouabain and 20–30 μg plasma membrane protein. After 5 min preincubation at 37°C, the reaction was started by the addition of Na-ATP to achieve a final concentration of 2 mM ATP. The reaction was terminated after 30 min at 37°C, with 1 ml of 14% trichloroacetic acid. Inorganic phosphate was ordinarily determined by extraction of the phosphomolybdate complex into butyl acetate according to the method of Sanui [9]. The enzyme determinations were carried out in duplicate: experiments were repeated in several preparations of plasma membranes. All ATPase values were corrected for P_i release measured in the absence of plasma membranes. Ca^{2+}-activated Mg^{2+}-ATPase was calculated as the increment in P_i above the Mg^{2+}-ATPase activity upon the addition of Ca^{2+} in the presence of 5 mM Mg^{2+}. Maximal Ca^{2+}-ATPase activity was observed at a total Ca^{2+} concentration of 160 μM, which provided 4.6 μM free Ca^{2+} in this assay. The free Ca^{2+} concentrations were calculated according to Schatzmann [10].

The ($Na^+ + K^+$)-ATPase activity was measured as the ouabain-sensitive portion of the ATPase in the presence of 3 mM $MgCl_2$/130 mM NaCl/20 mM KCl/3 mM Na-ATP/18 mM histidine/18 mM imidazole buffer (pH 6.8). This was calculated as the difference in P_i release in the absence and presence of 0.1 mM ouabain.

Calcium uptake. Ca^{2+} uptake by spermatozoa plasma membrane vesicles was measured as described for the Ca^{2+}-activated ATPase assay except that the final volume of incubation was 0.2 ml. To this incubation medium was added 1 μCi of ^{45}Ca. The reaction was started by the addition of plasma membranes after preincubation for 5 min at 37°C. The reaction mixture was incubated at 37°C. At appropriate time intervals, 180 μl samples were removed and vacuum-filtered on 0.45 μm pore Millipore filters that had been prewashed with water. The membrane vesicles trapped on the filter were washed three times with 5 ml cold water. The dry filters were placed in scintillation vials with 7 ml Insta-Gel II (Packard) solution for measurement of the β radioactivity. Ca^{2+} uptake was expressed in nmol Ca^{2+}/mg protein per h. Uptake values were corrected for radioactivity bound to the filter when an identical reaction mixture without plasma membranes was filtered. This accounted for less than 10% of the total measured radioactivity. ATP-dependent uptake was determined from the difference in radioactivity bound to the filter in the presence and absence of ATP.

Electron microscopy. Freshly isolated membrane vesicles were fixed in cold 1% glutaraldehyde in phosphate buffer (pH 7.4), then washed three times with the phosphate buffer and post-fixed in osmium tetroxide. The membranes were dehydrated in graded alcohols and embedded in Epon 812. Thin sections were cut with an LKB ultratome III stained with uranyl acetate/lead citrate and examined with a JEOL 100-C transmission electron microscope.

Materials: ATP (disodium), quercetin and EGTA were purchased from Sigma Chemical Co., St. Louis, MO, and ouabain from Fluka. Ionophore A23187 was purchased from Eli Lilly and the stock solution was prepared in ethanol. ^{45}Ca was purchased from New England Nuclear.

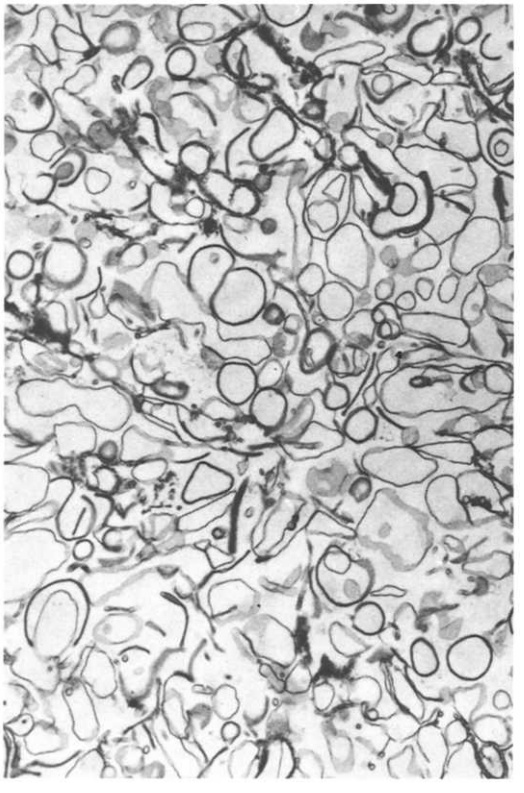

Fig. 1. Electron micrograph of thin section of the plasma membrane vesicles: Plasma membranes isolated by sucrose gradient fractionation were concentrated to a pellet by centrifugation, fixed and sliced as described in Materials and Methods. The majority of the vesicles appeared spherical with diameters from 2000 to 5000 Å. Magnification ×13200.

Results

Electron microscopic observations

Fig. 1 shows the thin section electronmicrograph of a pellet of the spermatozoa membranes. It can be seen that this membrane fraction is composed of relatively homogeneous vesicular structures with sizes ranging from 2000 to 5000 Å in diameter. The preparation is free of mitochondria. We also examined the sonicated cells in the transmission electron microscope. In these cells, the mitochondria were undamaged and were in their original location.

Calcium uptake by membrane vesicles

The time course of Ca^{2+} uptake by spermatozoa plasma membrane vesicles is shown in Fig. 2. In the absence of ATP, approximately 0.3 nmol Ca/mg protein bound to the plasma mem-

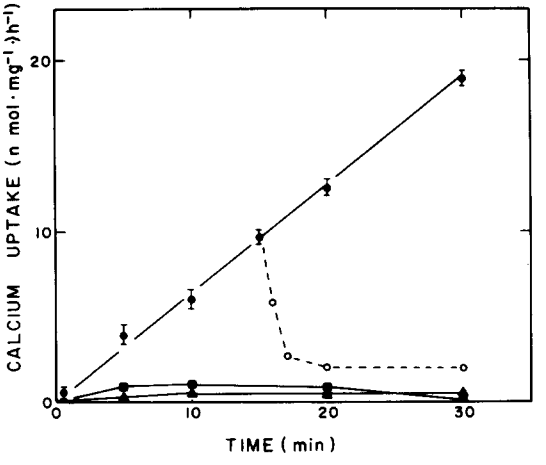

Fig. 2. Calcium uptake by plasma membrane vesicles. Ca^{2+} uptake activity was assayed in the medium described in Materials and Methods. Each point represents the mean ± S.E. of duplicate sample determinations from three membrane preparations. The S.E. was less than 0.2 nmol/mg of protein on all points without bars. The following conditions are shown: ●, optimal medium, ▲, Mg^{2+} or ATP omitted; ■, 1 μM A23187 added from the start; ○, A23187 added at 15 min. The A23187 was dissolved in ethanol, which had no effect on the measured uptake.

branes within 10 s. No significant additional uptake occurred in the absence of added ATP. No ATP-dependent uptake occurred when Mg^{2+} was omitted from the assay. In the presence of ATP and Mg^{2+}, Ca^{2+} uptake proceeded in a linear fashion for about 30 min. When 1.0 μM A23187 was present, no Ca^{2+} accumulation was observed. The addition of A23187 after 15 min of incubation caused the release of accumulated Ca^{2+}. The addition of 0.1 mM ouabain had no effect on the Ca^{2+} uptake activity.

Calcium uptake-kinetic features

The initial velocity of ATP-dependent Ca^{2+} uptake by spermatozoa plasma membrane vesicles was measured as a function of the free Ca^{2+} concentration in the medium (Fig. 3). The Ca^{2+} uptake reached a maximum at a free Ca^{2+} concentration of approx. 6 μM. A double-reciprocal plot of the data was used to calculate a maximal velocity (V_{max}) of 33 nmol Ca/mg protein per h and a K_m for free Ca^{2+} of 2.5 μM.

Fig. 4 shows the calcium uptake rate by membrane vesicles as a function of the ATP concentration. Calcium uptake reached maximum values at 0.2 mM ATP and the apparent K_m for ATP was 45 μM.

Calcium-ATPase activity by membrane vesicles

Ouabain-sensitive ($Na^+ + K^+$)-ATPase was assayed to confirm purity and to insure enzyme activity and integrity of the isolated plasma membranes. The mean ouabain-sensitive ATPase activity was 4.0 μmol P_i/mg protein per h in all membrane preparations. This represented a greater than 15-fold increase in specific activity over that measured in unfractionated sonicated cells.

The total Mg^{2+}-dependent, Ca^{2+}-activated Mg^{2+}-ATPase activities were linear with respect to time up to 60 min. Maximal activation of the Ca^{2+}-ATPase was measured at a free Ca^{2+} concentration of 10 μM (Fig. 5). Kinetic analysis revealed a K_m for Ca of 4.5 μM and a V_{max} of 1.6 μmol P_i/mg protein per h. Fig. 6 shows the Ca^{2+}-ATPase activity assayed at different ATP concentrations. Total Mg^{2+} was kept constant at 5

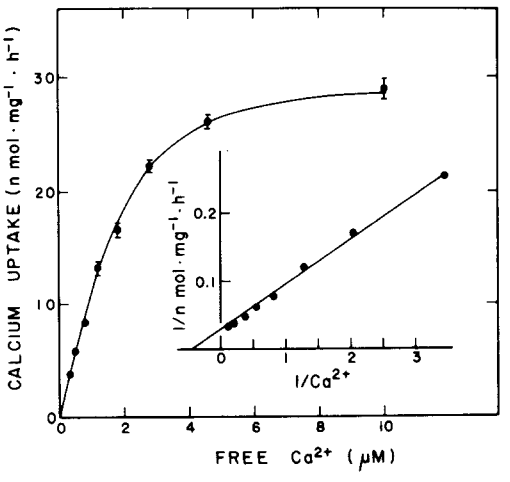

Fig. 3. Calcium uptake by plasma membrane vesicles: dependence on free Ca^{2+} concentration. Uptake was assayed after 20 min of incubation as described in Materials and Methods. The ordinate represents ATP-dependent Ca^{2+} uptake, i.e., the difference in Ca^{2+} uptake in the presence and absence of ATP. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations. The inset shows the double-reciprocal plot, which yields an apparent K_m of 2.5 μM.

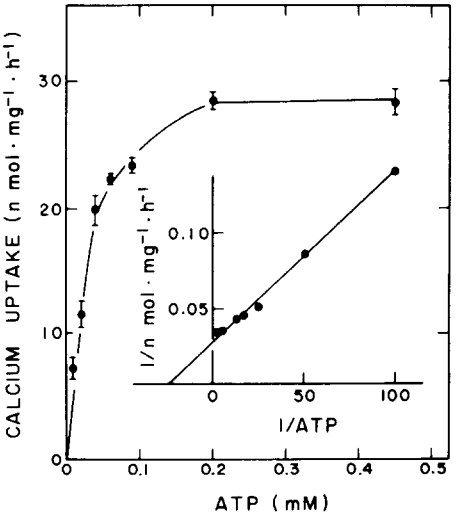

Fig. 4. Calcium uptake by plasma membrane vesicles: dependence on ATP concentration. Uptake was assayed as described in Methods, except that ATP concentration was varied as shown. The free Ca^{2+} concentration was maintained at 10 μM. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations. The inset shows the double-reciprocal plot which yields an apparent K_m of 45 μM.

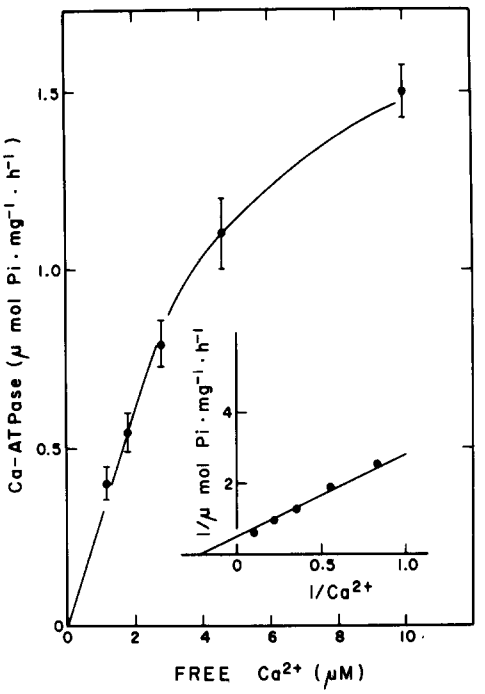

Fig. 5. Calcium-activated ATPase activity: dependence on free calcium concentration. ATPase activity was assayed after 30 min as described in Methods. The ordinate represents net Ca^{2+}-activated ATPase, i.e., the difference between ATPase in the presence and absence of Ca^{2+}. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations. The inset shows the double-reciprocal plot, which yields an apparent K_m of 4.5 μM.

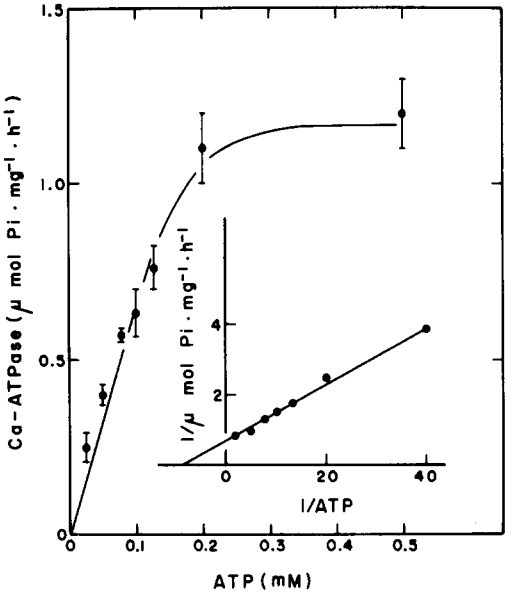

Fig. 6. Calcium-activated ATPase activity: dependence on ATP concentration. ATPase activity was assayed as described in Materials and Methods, except that ATP concentration was varied as shown. The free Ca^{2+} concentration was maintained at 10 μM. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations. The inset shows the double-reciprocal plot, which yields an apparent K_M of 110 μM.

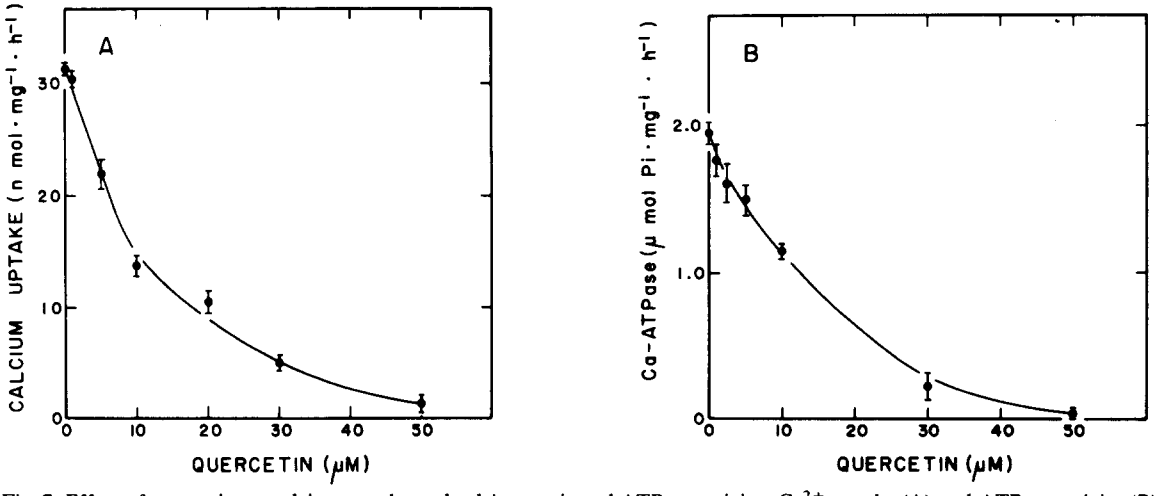

Fig. 7. Effect of quercetin on calcium uptake and calcium-activated ATPase activity: Ca^{2+} uptake (A) and ATPase activity (B) were assayed for 30 min (37°C) as described in Materials and Methods, except that various concentrations of quercetin were added to the incubation medium. The quercetin interferes in the ATPase assay; therefore, a blank without membranes was run in parallel for each quercetin concentration. The quercetin was dissolved in ethanol, and the final ethanol concentration in control and quercetin-containing samples never exceeded 0.5%. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations.

mM. The ATP concentration required for maximal Ca^{2+}-ATPase activity was approx. 0.2 mM. The K_m for ATP was 110 μM and the V_{max} was 1.4 μmol of P_i/mg protein per h.

When Mg^{2+} was omitted from the medium the ATPase could be activated by Ca^{2+} concentrations at the mM range.

Effect of quercetin

In order to characterize the Ca^{2+} transport and Ca^{2+}-activated ATP hydrolysis further, we tested the effects of quercetin on the membrane vesicles (Fig. 7). The flavonoid, quercetin, has been reported to inhibit $(Na^+ + K^+)$-ATPase from the electric organ of eel and kidney [11], the Mg^{2+}-ATPase of membrane vesicles from ram semen [12] and the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcoplasmic reticulum [13] and of red blood cells [14]. In the present study, it is shown in Fig. 7 that quercetin, at low concentrations, inhibits the ATP dependent calcium uptake and Ca^{2+}-ATPase activity. At quercetin concentrations above 50 μM, the activity of Mg^{2+}-ATPase (in absence of Ca^{2+}) was inhibited as well.

Discussion

We have described a spermatozoa plasma membrane ATP-dependent Ca^{2+} transport system with properties similar to the Ca^{2+} transport system described in plasma membranes of erythrocytes [15] macrophages [16], lymphocytes [17] and synaptic plasma membrane vesicles [18,19]. These properties include ATP and Mg^{2+} dependence, insensitivity to ouabain and a high affinity for Ca.

In order to study the energy transduction system responsible for net Ca^{2+} transport across the plasma membrane, presumably against an electrochemical gradient, we evaluated the Ca^{2+}-activated ATPase activity. This ATPase has characteristics similar to the Ca^{2+}-ATPase in the plasma membranes of erythrocytes [15] adipocytes [20] macrophages [21] neutrophils [22] and lymphocytes [17], all of which are thought to be Ca^{2+} transport systems. The spermatozoa ATPase has a high affinity for Ca^{2+} and is insensitive to ouabain. There are certain similarities between the ATPase and the transport activities which support the hypothesis that they are expressions of the same enzyme. The Ca^{2+} affinities (4.5 μM for ATPase and 2.5 μM for transport) are reasonably close in light of the differences in the experimental conditions and probably represent the same Ca^{2+}-binding site. The ATP activation curves for Ca^{2+} transport and Ca^{2+}-ATPase are also similar when measured under similar conditions with ATP affinities of 45 and 110 μM, respectively.

The maximal velocities of the Ca^{2+} uptake and Ca^{2+}-ATPase activities (33 nmol Ca/mg protein per h and 1.5 μmol P_i/mg protein per h, respectively) yield a very low ratio of Ca^{2+} transported per ATP hydrolyzed. The two assay systems differ, however. The leakiness of the vesicles to Ca^{2+}, the presence of right-side-out vesicles which may hydrolyze ATP but do not accumulate Ca^{2+}, and the inefficiency of membrane recovery during filtration could all contribute to a significant underestimation of Ca^{2+} translocation.

The Ca^{2+} transport activity was Mg^{2+}-dependent. When Mg^{2+} was omitted from the assay medium, no ATP-dependent Ca^{2+} uptake was observed. In contrast, when Mg^{2+} was removed from the ATPase assay, the enzyme could be activated by Ca^{2+}. Similarly, when Ca^{2+} was removed, Mg^{2+} alone stimulated the ATPase. The precise role of Mg^{2+} in Ca^{2+}-activated ATPase activity of erythrocyte membranes is also not clearly understood, since studies of the purified ATPase molecule have yielded results different from those found in intact membranes [23]. Taken together, the transport and ATPase data in sperm cells suggest that the nonselective Ca^{2+}- or Mg^{2+}-activated ATPase activities represent nonspecific divalent cation stimulation of phosphohydrolysis and that both ions are required for the Ca^{2+} transport ATPase.

The Ca^{2+}-ionophore A23187 inhibited the net Ca^{2+} uptake by the vesicles. This effect is consistent with the action of the ionophore in rendering the plasma membrane vesicles permeable to Ca^{2+}. With A23187, Ca^{2+} pumped into the vesicles leaks out rapidly enough to prevent the increase in intravesicular Ca^{2+} concentration necessary for calcium oxalate precipitation.

Quercetin has been reported to be an inhibitor of the Mg^{2+}- and Ca^{2+}-dependent ATPase connected with Ca^{2+} transport in erythrocytes [14] and in sarcoplasmic reticulum [13,27]. A relevant

finding in our study, therefore, was the inhibitory action of quercertin in the observed Ca^{2+}-ATPase, favoring the possibility that the ATPase is involved in Ca^{2+} translocation.

The Ca^{2+} transport system described above may be essential to Ca^{2+} homeostasis of sperm cells when plasma membrane permeability is altered during their maturation and their coincidental acquisition of motility. Furthermore, the pattern of motility also undergoes changes with advancing maturation, from non-propagated, flagellar twitches through oscillations to circular motions. Mature sperm cells exhibit progressive, more-and-more straight-line movement.

Sperm motility is influenced by deviations from normal values of extracellular calcium concentration, i.e., low concentrations support optimum motility, high concentrations causing aberrant swimming behavior, and chelation of environmental calcium completely suppressing movement of intact cells [24]. Small amounts of Ca^{2+} are essential for demembranated cells to retain their contractile power [25]. When intact spermatozoa are treated with the Ca^{2+}-ionophore A23187 in calcium-containing media, the resulting increase uptake of calcium into the cells causes changes in both motility and metabolism [26]. The Ca^{2+} transport system characterized here is presumably essential to maintain low level of intracellular calcium concentration.

Acknowledgement

We thank Mrs. B. Lederhendler for the skillful typing of this manuscript.

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