Biochimica et Biophysica Acta, 732 (1983) 464–468
Elsevier

BBA Report

BBA 70102

CALCIUM TRANSPORT BY BULL SPERMATOZOA PLASMA MEMBRANES

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(Received February 3rd, 1983)

Key words: Ca^{2+} transport; Plasma membrane; (Bull spermatozoa)

Plasma membrane isolated from frozen ejaculated bull spermatozoa were found to contain calcium transport activity. Thin-section electronmicrography of these membranes revealed relatively homogeneous vesicular membranes with sizes ranging from 2000 to 6000 Å in diameter. Membrane vesicles that were exposed to oxalate as a calcium-trapping agent accumulated Ca^{2+} in the presence of Mg^{2+} and ATP. One μM of the calcium-ionophore A23187, added initially, completely inhibited net Ca^{2+} uptake and, if added later, caused the release of Ca^{2+} accumulated previously. An Arrhemius plot for the rate of Ca^{2+} uptake revealed a break at 32–33°C, with E_a of 4.4 kcal/mol above the break and 32.2 kcal/mol below. The Ca^{2+} uptake was inhibited by low concentrations of quercetin, which is known to be an inhibitor of $(Ca^{2+} + Mg^{2+})$-ATPase in many systems.

The ability of mature mammalian spermatozoa to maintain a coordinated and forward motility is dependent upon intracellular free Ca^{2+} [1]. Control of intracellular free Ca^{2+} concentration thus appears to be crucial for the maintenance of normal cell function. In mammalian spermatozoa, the mechanism regulating intracellular Ca^{2+} concentration has not been fully characterized. Bradley and Forrester have shown the presence of ATP-requiring Ca^{2+} pump [2] and Na^+/Ca^{2+} antiporter [3] in ram spermatozoa plasma membranes. The plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase of ram spermatozoa has been characterized by us recently [4]. In the present representation we have characterized the basic properties of the ATP-dependent Ca^{2+}-pump of the plasma membrane from frozen bull spermatozoa.

Frozen bull sperm cells were thawed at 37°C by taking one frozen capsule ($5 \cdot 10^7$ cells) into 0.875 ml of medium comprising 155 mM NaCl/10 mM histidine (pH 7.4). The cells were transferred into ice, and washed from the diluent by three centrifugations at $3000 \times g$, 4°C for 10 min. The washed cells were resuspended in hypotonic medium comprising 10 mM histidine (pH 7.4)/0.5 mM EDTA. The cells were disrupted by ultraturrax and the plasma membranes were isolated by differential centrifugation and sucrose gradient, as described in our recent paper [4]. These membranes showed a 45-fold enrichment of the plasma membrane marker $(Na^+ + K^+)$-ATPase and no inhibition of Ca^{2+} uptake was found by azide, which is known to inhibit Ca^{2+} uptake by mitochondria. When examined by transmission electron microscopy, the membranes were vesicular and mitochondria were not identified.

The $(Na^+ + K^+)$-ATPase activity was measured as the ouabain-sensitive portion of the ATPase. The methods for Ca^{2+} uptake and electron microscopy of the spermatozoa plasma membrane vesicles were described in our recent paper [4]. The frozen cells were generously supplied by D. Kalay and M.

Abbrevation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

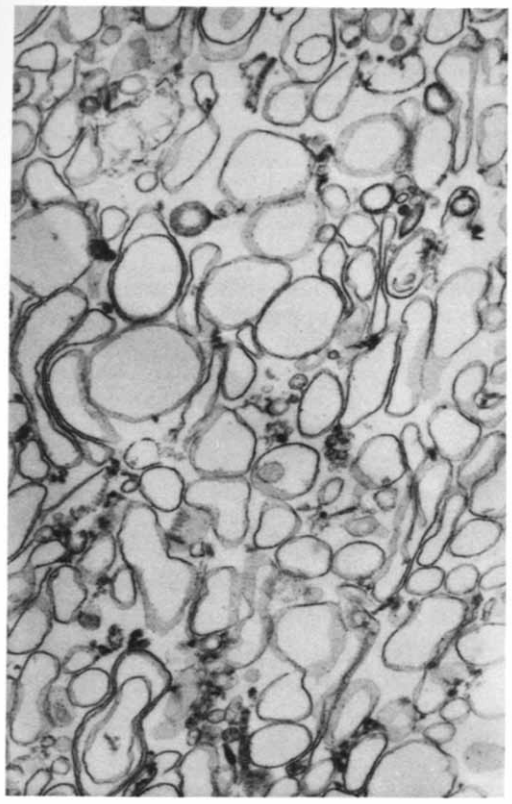

Fig. 1. Electron micrograph of thin section of the plasma membrane vesicles. Plasma membrane isolated by sucrose gradient fractionation were concentration to a pellet by centrifugation, fixed and sliced. The majority of the vesicles appeared spherical, with a diameter from 2000 to 6000 Å. Magnification ×16600.

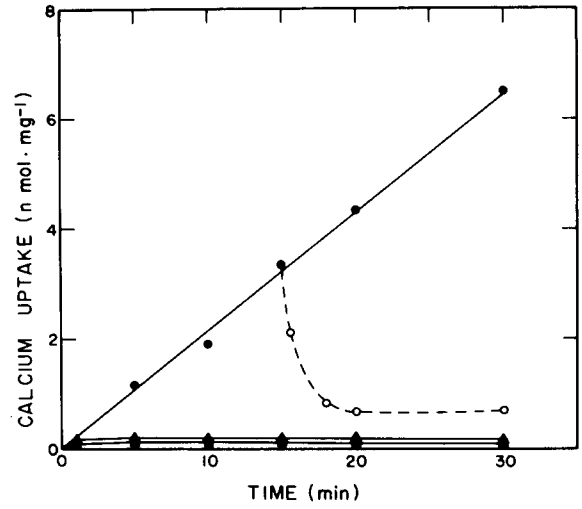

Fig. 2. Calcium uptake by plasma membrane vesicles. Calcium uptake activity was assayed in the medium as described [4]. Each point represents the mean from three membrane preparations. The following conditions are shown: ●, optimal medium; ▲, Mg^{2+} on ATP omitted; ■, 1 μM ionophore A23187 added from the start; ○, ionophore A23187 added at 15 min. The ionophore A23187 was dissolved in ethanol, which had no effect on measured uptake at the concentration of ethanol used in the experiment (0.1% ethanol).

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The thin-section electronmicrograph of a pellet of the spermatozoa membranes is shown in Fig. 1. It can be seen that this membrane fraction is composed of relatively homogeneous vesicular structures with sizes ranging from 2000 to 6000 Å in diameter. The preparation is free of mitchondria. We also examined the sonicated cells in the transmission electron microscope. In these cells, the mitochondria were undamaged and were located in their original location.

Fig. 2 shows the time-course of calcium uptake by spermatozoa plasma membrane vesicles. In the absence of ATP, approx. 0.2 nmol Ca^{2+} per mg protein bound to the plasma membranes within the first minute of incubation, and thereafter binding did not increase above this basal level. 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 ionophore A23187 was present, no Ca^{2+} accumulation was observed. The addition of ionophore A23187 after 15 min of incubation caused the release of accumulated Ca^{2+}. This result suggests that the accumulation of Ca^{2+} occurred against an electrochemical gradient [5]. UTP and CTP did not support Ca^{2+} uptake. A very small Ca^{2+} uptake was found with GTP, and ADP was considerably less effective than ATP (Table I). Sodium azide, at concentrations that almost totally inhibit calcium uptake by mitochondria from several tissues [6,7], had no effect on the uptake of calcium by spermatozoa plasma vesicles. Oligomycin and ouabain, which are known to affect Ca^{2+} transport in mitochondria and plasma membranes from other cells, did not significantly affect that Ca^{2+} uptake in our preparations.

TABLE I

CALCIUM UPTAKE BY PLASMA MEMBRANE VESICLES: NUCLEOTIDE REQUIREMENTS

The incubation medium described in the text, except for the nucleotides which were those shown at a concentration of 2 mM.

Nucleotide	Calcium uptake (nmol/mg per h)
Mg-ATP	13.25
Mg-ADP	8.01
Mg-GTP	2.35
Mg-UTP	1.61
Mg-CTP	0.05

The initial velocity of ATP-dependent Ca^{2+} uptake by the plasma membrane vesicles was measured as a function of the free Ca^{2+} concentration in the medium. Maximal activation of Ca^{2+} uptake was measured at a free Ca^{2+} concentration of 10 μM. Kinetic analysis revealed a K_m for Ca^{2+} of 2.5 μM and a V_{max} of 14.3 nmol Ca^{2+}/mg protein per h. Calcium-uptake reached maximum values at 1.0 mM ATP and the apparent K_m for ATP was 160 μM.

Under conditions that promoted maximal calcium uptake, the plasma membrane vesicles had a powerful Mg^{2+}-ATPase activity (7.8 μmol/mg per h) in the absence of added calcium, and calcium stimulation of ATP hydrolysis could not be detected with consistency.

Fig. 3 shows an Arrhenius plot for the rate of Ca^{2+} uptake. There is a clear break at about 32–33°C, and a decrease in uptake at 42°C. The energy of activation was calculated to be 4.42 kcal/mol above the break and 32.22 kcal/mol below. The break in the Arrhenius plot has been observed in many other enzyme systems [8–10] and has frequently been attributed to some phase transition of the lipid membrane components.

In order to characterize the Ca^{2+} transport further, we tested the effect of quercetin on the membrane vesicles (Fig. 4). It is shown that quercetin, at low concentrations, inhibits the ATP-dependent calcium uptake.

The calcium transport system of sperm plasma vesicles shares several properties with intracellular calcium transport systems, such as mitochondria

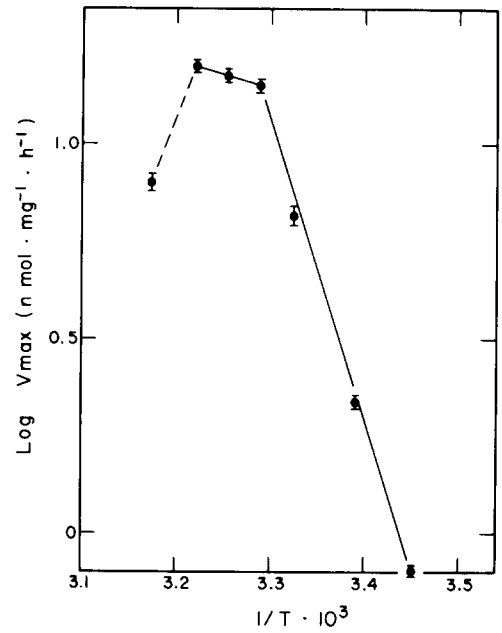

Fig. 3. Calcium uptake by plasma membrane vesicles. Arrhenius plot. Ca-uptake was determined at temperatures of 17–42°C using an incubation medium, as described [4]. Each point represents the mean ± S.E. of duplicate sample determinations in three membrane preparations.

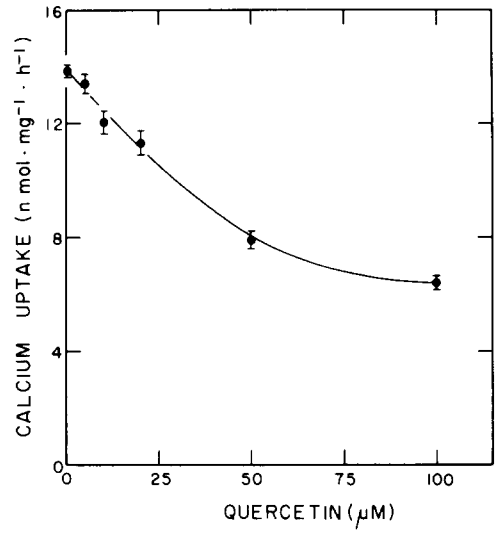

Fig. 4. Calcium uptake by plasma membrane vesicles. Effect of quercetin. Uptake was assayed as described [4], except that various concentrations of quercetin were added to the incubation medium. The quercetin was dissolved in dimethylsulfoxide. Each point represents the mean ± S.E. of duplicate sample determinations in two membrane preparations.

[11] and sarcoplasmic reticulum [12], including those of red cell membranes [13]. The spermatozoa system has a requirement for the presence of Mg^{2+}-ATP, although lower rates of calcium uptake were also detected in the presence of Mg^{2+}-ADP. Similar findings in macrophage [14] and renal plasma membrane preparations have been attributed to myokinase activity [7]. The calcium transport activity is enhanced by the permeant anion oxalate, and occurs against an electrochemical gradient, as demonstrated by the ionophore experiments. The calcium 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 ionophore 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.

The affinity of the calcium pump to free Ca^{2+} (K_m for free calcium, 2.5 μM) is similar to that found for ram spermatozoa plasma membranes [4], but is considerably higher than that described for microsomes and mitochondria [11,15]. This high affinity would seem to make the system in the vesicles fully operational under free calcium concentrations estimated to be in the cytoplasm under normal conditions. The affinity for ATP (K_m 160 μM) is lower in comparison to the Ca^{2+} pump in ram spermatozoa plasma membrane [4], but is considerably higher than that described in macrophage plasma membranes [14].

Due to the presence of a potent Mg^{2+}-ATPase activity associated with the vesicles, a parallel hydrolysis of ATP coupled to transport ((Ca^{2+} + Mg^{2+})-ATPase) could not be shown. We did show this (Ca^{2+} + Mg^{2+})-ATPase activity in ram spermatozoa plasma membranes [4]. Thus, it seems that we have here a technical problem that should be solved in the future.

Quercetin has been reported to be an inhibitor of the Mg^{2+}- and Ca^{2+}-dependent ATPase connected with calcium transport in erythrocytes [16], sarcoplasmic reticulum [17,18] and ram spermatozoa plasma membranes [4]. A relevant finding in our study, therefore, was the inhibitory action of quercetin in the observed calcium uptake, favoring the possibility that (Ca^{2+} + Mg^{2+})-ATPase is involved in calcium translocation.

A definite breakpoint was observed on the Arrhenius plot at 32°C. The energy of activation (E_a) below this point was 32.2 kcal/mol, while that above was 4.4 kcal/mol. A temperature-dependent change in E_a has also been reported for calcium transport system of human erythrocytes [19] and for (Ca^{2+} + Mg^{2+})-ATPase of the sarcoplasmic reticulum [20]. Studies of the physical state of the membrane lipids by high-angle X-ray diffraction and NMR suggested that the break in the Arrhenius plot is related to a thermally induced change in the membrane lipids different from a crystalline to liquid-crystalline transition.

The calcium transport system described above may be essential to sperm cell calcium homeostasis when plasma membrane permeability is altered during their maturation. The pattern of motility also undergoes changes with advancing maturation. When intact spermatozoa are treated with the calcium ionophore A23187 in calcium-containing media, the resulting increased uptake of calcium into the cells causes changes in both motility and metabolism [21]. The calcium transport system characterized here is presumbly essential to maintain low level of intracellular calcium concentration.

The demonstration of a Ca^{2+}-dependent ATPase on the outer acrosomal membrane of rabbit, human [22] and guinea pig [23], spermatozoa, implicated a role for this enzyme in the acrosome reaction. It was established that the memmalian acrosome reaction is dependent upon the presence of external Ca^{2+} [24] and that Ca^{2+} influx precedes or parallels its occurrence [25]. In addition, it was shown that the calcium ionophore A23187 can induce acrosome reaction in ram spermatozoa in the presence of Ca^{2+} [26]. Thus, it is possible that the Ca^{2+}-ATPase located on the outer acrosomal membrane represents calcium transport activity which is essential to maintain low level of calcium in the acrosomal space.

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

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