Biochimica et Biophysica Acta, 1108 (1992) 110–114

BBAMEM 75685

Changes in the nature of calcium transport systems on the porcine sperm plasma membrane during epididymal maturation

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(Received 6 February 1992)

Key words: Calcium transport; Sperm maturation; Lanthanum ion

Comparative studies of $^{45}Ca^{2+}$-transport across the plasma membrane were performed using porcine caput, corpus and cauda epididymal sperm. The Ca^{2+}-uptake is dependent on the presence of the substrates for respiration and is sensitive to verapamil. The Ca^{2+}-efflux is mediated by both Na^+-dependent and -independent systems. In the immature sperm in caput epididymis, Na^+-independent efflux is predominant, but it is gradually replaced by Na^+-dependent efflux during the epididymal transit. The net activity of Ca^{2+} accumulation into sperm increases with the epididymal maturation.

Introduction

The functional changes in the sperm plasma membrane are directly or indirectly responsible for epididymal sperm maturation leading to the acquisition of capacity for motility and fertilization. For example, the activity of adenylylcyclase on sperm plasma membrane increases during epididymal maturation, resulting in the increase in the intracellular cyclic AMP (cAMP) level [1,2]. Cyclic AMP is known as an intracellular messenger of sperm maturation [2].

In addition to cAMP, calcium is also well-known as an important intracellular regulator of various sperm functions including epididymal maturation. It has been proposed that various kinds of transport systems are involved in the regulation of Ca^{2+} concentration in mammalian sperm, such as the ATP-dependent Ca^{2+} pump [3,4], the Na^+/Ca^{2+} exchanger [5,6], the voltage-dependent Ca^{2+} channel [7], the calmodulin-dependent, energy-requiring Ca^{2+} transporter [8], and the receptor-operated Ca^{2+} channel [9]. But the detailed mechanisms of Ca^{2+} transport and regulation of its intracellular level are still unclear.

In the present study, changes in the activities and properties of calcium transport across the plasma membrane during epididymal maturation of porcine sperm were studied.

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Materials and Methods

Sperm preparation. Porcine caput, corpus and cauda epididymal sperm were collected by microperfusion of ductus epididymidis with 113 mM NaCl, 5 mM KCl, 5 mM glucose, 3 mM sodium pyruvate, 20 mM Tris-HCl, pH 7.4 (Pyr-Glu buffer) [10]. Sperm were washed three times by centrifugation and then suspended in the same buffer (10^9 cells/ml). Sperm suspension was stored at room temperature until use.

Determination of Ca^{2+} transport activity. In determination of Ca^{2+} uptake, sperm ($2 \cdot 10^8$ cells/ml) were incubated in the Pyr-Glu buffer containing 1 mM $^{45}CaCl_2$ at 30°C for appropriate time. 100 μl of the incubation mixture was added to 5 ml of the washing buffer (113 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.4)) layered on GF/C filter and immediately filtrated. The filter was washed twice more with 5 ml of the same buffer. The procedure was finished within 15 s. After the filter was dried up, radioactivity on the filter was counted in the scintillation cocktail. Ca^{2+} efflux activity was determined as follows. Sperm were incubated in the Pyr-Glu buffer containing 1 mM $^{45}CaCl_2$ at 30°C for 20 min and then washed with Pyr-Glu buffer by centrifugation. Sperm were resuspended in the Pyr-Glu buffer and further

incubated at 30°C. After 0 and 10 min, 100 μl of the incubation mixture was put on GF/C filter and washed as mentioned above. The efflux activity was expressed as the percentage of the radioactivity of $^{45}Ca^{2+}$ released from sperm during 10 min of the incubation, taking the radioactivity preloaded in sperm (the radioactivity at 0 min of the incubation for the efflux assay) as 100%.

Materials. $^{45}CaCl_2$ was purchased from New England Nuclear. Diltiazem, nifedipine, and caffeine were obtained from Wako Pure Chemical Industry, Japan. Verapamil, 3-isobutyl-1-methyl-xanthine, dibutyryl-adenosine 3′,5′-cyclic monophosphate, forskolin, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) were purchased from Sigma.

Results and Discussion

Although Ca^{2+} is well-known to play central roles in the regulation of many sperm functions, we have rather little knowledge on the Ca^{2+} transport systems as well as on the mechanism of the control of the intracellular Ca^{2+} concentrations in mammalian sperm. So, at first in this study, the basic properties of the Ca^{2+} transport across the porcine sperm plasma membrane were studied.

Fig. 1 shows the time course of the uptake of $^{45}Ca^{2+}$ by porcine cauda epididymal sperm under various conditions. In the Pyr-Glu buffer containing 1 mM $^{45}CaCl_2$, sperm linearly accumulated $^{45}Ca^{2+}$ within the cell during the first 4 min of the incubation; 1.18 pmol/10^6 cells per min, and the rate was then gradually decreased. The rate of Ca^{2+} uptake obtained in the present study is similar to the rates of boar and ram sperm reported by Simpson et al. [11], but lower than

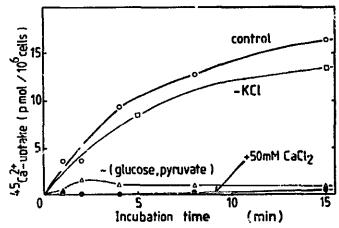

Fig. 1. Time course of Ca^{2+} uptake by porcine cauda epididymal sperm. $^{45}Ca^{2+}$ uptake was determined as described under Materials and Methods. ○, □, △, and ● show the $^{45}Ca^{2+}$ uptake activity in the complete Pyr-Glu buffer, in the absence of KCl, in the absence of glucose and pyruvate, and in the presence of unlabeled 50 mM $CaCl_2$, respectively. Data are means from three determinations.

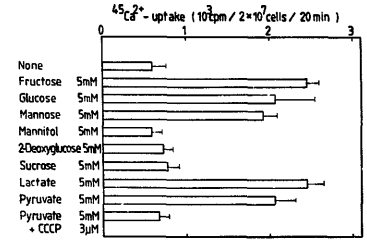

Fig. 2. Effects of exogenous substrates on Ca^{2+} uptake by cauda epididymal sperm. Sperm were incubated in 113 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4) in the presence of various exogenous substrates for the energy metabolism at 30°C for 20 min. 1 mM $^{45}CaCl_2$ was added to the incubation mixture and $^{45}Ca^{2+}$ uptake was determined as described under Materials and Methods. Data are means ± S.E. from eight determinations.

those reported for ram [12] and bovine [13,14] sperm, suggesting that the Ca^{2+} uptake activity of sperm varies among species and experimental conditions. Removal of K^+ from the medium did not influence the uptake activity. Ouabain did not either affect $^{45}Ca^{2+}$ uptake (data are not shown). These suggest that the membrane potential-dependent Ca^{2+} transport is not main pathway in porcine sperm.

On the other hand, $^{45}Ca^{2+}$ uptake is completely dependent on the presence of either glucose or pyruvate. Fig. 2 shows the effects of several substrates for energy metabolism on the $^{45}Ca^{2+}$ uptake activity. The metabolizable monosaccharides such as fructose, glucose and mannose increased the $^{45}Ca^{2+}$ uptake to the same extent as pyruvate and lactate did. But mannitol, which can not permeate the plasma membrane, and unmetabolizable saccharides such as 2-deoxyglucose and sucrose were not effective. It was also shown that CCCP, uncoupler of oxidative phosphorylation, completely suppressed the effect of pyruvate. These results suggest that the Ca^{2+} uptake by porcine sperm is highly dependent on energy levels and mitochondrial functions. In guinea pig epididymal sperm, though pyruvate and lactate were also reported to enhance the Ca^{2+} uptake, metabolizable monosaccharides were found to inhibit the uptake of Ca^{2+} by decreasing ATP levels [15]. Contrarily, porcine sperm seem to have high glycolytic activity enough to maintain ATP levels under the present conditions, which results in the similar extent of the stimulation of Ca^{2+} uptake by pyruvate, lactate, or the metabolizable monosaccharides. Alternatively, ATP levels in the cells may not directly correlate with Ca^{2+} transport activity, as reported by Breitbart et al. in bovine sperm [16].

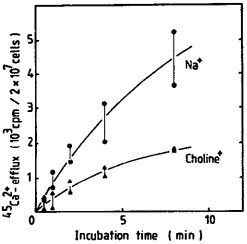

Fig. 3. Ca^{2+} efflux activity in the cauda epididymal sperm. Ca^{2+} efflux was determined in Pyr-Glu buffer (●) as described under Materials and Methods. ▲ shows the efflux activity when 113 mM NaCl is replaced with 113 mM choline chloride. Each point is the mean from duplicate determinations.

As shown in Fig. 3, the intracellular Ca^{2+} is excreted by at least two distinct systems; Na^+-dependent and -independent systems. Na^+-independent Ca^{2+} efflux activity is about 37% of the total efflux activity in the mature epididymal sperm. Na^+/Ca^{2+} antiporter and Ca^{2+} pump which were shown to exist in ram sperm are most probable for the exporters [5].

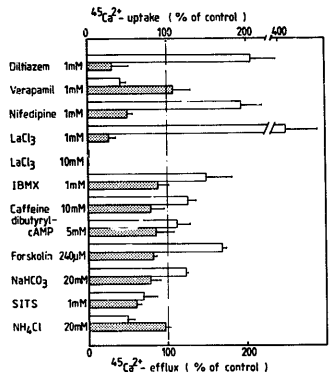

Fig. 4. Effects of several reagents on Ca^{2+} uptake and efflux. Cauda epididymal sperm were incubated with various effectors in the Pyr-Glu buffer at 30°C for 20 min. $^{45}Ca^{2+}$ uptake (open bar) was determined as Fig. 2. Sperm pre-loaded with $^{45}CaCl_2$ were incubated in Pyr-Glu buffer containing various effectors at 30°C for 10 min and the efflux activity (dotted bar) was determined as described under Materials and Methods. Data are means ± S.E. from eight determinations.

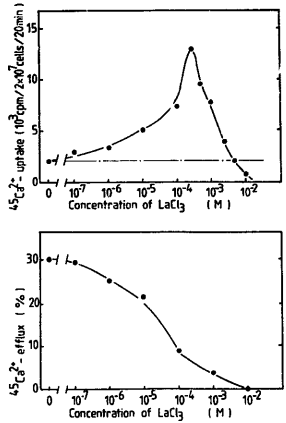

Fig. 5. Dependency of Ca^{2+} uptake and efflux on the concentration of $LaCl_3$. Cauda epididymal sperm were incubated at 30°C for 20 min with various concentrations of $LaCl_3$ and added to the incubation mixture for Ca^{2+} uptake assay. $^{45}Ca^{2+}$ uptake (upper) was determined as Fig. 2. $^{45}Ca^{2+}$-pre-loaded sperm were incubated in the Pyr-Glu buffer in the presence of various concentrations of $LaCl_3$ and Ca^{2+} efflux (lower) was determined as Fig. 4. Data are means from duplicate determinations.

Na^+/Ca^{2+} antiporter is also thought to be responsible for controlling Ca^{2+} uptake by bovine [6] and boar [17] sperm. Fig. 4 summarizes the effects of several reagents on influx and efflux of $^{45}Ca^{2+}$. Nifedipine and diltiazem, which are potent blockers of the voltage-operated calcium channels, increased $^{45}Ca^{2+}$ accumulation in sperm by strongly blocking Ca^{2+} efflux. On the other hand, 1 mM verapamil clearly inhibited $^{45}Ca^{2+}$ influx without affecting the efflux activity. Breitbart and Lardy [18] also reported the presence of a Ca^{2+} transporter sensitive to such rather high concentrations of verapamil in bovine sperm plasma membrane. So, at least three transporters were distinguished that are located on porcine sperm plasma membrane: verapamil-sensitive and voltage-insensitive importer and Na^+-sensitive and -insensitive exporters.

It is interesting that caffeine and bicarbonate, which activate various sperm functions through increasing cAMP concentration, weakly stimulated $^{45}Ca^{2+}$ uptake, though cAMP itself had no effect on Ca^{2+} transport. Methylxanthine-stimulated Ca^{2+} transport was reported in abalone sperm and it is also verapamil-sensitive [19]. SITS, an anion channel blocker, decreased both influx and efflux of Ca^{2+}, suggesting some inter-

actions between bicarbonate transporter [20] and Ca^{2+} transport systems. In this connection, it is interesting that bovine sperm plasma membrane contains two types of calcium transporter: a calcium-phosphate transporter which is stimulated by bicarbonate, and phosphate-independent calcium transporter which is inhibited by bicarbonate [16]. It was also found that forskolin enhanced Ca^{2+} influx activity and that NH_4Cl which is known to increase intracellular pH, decreased Ca^{2+} influx without affecting the efflux.

La^{3+} was found to have apparently biphasic effects on Ca^{2+} uptake. At lower concentrations than 2.5 mM, La^{3+} enhanced $^{45}Ca^{2+}$ uptake and the maximal enhancement was observed at 0.25 mM. This stimulatory effect was due to the inhibition of Ca^{2+} efflux, as also shown in Fig. 5. At higher concentrations than 0.25 mM, La^{3+} inhibited both influx and efflux, resulting in decrease of $^{45}Ca^{2+}$ uptake. Peterson et al. also observed the stimulation of Ca^{2+} binding to the plasma membrane vesicles of boar sperm by a low dose of La^{3+} (50 μM) [21]. Furthermore, 0.25 mM La^{3+} was found to enhance the acrosome reaction, which was induced by 1.8 mM $CaCl_2$ and 0.1% fatty acid-free BSA as reported by Nikolopoulou et al. [22]. It is strongly suggested that La^{3+} stimulates the acrosome reaction by increasing Ca^{2+} uptake and the intracellular Ca^{2+} levels (data are not shown).

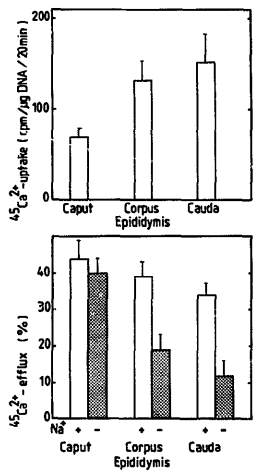

Fig. 6. Comparison of the activities of Ca^{2+} uptake and efflux among caput, corpus and cauda epididymal sperm. $^{45}Ca^{2+}$ uptake (upper) and efflux (lower) activities of caput, corpus and cauda epididymal sperm were determined as described under Materials and Methods. The dotted bars show the efflux when NaCl in the Pyr-Glu buffer was replaced with choline chloride. Data are means ± S.E. from 12 different experiments each with duplicate determinations.

The functional and structural modifications of the sperm surface occur during the epididymal maturation. This partly causes the changes in the activities of ion transport across the plasma membrane. We have already shown that bicarbonate transport activity decreases during the epididymal maturation of porcine sperm [20]. Fig. 6 shows that the net activities of $^{45}Ca^{2+}$ uptake increase while sperm are transported from caput to corpus epididymis, where sperm acquire motility and fertile ability. On the other hand, the total activities of $^{45}Ca^{2+}$ efflux decreased slightly, and the ratio of the Na^+-independent efflux activity to the total activity greatly decreased as sperm matured (93% in caput to 37% in cauda epididymal sperm). These results indicate that the accumulation of Ca^{2+} by sperm becomes more feasible and more dependent on the concentration of extracellular Na^+ during epididymal transit. Vijayaraghavan and Hoskins [13] reported that the Ca^{2+} uptake capacity of bovine sperm declines during epididymal maturation, but the reason for the discrepancy between the results for porcine and bovine sperm is not clear.

It is very interesting that Ca^{2+} transport activity further changes upon ejaculation [23] and during capacitation [15,24–26]. Although the mechanisms for the changes in the nature of Ca^{2+} transport remains to be elucidated, such changes must influence the sperm activities controlled by Ca^{2+}. The Na^+-dependency of Ca^{2+} efflux may be used as a good indicator for porcine sperm maturation in epididymis.

Acknowledgments

We thank Dr Nobuo Makino and Dr. Robin A.P. Harrison for their critical reading of the manuscript and discussions. This work was supported in part by Science Research Grants 03640594 and 03207101 from the Ministry of Education of Japan to N.O.

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