A SODIUM–CALCIUM EXCHANGE MECHANISM IN PLASMA MEMBRANE VESICLES ISOLATED FROM RAM SPERM FLAGELLA

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Received 19 September 1980

1. Introduction

The ability of mature mammalian spermatozoa to maintain a co-ordinated and forward motility is dependent upon intracellular free Ca^{2+} being maintained within a narrow concentration range [1]. At the level of the flagellar plasma membrane this could be achieved by two systems: (a) an ATP requiring Ca^{2+}-pump; or (b) a Na^+/Ca^{2+} antiporter. Although such plasma membrane Ca^{2+}-extrusion mechanisms have been found in a variety of cell types [2] they have only recently been experimentally determined in spermatozoa. Using a procedure to selectively fractionate mammalian sperm we have been able to show that there exists in the flagellar plasma membrane of ram sperm a Ca^{2+}-dependent Mg^{2+}-ATPase or Ca^{2+}-pump [3,4]. The report demonstrating the presence of both a Ca^{2+}-pump and a Na^+/Ca^{2+} antiporter in membranes isolated from dog sarcolemma [5], has now prompted us to examine sperm plasma membranes for the existence of such an antiporter mechanism. From these studies we hope to obtain a more detailed understanding of Ca^{2+} regulation in mammalian spermatozoa.

2. Materials and methods

2.1. *Preparation of flagella plasma membranes*

Membrane vesicles were isolated from ram sperm flagella preparations using hypotonic shock followed by sucrose gradient centrifugation [3]. Membrane vesicles were finally resuspended in 18–18 mM histidine–imidazole buffer (pH 7.1) and either used immediately or stored in liquid N_2.

2.2. *Transport assays*

To determine Ca^{2+} influx, membrane vesicles (200 μl) containing 200–300 μg protein were preincubated with 500 μl 160 mM NaCl for 1 h at 37°C [6]. Samples (30 μl) were then removed and diluted 30-fold into 160 mM LiCl, 20 mM morpholinopropane sulphonic acid (MOPS) adjusted to pH 7.4 with Tris–HCl which contained 40 μM $^{45}CaCl_2$ (0.25 mCi/mmol). The uptake of $^{45}Ca^{2+}$ was terminated at various time intervals by the addition of ice-cold medium (5 ml) containing 200 mM KCl, 5 mM KH_2PO_4, 10 mM EGTA (pH 7.4). These membrane vesicles were then harvested on Millipore filters (0.45 μm) and washed 2 times with 5.0 ml of the same medium. Filters were dried, suspended in scintillation fluid and then counted in a liquid scintillation counter. In Ca^{2+} efflux experiments, membrane vesicles were preincubated with 40 μM $^{45}CaCl_2$ in 20 mM MOPS/Tris–HCl (pH 7.4) (500 μl) for 1 h at 37°C. Efflux was then initiated by the addition of various components, followed by rapid removal of aliquots over 2 min. Protein determinations were by the Bradford method [7]. Verapamil and D600 were generous gifts from Knoll Pharmaceutical Co., and A23187 a gift from Dr R. J. Hosley, Eli Lilly and Co.

3. Results

Fig.1 illustrates $^{45}Ca^{2+}$ uptake into plasma membrane vesicles which had been preloaded with 160 mM NaCl, then diluted 30-fold into buffered 160 mM LiCl containing 40 μM $^{45}CaCl_2$. The $^{45}Ca^{2+}$ uptake is very rapid over the first 15 s interval with ~550 nmol Ca^{2+}/mg protein accumulated. The rate of uptake is less rapid over the remainder of the 2 min period, with

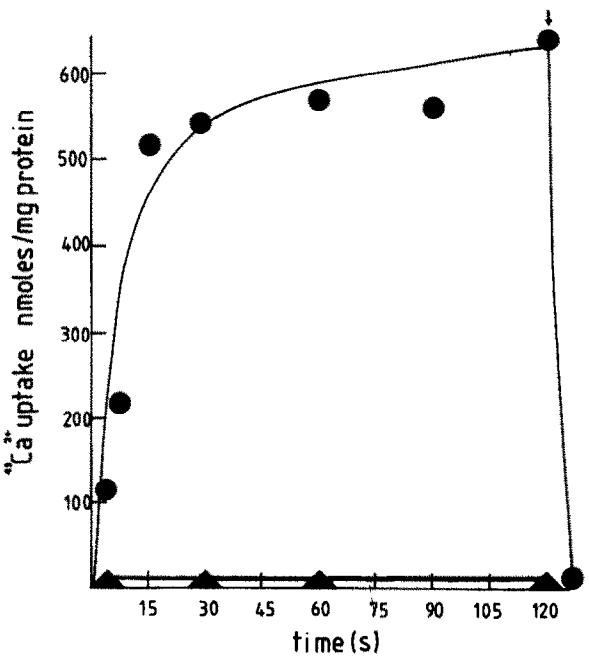

Fig.1. Sodium-dependent calcium uptake by sperm membrane vesicles. Membrane vesicles (240 μg protein) were preloaded with 160 mM NaCl in 20 mM MOPS/Tris buffer (pH 7.4) then diluted 30-fold into 160 mM LiCl, 20 mM MOPS/Tris buffer (pH 7.4) containing 40 μM $^{45}CaCl_2$. Ca^{2+} uptake was measured with respect to time (●——●). A control experiment was performed where vesicles were preloaded with 160 mM LiCl in 20 mM MOPS/Tris buffer (pH 7.4) (▲——▲). NaCl (60 mM) or A23187 (9 μM) were added as indicated by the arrow.

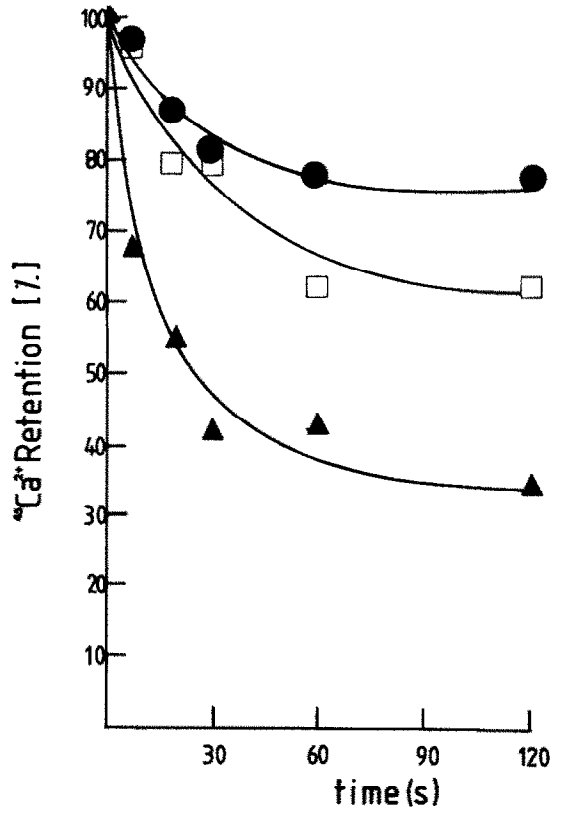

Fig.2. Sodium-induced calcium efflux by sperm membrane vesicles. Membrane vesicles (240 μg protein) were preloaded with 40 μM $^{45}CaCl_2$ in 20 mM MOPS/Tris buffer (pH 7.4). Ca^{2+} efflux was induced by the addition at time zero of either 60 mM NaCl (▲——▲) or 60 mM KCl (●——●). Samples removed at the various time points were washed in KCl/KH_2PO_4/EGTA as in section 2. Results are expressed as Ca^{2+} retention (%) determined from the ratio of Ca^{2+} in the vesicles at each experimental time point compared to the zero time value.

Ca^{2+} uptake finally reaching 600 nmol/mg protein. The Ca^{2+} uptake would appear to truly represent sequestration into a membrane-enclosed compartment since on addition of the Ca^{2+} ionophore A23187 (9 μM) an immediate and total loss of Ca^{2+} from the vesicles is recorded. When membrane vesicles were preloaded with either 160 mM LiCl or 160 mM KCl, no significant $^{45}Ca^{2+}$ uptake was measured on dilution of the vesicles into the $^{45}Ca^{2+}$ containing medium. The interdependence and reversibility of the membrane Ca^{2+}/Na^+ flux is indicated by the rapid loss of Ca^{2+} from the vesicles following addition of 60 mM NaCl to the incubation media (fig.1).

To further investigate the dependence of membrane Ca^{2+} movements on Na^+ fluxes the experimental conditions were modified to preload sperm plasma membrane vesicles with $^{45}CaCl_2$ instead of NaCl. In these experiments the subsequent addition of 60 mM NaCl to Ca^{2+}-loaded vesicles resulted in a substantial $^{45}Ca^{2+}$ efflux (fig.2). The efflux was found to be most rapid over the initial 15 s incubation period and resulted in a 40% loss of $^{45}Ca^{2+}$ from the membrane vesicles. At the end of the 2 min assay period 60% of the total vesicular $^{45}Ca^{2+}$ was released. The Na^+-induced efflux was also found to be dependent upon the concentration of Na^+ added to the Ca^{2+}-loaded vesicles. In experiments using 15 mM NaCl in the external medium, only a low (<10%) $^{45}Ca^{2+}$ efflux was detected. However, at higher levels of NaCl (20–60 mM) the degree of $^{45}Ca^{2+}$ efflux was found to be directly proportional to the amount of Na^+ added.

The effect of two Ca^{2+} antagonists, verapamil and

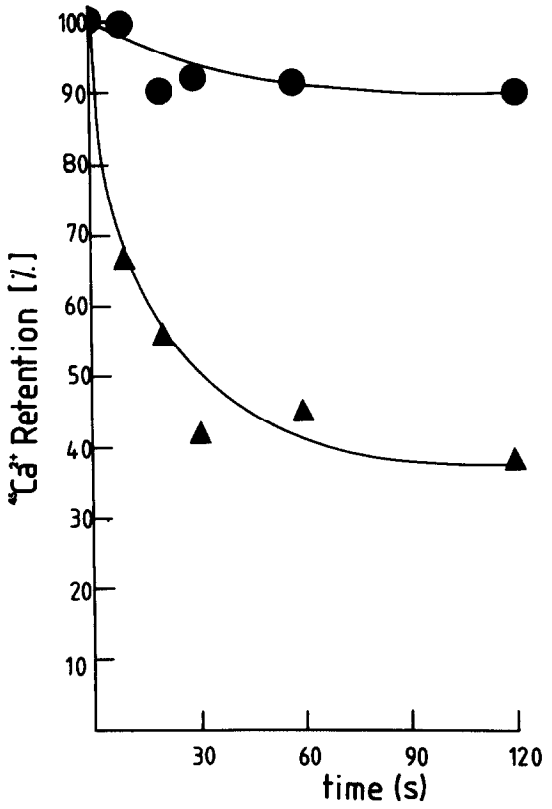

Fig.3. The effects of verapamil and D600 on Na^+-induced Ca^{2+} efflux. Membrane vesicles (240 μg protein) were preloaded with $^{45}CaCl_2$ as in fig.2 and Ca^{2+} efflux induced by the addition of 60 mM NaCl (▲——▲), 60 mM NaCl + 10 μg/ml verapamil (□——□), 60 mM NaCl + 10 μg/ml D600 (●——●). Results are expressed as Ca^{2+} retention (%) as in fig.2.

D600, on Na^+-induced Ca^{2+} efflux from $^{45}CaCl_2$ preloaded membrane vesicles is demonstrated in fig.3. When either verapamil or D600 were added to vesicles preloaded with $^{45}Ca^{2+}$, the subsequent efflux of Ca^{2+} on addition of 60 mM NaCl was decreased. In the presence of verapamil a 40% Ca^{2+} loss was observed over 2 min. In comparison when D600 was present only a 20% Ca^{2+} release was detected over the same period. These changes in Ca^{2+} efflux represent ~33% and ~66% inhibition, respectively, of Ca^{2+} efflux as compared to the control situation. When either of these compounds were added to the membrane vesicles during the 1 h period of passive loading, the level of Ca^{2+} accumulated was unaffected.

4. Discussion

Several reports now exist which show that the plasma membrane of cells from excitable tissues, such as heart and brain, exhibit a Na^+/Ca^{2+} exchange antiporter [5,6,8]. We have accumulated data demonstrating that mammalian spermatozoa have many characteristics in common with cells from excitable tissues. For example, sperm mitochondrial Ca^{2+}-transport responds to ruthenium red, Na^+ and Mg^{2+} in a similar manner to that reported for heart mitochondria [9,10]. In addition, sperm cells have been found to contain both the synthetic and degradative enzymes required for acetylcholine metabolism as well as possessing a nicotinic type acetylcholine receptor [11,12]. This concept of sperm metabolism is further reinforced by these results, which demonstrate the existence of a flagellar plasma membrane Na^+/Ca^{2+} antiporter, similar in properties to Na^+/Ca^{2+} antiporter systems identified in the plasma membranes of heart, brain and squid axons [2].

In membrane vesicles isolated from ram sperm flagella we have shown that the two Ca^{2+} antagonists and D600 do not effect the passive loading of the vesicles with $^{45}Ca^{2+}$ but do reduce both the rate and extent of Na^+-induced Ca^{2+} fluxes. Verapamil and D600 decrease the cardiac excitation–contraction cycle [13], and are thought to exert their antagonistic effect by blocking some component of the Ca^{2+}-transport system in these tissues [14]. However, the exact mode of action of these drugs is still uncertain. From these results it would appear that in mammalian sperm, verapamil and D600 specifically block the Na^+/Ca^{2+} antiporter.

The regulation of intracellular Ca^{2+} by many cells is thought to occur primarily by plasma membrane-located Ca^{2+}-pumps [2]. In cells which also contain a plasma membrane, Na^+/Ca^{2+} exchange systems the two Ca^{2+} efflux processes may work in concert to control cytostolic Ca^{2+}. It has been proposed that in these situations the high-capacity, low-affinity Na^+/Ca^{2+} antiporter is the predominant plasma membrane Ca^{2+}-extruding system [5]. In which case the low-capacity high-affinity plasma membrane Ca^{2+}-pump functions more as a system for the fine control of intracellular Ca^{2+}. If this is also the arrangement in mammalian sperm then it is possible to postulate a model for intrasperm Ca^{2+} regulation. Sperm are normally subjected to a continuous Na^+ influx, in response to the high levels (70–100 mM) of seminal plasma Na^+ [15]. This condition would generate via the Na^+/Ca^{2+} antiporter an opposite and continuous efflux of Ca^{2+} from the sperm to lower intrasperm

Ca^{2+}. The plasma membrane (Ca^{2+} + Mg^{2+}) ATPase may further facilitate this process by pumping out Ca^{2+} in excess of that normally handled by the Na^{+}/Ca^{2+} antiporter. According to this model the plasma membrane Ca^{2+}-pump of spermatozoa may play only a modest but nonetheless important role in contributing to the Ca^{2+} homeostasis of the sperm in vivo. In comparison, the bulk transport of Ca^{2+} across the sperm plasma membrane would be the responsibility of the Na^{+}/Ca^{2+} antiporter.

Acknowledgements

This work was supported by the Ford Foundation of New York and Medical Research Council of New Zealand. We are grateful to Dr A. J. Allison of the Invermay Agricultural Research Centre for the supply of ram semen.

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