PLASMA MEMBRANE BOUND Ca^{2+}-ATPase ACTIVITY IN BULL SPERM

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1. Introduction

The acrosome reaction in vertebrate sperm has been shown to be essential for fertilisation [1]. The process involves fusion and vesiculation of sperm head plasma membranes and the underlying outer acrosomal membrane, resulting in the exocytotic release of acrosomal enzymes. Yanagamachi and Usui have demonstrated that sperm have an absolute requirement for Ca^{2+} during the acrosome reaction [2]. A net uptake of Ca^{2+} has been identified [3] and experiments with the Ca^{2+} ionophore A23187 suggest that Ca^{2+} transmembrane fluxes govern the induction of the acrosome reaction [4]. On the basis of electron microscopic observations Gordon has suggested that an ATPase, which is not stimulated by Ca^{2+}, transports Ca^{2+} into the periacrosomal space and a Ca^{2+} dependent ATPase located on the outer acrosomal membrane transport this Ca^{2+} into the acrosome, thus playing a crucial role in the induction of the acrosome reaction [5,6]. However, this hypothesis has been challenged by reports of the absence of Ca^{2+}-ATPase activity in guinea pig sperm [2], though earlier reports identified Ca^{2+}-ATPase activity on sperm membranes by cytochemical methods [5].

In the course of biochemical characterisation of the various subcellular fractions of bull sperm, we have detected the presence of a Ca^{2+}-ATPase in the plasma membrane fraction. In this communication we report the observation of Mg^{2+}-independent Ca^{2+}-ATPase activity, confined primarily to a fraction enriched in sperm plasma membranes. This enzyme is inhibited by the anionic, hydrophobic (fluorescent probes 1-anilino-8-naphthalene sulfonate (ANS) and 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS), and also *N,N'*-dicyclohexylcarbodiimide (DCCD), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) which are carboxyl and sulfhydryl group modifiers, respectively.

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2. Experimental

Bull semen, obtained from the Centralised Semen Center, Hebbal, was diluted with 2 vol. of neutral Hepes buffer, containing 0.15 M NaCl. Sperm cells were isolated from the diluted semen by a modification of Garber's procedure [7], as adapted by Zahler and Doak [8]. The procedure followed for subcellular fractionation of bull sperm was essentially that of Zahler and Doak except that it was extended to obtain plasma membranes, in addition to acrosomal membranes as described in [8]. Sperm cells were homogenised at pH 7 and layered on a discontinuous gradient (1.3 M/1.75 M sucrose) and centrifuged at 100 000 × *g* for 3 h at 0–5°C. Details of isolation and biochemical characterisation of the various subcellular fractions will be published elsewhere.

Protein was estimated by the method of Lowry et al. [9]. Inorganic phosphate was measured according to the method of Chen et al. [10]. The enzymes, $(Na^+ + K^+)$-ATPase [11], alkaline phosphatase [8], 5'-nucleotidase [12], acetylcholinesterase [13] were assayed using standard methods. Ca^{2+}-ATPase [14] was monitored in the presence and absence of ouabain. The $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured by the method of Farrance and Vincenzi [15] and enzyme activity was taken to be the 'extra ATP splitting' induced by Ca^{2+} addition in the presence of Mg^{2+} and ouabain.

3. Results

A high alkaline phosphatase activity was observed in the membrane fraction which bands above 1.3 M sucrose and based on this finding Zahler and Doak [8] suggested that this fraction may be useful in studies of plasma membranes. However, a further characterisation was not reported. This fraction has been enzymatically characterised in greater detail in the present study. The distribution of putative plasma membrane marker enzymes, in the sperm subcellular fraction, is summarised in table 1. There is an appreciable enrichment of Ca^{2+}-ATPase, Na^+, K^+-ATPase, 5′-nucleotidase, alkaline phosphatase and acetylcholinesterase in the sperm plasma membrane fraction (SPM) relative to the outer acrosomal membranes (SAM), supernatant (SS), sperm pellet (SPT) and the homogenate (SH). The results also indicate that Ca^{2+}-ATPase is exclusively localised in the plasma membrane fraction and is completely absent in the outer acrosomal membranes and the supernatant. This Ca^{2+}-ATPase was not affected by ouabain or stimulated by the simultaneous presence of Na^+ and K^+. Mg^{2+} was also without effect, suggesting an Mg^{2+}-independent Ca^{2+}-ATPase activity. The enzyme activity was dependent on Ca^{2+} concentration. Maximal activity was attained at $\geqslant$3 mM Ca^{2+}, with a relatively broad pH optimum around pH 7.4. The enzyme levels decreased on storage of membrane samples, with only 50% of the original activity being observed after 24 h at 4°C. The hydrophobic, anionic fluorescent probes TNS (1 mM), ANS (1 mM) and DTNB (2 mM) an –SH group blocker inhibited the Ca^{2+}-ATPase activity by 70, 76 and 74%, respectively, DCCD (1 mM) a carboxyl group modifier completely blocked enzyme activity.

4. Discussion

As suggested earlier [8] it appears that the membranes which band on top of 1.3 M sucrose constitute bull sperm plasma membranes. The relatively high enrichments of Na^+, K^+-ATPase, acetylcholinesterase, 5′-nucleotidase and alkaline phosphatase, which are considered to be general markers for plasma membranes [16], support the view that the isolated membrane fraction is indeed enriched in bull sperm plasma membranes. The localisation of the Ca^{2+}-ATPase activity in this fraction is of particular interest since Ca^{2+} has been implicated in the mediation of many biochemical events, which occur during the reproductive process [17–20] and also in the ATP dependent head-to-head association of bull sperm [21]. Gordon, based on cytochemical investigations on guinea pig, human and rabbit sperm suggested the involvement of an SAM bound Ca^{2+}-ATPase [5,6]. However, Yanagimachi and Usui were unable to detect the presence of this enzyme [2]. Since most investigations on this enzyme have been cytochemical, there exists considerable uncertainty concerning its presence and location. This report establishes the

Table 1
Enzyme distribution in subcellular fractions of bull sperm[a]

Enzyme	SPM	SAM	SS	SPT	SH
Ca^{2+}-ATPase[b]	47.03 (6)[e]	0 (9)	0 (6)	9.25 (6)	3.57 (3)
Na^+, K^+-ATPase[b]	19.54 (5)	5.05 (5)	0.35 (5)	1.25 (5)	2.60 (5)
5′-Nucleotidase[b]	18.47 (5)	4.25 (3)	0.20 (7)	0.94 (5)	3.74 (3)
Alkaline phosphatase[c]	5.27 (7)	1.48 (7)	0.25 (7)	2.10 (7)	0.94 (4)
Acetylcholinesterase[d]	8.36 (5)	1.86 (3)	0.82 (4)	0.53 (4)	0.30 (3)

[a] SPM, material collected from the sample/1.3 M sucrose interface enriched in sperm plasma membrane; SAM, fraction removed from the 1.3 M/1.75 M interface containing outer acrosomal membrane; SS, soluble fraction obtained from the sample volume on top of the gradient; SPT, residual pellet containing sperm fragments and intact sperm; SH, homogenised sperm cells

[b] Activity expressed in μmol of Pi liberated/mg protein/h

[c] Activity in nmol of *p*-nitrophenol released/mg protein/h

[d] Specific activity in units of enzyme/mg protein/h

[e] Numbers in parentheses are the number of different samples, averaged to yield reported values

presence of a Ca^{2+}-ATPase in the fraction enriched in plasma membranes of bull sperm and its absence in the SAM fraction. Identical results have been obtained with hamster sperm (unpublished results). It is likely that the membrane-bound Ca^{2+}-ATPase could serve as a marker for SPM.

The sperm enzyme is not influenced by Mg^{2+}, an observation similar to that reported for membrane bound Ca^{2+}-ATPase in skeletal muscle [22], cardiac muscle [23] and uterine smooth muscle [24]. The pH optimum of 7.4 obtained in this study is in contrast to the value of 9.0 reported for outer acrosomal membrane bound Ca^{2+}-ATPase [6]. In our experiments no Ca^{2+}-ATPase activity was detected in the acrosomal membrane fractions, even at pH 9.0. It is however difficult to correlate the results of the biochemical fractionation described in this report with earlier cytochemical studies [5,6]. The sperm Ca^{2+}-ATPase is inhibited by DCC and DTNB, a result analogous to that obtained for the Ca^{2+}-ATPase of sarcoplasmic reticulum membranes [25,26]. It is interesting to note that the hydrophobic fluorescent probes ANS and TNS, which cause immediate immobilisation of motile sperm cells [27], also effectively inhibit the sperm Ca^{2+}-ATPase. These inhibition studies suggest that hydrophobic binding sites and at least one carboxylic and sulfhydryl group may be implicated at the enzyme active site. It is tempting to speculate that this enzyme is coupled to an energy-linked Ca^{2+} transport system in sperm. The existence of a Ca^{2+} pump and the functional significance of Ca^{2+}-ATPase in Ca^{2+} transport and the acrosome reaction remain to be established.

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