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IDENTIFICATION OF CALMODULIN-LIKE ACTIVITY IN HUMAN SEMINAL PLASMA

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SUMMARY: Human seminal plasma was found to contain relatively high levels of a heat stable proteinaceous factor with properties similar to that of the calcium-binding protein calmodulin. The seminal plasma factor increases the $(Ca^{2+} + Mg^{2+})$-ATPase activity found in human red blood cell plasma membranes by 370% and the activation was completely abolished by chlorpromazine, amitriptyline and theophylline. A similar calmodulin-activated Ca^{2+} pump, has been found in the plasma membrane of ram sperm tails. The existence of calmodulin in mammalian seminal plasma may be responsible for some of the metabolic changes associated with sperm maturation.

INTRODUCTION

Mammalian spermatozoa undergo many important functional changes during the migration through the epididymal tract. The most critical of these changes is associated with the capacity for coordinated forward motility and fertilizing ability (1,2). These functional changes have been linked to other metabolic and structural changes occurring in the epididymal phase of sperm maturation, for example, increased levels of cAMP (3) decreased levels of phosphodiesterase activity (4) and increased levels of membrane disulphide bonds (1,5). Further modifications occur with ejaculation of the sperm as demonstrated by the relatively impermeable nature of ejaculated mammalian spermatozoa to Ca^{2+}, compared to epididymal spermatozoa (6,7). The actual control mechanisms associated with many of these sperm maturation changes is still uncertain although evidence indicates that some sperm functions are modulated by the absorption or integration of specific membrane proteins (6,8).

Abbreviations used are: EGTA, ethyleneglycol-bis-(β-amino-ethyl ether)-N,N'-tetraacetic acid; FMP, forward motility protein; RBC, red blood cell.

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Recently calmodulin has been shown to be the vital modulating protein for many diverse Ca^{2+}-dependent reactions (9-11) in a very wide range of tissues (12-15), including testes and spermatozoa (16). These observations combined with the well documented importance of Ca^{2+} in sperm motility (17,18) prompted us to investigate mammalian seminal plasma for calmodulin activity. In this paper we show that human seminal plasma contains high levels of a heat-stable proteinaceous factor with similar properties to that of calmodulin and discuss the implications of these findings with respect to sperm maturation.

MATERIALS AND METHODS

Preparation of RBC Membranes and Hemolysate. Recently outdated human red blood cells (RBCs) in citrate buffer was obtained from the local blood bank, and plasma membranes prepared as previously described (19). The freshly prepared membranes were finally suspended in 18mM-18mM histidine-imidazole buffer, pH 7.1 before storage at 4°C. The hemolysate, obtained from the lysis step during the preparation of RBC membranes (19), was dialysed for 12hr against 15.2mM imidazole buffer, pH 7.4 at 4°C before use.

$(Ca^{2+} + Mg^{2+})$-ATPase Assay. The assay used was the same as that described by Gopinath and Vincenzi (11). The reaction mixture, final volume 1 ml, contained 3mM ATP, 18mM-18mM histidine-imidazole buffer, pH 7.1, 3mM $MgCl_2$, 80mM NaCl, 15mM KCl, 0.1mM ouabain, 0.1mM EGTA, and between 0.1 - 0.2mg of RBC membrane protein. $CaCl_2$ (0.2mM) was present in all tubes, except in those used for the determination of Mg^{2+}-ATPase activity. When added modulators were present at the following final concentrations; RBC hemolysate (8.25 mg/ml), heat-treated human seminal plasma (8.25 µg/ml), chlorpromazine (5.25 µg/ml), amitriptyline (5.25 µg/ml) and theophylline (3mM). The reaction, started by addition of ATP, was carried out at 37°C for 60 min and terminated by addition 10% TCA (0.5 ml). ATPase activity was then determined by an enzyme-linked assay, estimating the amount of ADP produced in the assay period (20). Protein was determined by the method of Bradford (21).

Seminal Plasma Preparation. Semen obtained from healthy human donors was diluted with two volumes of isotonic buffer containing 0.03M Tris-HCl, pH 7.4, 0.103M NaCl, 12.5mM KH_2PO_4, 2.5mM K_2HPO_4, 3.0mM $MgCl_2$, 0.4mM EDTA and centrifuged at 1500g for 15 min at room temperature. The supernatant was then removed and centrifuged at 5,000g for 15 min. at 4°C. The cell free supernatant was then heat-treated at 85°C for 10 min and finally centrifuged for 15 min at 40,000g and stored at 4°C.

Preparation of Sperm Plasma Membranes. Sperm were separated from ram semen by centrifugation at 3,000g for 10 min and washed a further 3X in isotonic buffer as previously described (22). Sperm tails were then isolated (23) and suspended in hypotonic buffer to release plasma membranes (22,24).

SDS-Polyacrylamide Gel Electrophoresis. Protein samples were analyzed on 10-13.5% linear gradient polyacrylamide slab gels, containing 0.1% SDS. Following electrophoresis at room temperature for 7.5hr at 25 mA the gels were fixed, stained in Coomassie blue and then destained as described by Owens and Haley (25). Standard protein molecular weight markers (Sigma Chemical Co.) were used for the determination of molecular weights.

Chlorpromazine was a gift from Kempthorne Prosser and Co. and amitriptyline a gift from LaRoche (N.Z.) Ltd.

RESULTS AND DISCUSSION

Human seminal plasma was heated at 85°C for 10 min and then assayed for its ability to activate the $(Ca^{2+}+Mg^{2+})$-ATPase, or Ca^{2+} pump, found in RBC plasma membranes (26,27). As shown in Table I, heat-treated seminal plasma increases the $(Ca^{2+}+Mg^{2+})$-ATPase activity by almost 400%. This compares favourably to the approximately 350% increase obtained with RBC hemolysate, a well defined source of calmodulin (11-13, 28). To examine the chemical nature of the $(Ca^{2+}+Mg^{2+})$-ATPase activating component present in human seminal plasma, a sample of heat-treated seminal plasma was

TABLE I

Effects of Seminal Plasma and Hemolysate on Erythrocyte Membrane ATPase Activity

Extract added	$(Ca^{2+}+Mg^{2+})$-ATPase Specific Activity[a]	Activation (% Change)[b]
--	1.25 ± 0.11[c]	--
RBC Hemolysate	5.60 ± 0.33	+348
Heat-treated seminal plasma[d]	5.89 ± 0.44	+371
Trypsin treated seminal plasma[e]	0.98 ± 0.08	-22

[a] Expressed as μmol ADP produced/mg RBC membrane protein/hr. The figures are the mean ±SD of at least 4 separate determinations.

[b] Compared to the basal level of activity, obtained in the absence of any exogenous modulators.

[c] In the absence of Ca^{2+} the specific activity was 0.10 ± 0.02 μmol ADP produced/mg RBC membrane protein/hr. Similar values were also obtained in the presence of excess EGTA (0.2mM).

[d] Heat-treated as described in Methods.

[e] Heat-treated seminal plasma was incubated with trypsin (1 mg/ml) at 25°C for 45 min. After addition of soybean trypsin inhibitor (1 mg/ml) the sample was centrifuged at 5,000g for 10 min and the supernatant tested as described in Methods.

TABLE II

The Effect of Various Compounds on Seminal Plasma Induced Activation of Erythrocyte Membrane ATPase

Additions[a]	$(Ca^{2+}+Mg^{2+})$-ATPase Specific Activity[b]	Activation (% Change)[c]
Heat-treated seminal plasma	5.89 ± 0.44	+371
Heat-treated seminal plasma plus Chlorpromazine	1.33 ± 0.11	+6
Heat-treated seminal plasma plus Amitriptyline	1.30 ± 0.11	+4
Heat-treated seminal plasma plus Theophylline	0.92 ± 0.09	-26

[a] Present at the concentrations detailed in Methods.

[b] Expressed as μmol ADP produced/mg RBC membrane protein/hr. The figures are the mean ±SD of at least 4 separate determinations.

[c] Calculated as for Table I.

subjected to digestion with trypsin. Following incubation with trypsin heat-treated seminal plasma no longer increased the RBC $(Ca^{2+}+Mg2^{+})$-ATPase activity (Table I), indicating that the seminal plasma plasma $(Ca^{2+}+Mg^{2+})$-ATPase stimulating factor is proteinaceous.

Activation of the RBC plasma membrane Ca^{2+} pump by calmodulin requires a Ca^{2+}-dependent protein-protein association (9). This activation can be blocked by using either EGTA to chelate Ca^{2+} ions or the tricyclic drugs, chlorpromazine and amitriptyline, to inhibit calmodulin interaction with $(Ca^{2+}+Mg^{2+})$-ATPase (29). In our investigations we found that both of these treatments eliminated the ability of the human seminal factor to activate the RBC plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase. RBC $(Ca^{2+}+Mg^{2+})$-ATPase activity was reduced to near basal levels of activity (Table II) in the presence of heat-treated seminal plasma when chlorpromazine was added. Amitriptyline produced similarly low levels of activity (Table II). Experiments using purified ram sperm tail membranes confirm the presence of a $(Ca^{2+}+Mg^{2+})$-ATPase in sperm plasma membranes (24). Heat-treated

human seminal plasma increases the activity of this enzyme while chlorpromazine and amitriptyline again reduce activity to near basal levels.

The above results show that a component of human seminal plasma has many of the properties associated with calmodulin. These include (a) heat stability (b) sensitivity to trypsin digestion (c) ability to activate the $(Ca^{2+}+Mg^{2+})$-ATPase, or Ca^{2+} pump, present in many plasma membranes and (d) an abolition of $(Ca^{2+}+Mg^{2+})$-ATPase activation when incubated with chlorpromazine or amitriptyline. Although the identification of calmodulin-like activity in seminal plasma is interesting our results have far greater implications. Mammalian sperm taken from the caput region of the epididymis are quite different from sperm found in either the caudal epididymis or in the ejaculate (1,3,30). Only the two last named sperm types are capable of coordinated forward motility. Sperm from the caput region of the epididymis require the addition of both seminal plasma proteins plus methylxanthines, e.g., theophylline or caffeine, to achieve coordinated motility (2,31,32). Publications by Hoskins and his coworkers (2,32-34) have supported the concept of a seminal plasma protein factor (forward motility protein or FMP) which is largely responsible for inducing sperm motility. We propose that it is very possible that the three proteins calmodulin, FMP and the heat stable seminal plasma protein, found in our experiments, are one and the same. Support for this hypothesis is provided by the common physical properties of all three protein factors. For example FMP is a heat stable, acidic protein known to occur in a wide range of tissues, but with highest specific activity in seminal plasma, and caudal epididymal fluid (32). The molecular weight of approximately 37,000 published for FMP (32-34) was determined by Sepharose column chromatography and is about double the molecular weight of calmodulin (10). Because calmodulin is a rod-like molecule, determination of its molecular weight by molecular sieve chromatography will give anomalously high values (10), which would resolve this disparity in molecular

weights. When we examined heat-treated human seminal plasma using electrophoresis in linear 10-13.5% polyacrylamide gels, more than 15 protein species were identified but by far the most predominant species had a molecular weight of approximately 18,200. This is consistent with previously published values of 17,000 to 19,000 for calmodulin (10).

Assays to determine FMP activity, generally involve using high concentrations of theophylline, (15 - 33mM) to increase intrasperm levels of cAMP (32,33). However we found that 3mM theophylline totally eliminates the ability of heat-treated seminal plasma to activate the RBC plasma membrane (Ca^{2+}+Mg^{2+})-ATPase (Table II). In fact, the activity with added theophylline was 26% lower than the basal (i.e., no factor added) value for RBC plasma membrane (Ca^{2+}+Mg^{2+})-ATPase activity (Table I). At present we cannot explain this effect.

Assuming calmodulin is a critical factor incorporated into mammalian sperm during epididymal maturation, an explanation for some of the concomitant metabolic changes is possible. The increased impermeability of ejaculated sperm to Ca^{2+} would reflect calmodulin-induced activation of the sperm plasma membrane (Ca^{2+}+Mg^{2+})-ATPase. Activation of this enzyme would lower intrasperm Ca^{2+} levels which, in turn, would have a direct stimulatory effect upon the dynein-ATPase of the sperm flagella (35). Similarly the increased levels of intrasperm cAMP associated with sperm maturation would be consistent with a calmodulin-induced stimulation of adenylate cyclase (9). Our laboratory is currently investigating these metabolic interactions.

Our contention that calmodulin enters or binds to the plasma membrane of mammalian sperm from the seminal plasma (and probably also from the fluids of the caudal epididymis) is contrary to the normally accepted view of calmodulin being a cytoplasmic protein, but the spermatozoon is a highly differentiated cell virtually devoid of a normal cytosolic compartment. This proposal is supported by the report that in some mammalian

sperm the only detectable calmodulin is bound to the acrosomal membrane (16). The molecular modifications required to facilitate extracellular incorporation of calmodulin into the sperm plasma membrane may well explain the reported existence of a carbohydrate moiety in FMP (32,33).

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