Acrosome membrane associated protein kinase activity in hamster spermatozoa

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Abstract Acrosomal membranes isolated from the caput and cauda epididymal spermatozoa of hamster exhibited protein kinase activity and the endogenous protein substrates that were phosphorylated in the acrosomal membranes of caput and cauda spermatozoa were not all the same. The kinase activity was identified as a cAMP independent type and the use of specific stimulators and inhibitors indicated that the activity was not due to casein kinase, protein kinase A or protein kinase C but due to a tyrosine specific protein kinase that was not inhibited by Genistein. Phosphotyrosine was identified as the predominant phosphorylated residue in the proteins.

Key words: Phosphorylation; Sperm maturation; Membrane protein; Protein kinase; Acrosomal membrane; Hamster spermatozoa; Tyrosine specific protein kinase

1. Introduction

The mammalian sperm acrosome reaction is essential for fertilization. It is an exocytotic event involving the fusion of the outer acrosomal membrane with the distal sperm head plasma membrane and the simultaneous activation and release of hydrolytic enzymes. However, as yet, the molecular basis underlying the sperm acrosome reaction is not clearly understood. Recent studies have clearly indicated that phosphorylation of proteins in spermatozoa, especially at the tyrosine residue, is an important regulatory event that modulates events associated with capacitation such as the acrosome reaction and fertilizing ability of spermatozoa [1–3]. Further, inhibition of protein tyrosine kinase activity was observed to prevent acrosomal exocytosis [4] and the fertilizing ability of spermatozoa. Thus, it is likely that phosphorylation of proteins in both the plasma membrane and acrosomal membrane, the two membranes involved in acrosomal exocytosis, may be a prerequisite for the acrosome reaction. Though studies have been directed specifically towards identification of the enzyme tyrosine protein kinase and its substrates in the plasma membrane of spermatozoa [1,2,5,6] nothing is known about either the enzyme or its substrates in the acrosomal membranes of spermatozoa.

The present study demonstrates for the first time that the acrosomal membranes from caput and cauda epididymal spermatozoa of hamster possess protein kinase activity and the endogenous proteins that are phosphorylated vary during epididymal transition. The kinases in the acrosomal membranes of both the caput and cauda epididymal spermatozoa were of cAMP independent type and analysis of the phosphorylated proteins indicated that in the majority of the proteins the predominant phosphorylated amino acid residue was tyrosine.

2. Materials and methods

2.1. Chemicals

Phosvitin, casein, histones, spermidine, phosphatidylserine (PS), diolein (DO), ATP, cAMP, and phosphoamino acid standards (pY, pT, pS) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other reagents were of the highest analytical grade.

2.2. Isolation of membranes

The plasma and acrosomal membranes of the caput and cauda epididymal spermatozoa of hamster were isolated following essentially the method described by Zahler and Doak [7]. The purity of the above membranes was judged by assaying the activity of various membrane bound enzymes which served as markers of sperm plasma and acrosomal membranes [8–10].

2.3. Protein kinase assay

The method used was essentially according to Noland et al. [5]. The assay mixture contained 32 mM Tris pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 20 μM ATP, 10 μCi [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol) and 100 μg of membrane protein and was incubated at 30°C for 5 min. The reaction was terminated by the addition of 25 μl of 5×SDS-PAGE sample buffer and subsequently the samples were electrophoresed on a 10% SDS-polyacrylamide gel [11], stained, dried and the phosphorylated proteins visualized by autoradiography. In experiments where the protein kinase activity of the membranes was checked using exogenous substrates such as casein, histone or phosvitin the membrane concentration was reduced to 50 μg so that the phosphorylated membrane proteins would not interfere with the exogenous substrate bands in the autoradiogram.

In order to quantitate the kinase activity the reaction was stopped by adding TCA to a final concentration of 25%. The resulting precipitate was then washed with 5% TCA (3 or 4 times), dried with diethyl ether and the radioactive counts in the absence of a scintillant were determined using a scintillation counter for Cerenkov radiation [12].

2.4. Analysis of phosphoamino acids

Phosphoamino acids of the phosphorylated proteins were determined following partial acid hydrolysis (6 N HCl for 3 h at 110°C) of the proteins after which the HCl was removed and the hydrolysate (1 μl) was analyzed by ascending chromatography on a precoated silica gel plate (Merck) by the method of Munoz and Marshall [13]. The plate was dried, autoradiographed and the resolved phosphoamino acids were identified based on the mobilities of phosphoamino acid standards (phospho-ser, phospho-thr and phospho-tyr 5 mg/ml) which were run simultaneously.

2.5. Western blot analysis

Acrosomal membrane proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, incubated with monoclonal anti-phosphotyrosine antibody (Promega, USA), washed and the antibody was detected using alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, USA) [14].

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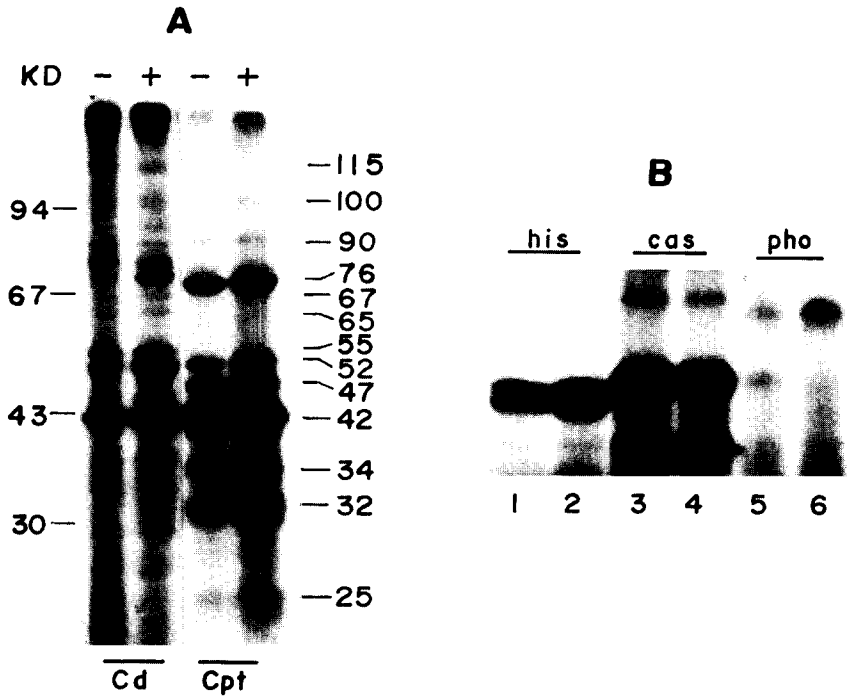

Fig. 1. A: Phosphorylation of the endogenous proteins of the acrosomal membranes of the Caput (Cpt) and Cauda (Cd) epididymal spermatozoa of hamster in the presence (+) or absence (−) of NP-40 (0.05%). The protein molecular weight markers used were phosphorylase b (94 kDa), bovine serum albumin (66 kDa), egg albumin (42 kDa) and carbonic anhydrase (30 kDa). B: Phosphorylation of histones (his), casein (cas) and phosvitin (pho) by the acrosomal membranes (50 μg) of the caput (lanes 1, 3 and 5) and cauda (lanes 2, 4 and 6) epididymal spermatozoa of hamster. The autoradiograms were developed in such a way as to avoid interference by the phosphorylated proteins of the acrosomal membranes.

3. Results and discussion

Acrosomal membranes isolated from hamster epididymal spermatozoa were enriched with respect to the enzymes Na^+/K^+-ATPase, Mg^{2+}-ATPase, 5′-nucleotidase and alkaline phosphatase which are general marker enzymes of membranes. In addition, the membranes were associated with high activity of acrosin and hyaluronidase which are marker enzymes for acrosomal membranes and lacked Ca^{2+}-ATPase activity, a specific marker for hamster sperm plasma membrane [10]. These purified acrosomal membranes of hamster spermatozoa upon incubation with [γ-^{32}P]ATP, incorporated ^{32}P label into a number of membrane proteins in the absence of any exogenous kinase indicating that the acrosomal membrane possessed a protein kinase activity that caused the transfer of the terminal phosphate of ATP to the endogenous proteins in the membrane (Fig. 1A). Further, the time dependent phosphorylation studies indicated maximum phosphorylation within 3 min in the acrosomal membranes of both the caput and cauda epididymal spermatozoa.

Earlier investigators had provided evidence for the presence of protein kinases on the surface of goat, ram, boar, bovine

Table 1
Effect of various activators and inhibitors of protein kinases on the incorporation of ^{32}P into acrosomal membrane proteins of caput and cauda epididymal spermatozoa of hamster[a]

Effectors	^{32}P Incorporation into membrane proteins		
	Concentration	Caput sperm acrosomal membrane	Cauda sperm acrosomal membrane
		Activity (% control)	Activity (% control)
cAMP[b]	100 μM	109 ± 4	92 ± 8
H8[b]	20 μM	84 ± 12	87 ± 10
Spermine	200 μM	100 ± 3	104 ± 19
Spermidine	200 μM	105 ± 1	90 ± 9
Heparin	2 μg	100 ± 3	103 ± 5
H7[c]	20 μM	97 ± 11	105 ± 14
Genistein[d]	6 μg	98 ± 3	103 ± 6
Vanadate[d]	100 μM	102 ± 6	109 ± 5

[a]The caput and cauda sperm acrosomal membranes were incubated separately in the presence and absence of various effectors. The values are a mean of triplicate measurements from a single experiment and representative of three other experiments that gave similar results. The counts in the controls were in the range of 2–2.2 × 10^4 cpm/mg protein/min.
[b]Assay was done in the presence of 20 μM cAMP.
[c]Assay mixture included 10 μg of phosphatidyl serine and 0.2 μg of diolein.
[d]Assay mixture included 5 mM Mn^{2+} and 100 μg of poly glu-tyr.

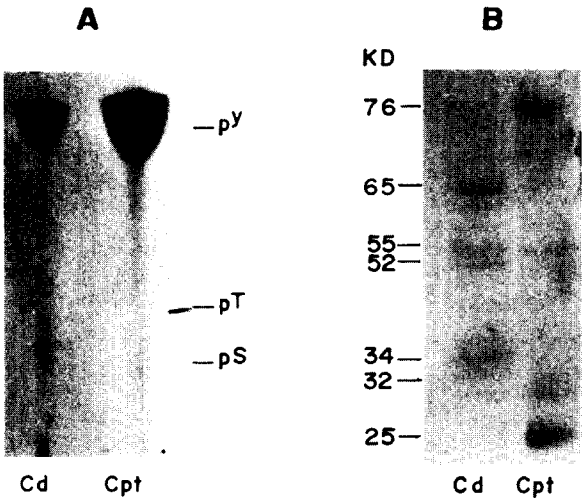

Fig. 2. A: Phosphoamino acid analysis of the phosphorylated proteins of the acrosomal membranes of caput (Cpt) and cauda (Cd) epididymal spermatozoa of hamster. The resolved amino acids were identified based on the mobilities of the phosphoamino acid standards (phospho-ser, pS; phospho-thr, pT and phospho-tyr, pY). B: Western blot analysis of the acrosomal membrane proteins of caput (Cpt) and cauda (Cd) epididymal spermatozoa of hamster using antiphosphotyrosine antibody. The molecular weights of the proteins detected by the antibody are indicated on the left.

and human spermatozoa [5,6,15–18]. But in this report, we provide evidence for the first time for an acrosomal membrane associated protein kinase activity in hamster spermatozoa. Our data clearly indicate that in the acrosomal membranes of caput epididymal spermatozoa, nine proteins are phosphorylated (100, 90, 76, 52, 47, 42, 34, 32 and 25 kDa) (Fig. 1A). In the acrosomal membranes of cauda epididymal spermatozoa also nine proteins are phosphorylated out of which five proteins (100, 90, 76, 52 and 42 kDa) are also observed in the caput and four other proteins (115, 67, 65 and 55 kDa) were found to be phosphorylated only in the acrosomal membranes of cauda spermatozoa (Fig. 1A). This differential phosphorylation of the proteins in the acrosomal membrane of spermatozoa upon maturation is probably indicative of a modification of the kinase activities or the substrate proteins or both in the membranes. It was also observed that in the presence of 0.05% NP-40 the phosphorylation of the acrosomal membrane proteins of the caput and cauda epididymal spermatozoa increased distinctly possibly due to the removal of an endogenous protein kinase inhibitor, activation of the kinase by the detergent or the inhibition of a possible protein phosphatase. Further, the acrosomal membranes of caput and cauda spermatozoa were capable of phosphorylating the exogenous substrates histone, casein and phosvitin (Fig. 1B).

Earlier studies on the intact spermatozoa of rat [19], dog [20,21], goat [22], bull [5,23] and man [16] had provided conclusive evidence for the presence of cAMP independent protein kinases in these spermatozoa, and they were also shown to be associated with the sperm plasma membrane [29,21]. Protein kinase activity in the acrosomal membranes of hamster spermatozoa was also not seen to be stimulated by cAMP or inhibited by H8 (Table 1), an inhibitor of protein kinase A, thus suggesting that in the hamster sperm acrosomal membranes the protein kinase activity was of the cAMP independent type. Since cAMP dependent kinases have been identified in both the membrane and soluble fractions of mammalian spermatozoa and have been shown to be involved in the regulation of motility [29,21], it is not surprising that cAMP dependent kinases were absent in the acrosomal membrane of hamster spermatozoa which has nothing to do with motility.

Polyamines like spermine and spermidine are known to be present in the mammalian spermatozoa and have been implicated in sperm acrosome reaction [24] probably by activating a polyamine dependent protein kinase like casein kinase. However, in the present investigation, it was observed that spermine and spermidine which are stimulators of casein kinase II did not stimulate the overall phosphorylation of the acrosomal membrane proteins of caput and cauda epididymal spermatozoa of hamster, thus implying the absence of casein kinase II in both membranes. Heparin, an inhibitor of casein kinase, also had no effect on the kinase activity of the acrosomal membranes.

Protein kinase C, a kinase which is calcium activated and phospholipid dependent, has been implicated in the motility and acrosome reaction of spermatozoa [25,26]. However, in the present study it was observed that H7, an inhibitor of the same enzyme, did not inhibit the protein kinase activity of hamster sperm acrosomal membranes.

Very few studies have been carried out so far to identify the amino acid residues which are phosphorylated in the proteins of spermatozoa. In goat spermatozoa, the protein kinases phosphorylated only serine and threonine residues [18,22] but in addition to these residues phosphotyrosine was also detected in certain phosphoproteins of spermatozoa of mouse, rat, rabbit, boar and man [1,18,27]. In the present study, phosphoamino acid analysis of the phosphorylated acrosomal membrane proteins of hamster spermatozoa indicated that phospho-tyr was the predominant residue although phospho-ser and phospho-thr were also present in trace amounts (Fig. 2A). Thus it appears that protein tyrosine kinase is the predominant kinase activity in the acrosomal membranes of hamster spermatozoa. However, Genistein, an inhibitor of protein tyrosine kinase, did not inhibit this activity. Though the reason for this discrepancy is not clear it is worthwhile to mention that it has been reported that Genistein does not inhibit all types of protein tyrosine kinases [28]. The activity of protein tyrosine kinases is known to be regulated by specific protein tyrosine phosphatases and these phosphatases are known to be sensitive to vanadate. However, the kinase activity of the acrosomal membranes was not sensitive to vanadate (Table 1) indicating the absence of any tyrosine specific phosphatase activity that could be responsible for the differential phosphorylation observed in the acrosomal membranes of the caput and cauda spermatozoa.

The presence of phosphotyrosine containing phosphoproteins in the acrosomal membranes of both caput and cauda epididymal spermatozoa (Fig. 2B) was further confirmed by Western blot analysis using monoclonal antiphosphotyrosine antibody. The predominant proteins in the caput acrosomal membrane that cross-reacted with the antibody had molecular weights of 25 and 76 kDa whereas in the cauda membranes, proteins of 34, 55 and 65 kDa cross-reacted with the antibody. The antibody was specific as evidenced by the fact that its binding was not observed in the presence of excess of *O*-phospho-L-tyrosine. Further, the second antibody (alkaline phos-

phatase conjugated goat anti-rabbit IgG) by itself also did not cross-react with any of the acrosomal proteins. Recent studies have suggested that phospho-tyr containing phosphoproteins are involved in spermatozoal functions such as capacitation, acrosome reaction and fertilizing ability [1–3,29–32].

In conclusion, this report establishes for the first time the presence of a cAMP independent protein kinase activity in the acrosomal membranes of hamster spermatozoa. The protein kinase was neither a casein kinase, protein kinase A nor protein kinase C but a tyrosine specific protein kinase. These observations are significant considering that protein tyrosine kinases have been implicated in the acrosome reaction of spermatozoa [1–4].

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