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Cell surface phosphorylation by a novel ecto-protein kinase: A key regulator of cellular functions in spermatozoa

Debjani Nath [a,*], Arunima Maiti [b], Gopal C. Majumder [b]

[a] Department of Zoology, University of Kalyani, Kalyani, Nadia, PIN. 741235, West Bengal India
[b] Indian Institute of Chemical Biology, 4 Raja S C Mullick Road, Jadavpur, Kolkata 700032, India

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Abstract

Since 1976 many studies have been reported on the occurrence and functional significance of ecto-protein kinases in a variety of cell types although their precise biochemical identity is largely unknown. This study reports for the first time purification to apparent homogeneity of an ecto-protein kinase (ecto-CIK) and some of its characteristics using caprine sperm as the cell model. The ecto-CIK is a unique membrane-specific serine/threonine protein kinase. It is a strongly basic 115 kDa protein made up of two subunits: 63 and 55 kDa. The ecto-kinase undergoes a remarkable lateral movement on the outer cell surface culminating in capping on the sperm acrosomal tip. MPS, its major protein substrate is also located on the acrosomal tip. Both ecto CIK and MPS serve as potential regulators of flagellar motility. This novel enzyme appears to be major kinase responsible for the reported regulation of mammalian cellular functions by modulating phosphorylation of the membrane-bound proteins.

1. Introduction

Recent studies from our laboratory have shown the epididymal spermatozoa is a potentially important model for investigation on the role of cell surface enzymes that modulate the phosphorylated states of proteins [1]. Cyclic AMP-dependent protein kinases have been demonstrated on the external surface of spermatozoa derived from rat [2,3], human [4,5] and goat [6]. However, these ecto-protein kinases are incapable of phosphorylating the endogenous PM-bound proteins [6,7]. Previous studies from our laboratory provided several lines of evidences for the occurrence of a cAMP-independent protein kinase (ecto-CIK) on the external surface of goat epididymal spermatozoa that causes phosphorylation of the serine and threonine residues of multiple membrane-bound phosphoproteins [7–9]. A preliminary study has been reported on the partial purification of the sperm ecto-CIK [10]. An ecto-phosphoprotein phosphatase has also been demonstrated on the sperm surface [11–13]. Another study has shown that this ecto-CIK and a phosphoprotein phosphatase (PPase) act in concert as a coupled-enzyme system to modulate the phosphorylated states of the ecto-phosphoprotein substrates[14]. The specific activities of these ecto-CIK and PPase have been found to increase markedly during forward progression of spermatozoa, suggesting thereby that these ecto-enzymes and their substrate proteins may have a role in modulating sperm motility [7,8,12].

We present here for the first time the purification of an ecto-CIK to apparent homogeneity from the plasma membrane of goat spermatozoa that has high affinity for the phosphorylation of membrane associated proteins. Studies have also been carried out to elucidate some of its biochemical characteristics and functional significance.

2. Materials and methods

ATP (horse muscle), GTP, dATP, ADP, cyclic GMP, cyclic AMP, casein, phosvitin, protamine, calf thymus histone, O-phosphoserine, O-phosphothreonine, O-phosphotyrosine, rabbit skeletal muscle protein kinase inhibitor, phenyl methyl sulphonyl fluoride (PMSF), β-mercaptoethanol, Triton X-100, SDS, Polyethylene glycol, DEAE-cellulose, ethanolamine, EGTA, spermine, spermidine, putrescine, poly (Glu: Tyr, 4:1), TEMED, ammonium persulphate, heparin, phospholipids, diolein, calmodulin, SDS markers (MW-SDS-200), gel filtration markers (MW-GH-200), ampholine (pH 8–10.8) and Sephacryl S-300 (obtained

* Corresponding author. Tel.: +91 33 24109031; fax: +91 33 25828282.
E-mail address: nath_debjani@yahoo.co.in (D. Nath).

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from Sigma Chemical Co). Poly buffer exchangers (PBE-118) and (PBE-74) were obtained from Pharmacia fine chemicals, Uppsala, Sweden; Sepharose 4B (activated with CNBr and coupled with casein); [γ^{32}-P] ATP (prepared according to the procedure described earlier [15]. Bovine serum albumin, Tween-20, orthophenyl-diamine, 4-chloro-1-napthol, hydrogen peroxide, HRP-conjugated anti rabbit IgG, orthophenylendiamine, ficoll-400, complete and incomplete Freunds adjuvants, gelatine, ammonium sulphate.

2.1. Isolation of goat epididymal mature sperm

Goat cauda-epididymal mature spermatozoa were isolated within 2 h of slaughter [16,17]. Each part of epididymis was cut to 4-5 pieces with a sharp razor blade and then suspended in a modified Ringer's solution (RPS medium: 119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate, 50 unit/ml penicillin, pH 6.9) with a gentle stirring. After 5 min, the suspension was filtered through four layers of cheesecloth and spermatozoa were sedimented by centrifugation at 500×*g* for 1 min. The cell pellet was washed two times with RPS medium and finally dispersed in the same medium. The isolated spermatozoa were highly pure as judged by the phase-contrast microscopy. There was no detectable contamination with other cells or cell-fragments.

2.2. Isolation of sperm plasma membrane

Highly purified plasma membrane was isolated from the mature cauda and maturing corpus and caput spermatozoa by an aqueous two-phase-polymer method [16,18]. Membrane purity was judged by estimating marker enzymes: alkaline phosphatase, 5-nucleotidase, acrosin, cytochrome C-oxidase, glucose-6-phosphatase and by electron microscopic study. The specific activities of the plasma membrane bound 5-nucleotidase and alkaline phosphatase were 10–14 fold higher in the isolated PM than in the cell debris, indicating marked membrane enrichment. There was no detectable amount of acrosin and glucose-6-phosphatase in the isolated PM and specific activity of cytochrome oxidase was nearly 7-fold lower in membrane than in the cell debris. The data show that there is little contamination of PM with acrosome, mitochondria and endoplasmic reticulum. Electron microscopic studies also showed high degree of purity of the isolated sperm PM. The membrane preparation was finally dispersed in 25 mM potassium phosphate buffer, pH 7.0, containing 1 mM PMSF, 2 mM β-mercaptoethanol, 1 mM EDTA 30% (v/v) glycerol and were stored at −20 °C. The protein content of the plasma membrane was estimated using BSA standard [19].

2.3. Purification of goat sperm membrane ClK

Plasma membrane after isolation was dissolved in 25 mM K-Po4, pH 7.0 containing 1 mM PMSF, 2 mM β -mercaptoethanol, 30% glycerol and stored at −20 °C to maintain the stability of the enzymatic activity. All the purification steps were carried out at 0–4 °C.

The isolated plasma membrane (concentrated approximately 10 mg/ml) were solubilized using 1% Triton X-100 and kept in ice for 1 h with intermittent stirring. Then the sample was centrifuged at 27,000×*g* for 90 min at 4 °C. The resulting supernatant was loaded on a DEAE cellulose column (1 × 10 cm) equilibrated with 5 mM K-Po4 pH 7.0 containing 1 mM PMSF, 2 mM β-mercaptoethanol, 20% glycerol and 0.1% triton X-100 (buffer A). The activity peak was eluted in unretained fraction. The unretained fraction was then chromatographed over a casein-Sepharose 4B column (0.9 × 5 cm), primarily equilibrated with the same buffer used in DEAE column or buffer "A". The column was eluted with discontinuous gradient of 0.1 M, 0.2 0.3 M, 0.5 M and 1 M NaCl. The activity peak was eluted with 0.2 M NaCl-buffer A.

Pooled fraction of isolated kinase, eluted at 0.2 M NaCl were concentrated by Diaflo Ultrafiltration using PM 30 membrane (Amicon). The concentrated elute was dialysed against pharmalyte–HCl pH 8.0 (1:45) or eluent buffer and loaded on a ion exchange resin PBE 118 (0.7 × 10 cm or 3 ml), equilibrated with 0.025 M triethylamine–HCl pH 11.0 [20]. After passing the sample, the column was washed with eluent buffer in 1 ml fraction. The elution was monitored by measuring pH of each fraction using a pH meter as well as activity of enzyme. The CIK activity fractions were pooled and concentrated using PM 30 Diaflo ultrafiltration membrane.

The concentrated fraction, containing the CIK activity of step IV was subjected to Casein-Sepharose 4B column again to remove the ampholyte as well as for further enrichment of CIK activity. The affinity column was primarily equilibrated with buffer A. After passing the sample the column was washed thoroughly with buffer A and activity fraction was eluted using 0.2 M NaCl-buffer A like step III. The fractions containing CIK activity were concentrated using PM-30 diaflo ultrafiltration membrane (Amicon) and dialysed against buffer A and supplemented with 50% glycerol and kept at −20 °C until used. The first three steps of the purification method were developed as mentioned earlier with minor modification [10].

2.4. Assay of isolated CIK

The standard assay system contained 200 nmol of ATP containing 20–50.10^4 cpm, 2 μmol of magnesium chloride, 1 mg of casein, and 200 ng of isolated enzyme in a total volume of 0.2 ml of 50 mM tris–HCl buffer pH 9.0. The incubation was carried out at 37 °C for 5 min. When casein was used as substrate, the reaction was stopped by adding 0.1 ml 0.5% casein as carrier protein containing 250 mM K-phosphate–10 mM ATP and 2 ml 10% TCA. The radiolabelled protein was recovered by filtration through whatman no 1 filter paper washed with 5% TCA dissolved in scintillation fluid and counted for radioactivity.

One unit of CIK activity has been defined as the amount of the enzyme that catalyzes transfer of 10 pmol of ^{32}P from [γ ^{32}P] ATP to casein.

2.5. Purification of MPS

The major physiological substrate localized externally was purified from goat sperm plasma membrane [21]. The plasma membrane was phosphorylated by endogenous ecto-CIK. The assay medium contained 5 nmol γ-^{32}P-ATP containing 8–10 × 10^6 cpm, 2 μmol $MgCl_2$/l, 0.2 μmol EGTA/l, and 150–200 μg of plasma membrane in a total volume of 0.2 ml 50 nmol Tris–HCl l^{-1}, pH 8.5. The reaction mixture was incubated for 1 min at 37 °C. The reaction was stopped with 125 μmol l^{-1} potassium phosphate buffer (pH 7), 4 μmol l^{-1} of ATP to a final volume of 2 ml water.

The labelled membrane proteins ware solubilised with 1% Triton X-100 in 5 mM potassium-phosphate buffer (pH 7) containing 1 mM PMSF, 1 mM EDTA, 2 mM β-mercaptoethanol, 20%(v/v) glycerol. The solubilised labelled plasma membrane was applied to Sephacryl S-300 column (1.4 × 75 cm) equilibrated previously with 5 mM Tris–Cl, pH 8 containing 20%(v/v) glycerol, 1 mM PMSF , 2 mM β-mercaptoethanol, 1 mM EDTA and 0.05% (v/v) Triton X-100 (Buffer A). The fractions containing the highest radioactive protein peak were then subjected to DEAE cellulose column (1 × 5 cm) previously equilibrated with buffer A. The major radioactive peak was eluted at 60 mM NaCl, concentrated and dialysed against PBE-74-HCl (pH 4) or eluent buffer and subjected to chromatofocusing using PBE-94 (0.7 × 10 cm) equilibrated with 0.025 M imidazole–HCl, pH 7. The radioactive fractions were concentrated by ultrafiltration and used for further studies. All procedures ware performed at 4 °C and the concentrated MPS were preserved in buffer A at −20 °C until further use.

2.6. Polyacrylamide gel electrophoresis under non-denaturing conditions

To check the homogeneity, the isolated cyclic AMP independent protein kinase (CIK) obtained from step V was analyzed by non denaturing (Without SDS) polyacrylamide gel electrophoresis, with suitable modification. Before application of the sample the gel was pre-electrophoresed for 30 min with a constant current of 20 mA. The purified enzyme was given at different dose (3 μg, 6 μg, 15 μg and 30 μg) on the polyacrylamide gel and electrophoresis towards the cathode was carried at 6 °C with a constant current of 20 mA/gel using pyronine Y as the tracking dye. Electrophoresis was generally completed within 8 h. After completion, one lane of the gel was sectioned for the assay of activity. For elution of the kinase activity, each gel slice (2 mm thickness) was dispersed in 0.2 ml of buffer A overnight at 6 °C and the eluates were assayed for kinase activity. Remaining portion of the gel was stained with silver nitrate [22] for the detection of the protein band.

Table 1
Purification of CIK from isolated plasma membrane

Step	Total activity Units $\times 10^{-3}$	Total protein (mg)	Specific activity Unit/mg $\times 10^{-3}$	Recovery (%)	Fold purification
Plasma membrane	300	142	2.11	100	1
Triton extract	182	71	2.56	60.67	1.2
DEAE unbound	170	35.5	4.8	56.67	2.2
Casein -Sepharose 4B affinity Chromatography	166	1.8	91	55.33	43.2
Chromatofocusing	153	1.5	298	51	141.6
Re-affinity chromatography	145	0.43	326	48.33	154.7

2.7. Determination of molecular weight and subunit composition

The native molecular weight of CIK was estimated using a column of Sephacryl S-200 (0.9 × 50). The gel was equilibrated with tris–HCl buffer pH 9.0 containing 5% glycerol. 50 μg of isolated CIK was loaded on the column. Elution was carried out with the equilibration buffer at a flow rate of 3 ml/h. Fractions (1 ml) were collected and protein was monitored by activity measurement peak. The column was calibrated with known molecular weight markers like β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa).

To determine the subunit composition the isolated kinase the factor was subjected to SDS-PAGE according to Laemmli et al. [23]. Markers, used for determination of molecular weight of the CIK subunits, were β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (45 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa). To specify the purity the isolated CIK was applied in three successive lanes (5 μg, 10 μg, 25 μg). After completion of run the protein bands were detected by silver staining.

2.8. Determination of isoelectric point

To determine the isoelectric pH of the isolated CIK, isoelectric focusing in gel tube (0.6 × 11 cm) was carried out using a discontinuous sucrose density gradient containing 2% ampholine, pH 8–10.5. A 10% polyacrylamide gel base containing 2% ampholine was prepared at the bottom of the gel tubes to support the sucrose gradient. CIK activity (from step V) was added with 20% and 15% sucrose. Sucrose gradient was formed by layering successively 1 ml each of 22%, 15% and 10% sucrose on top of the polyacrylamide gel. Approximately 50 μg protein was loaded on each tube. Isoelectric focusing was carried out for 4 h at 200 V and 2 h at 400 V at 6 °C, using 0.1 N NaOH and 0.1 M acetic acid as the cathode and anode buffers respectively. After the run the gel base was punctured and fractions of 2 drops each were collected. An aliquot of the fraction was assayed for activity and the rest was diluted ten times with distilled water and pH of each fraction was measured by microelectrode pH meter.

2.9. Analysis of phosphoaminoacid of casein and MPS

The enzymatic phosphorylation of casein (whole casein) and boiled plasma membrane protein were performed by a 5 min incubation (unless indicated otherwise) in the presence of isolated CIK (5 μg), 200 nmol of ATP (60×10^4 cpm) and 2 μmol $MgCl_2$ at pH 9.0 under the condition previously described in standard assay condition. Casein and PM proteins were added as 0.5 mg/assay (unless otherwise indicated). The ^{32}P-products were precipitated with 15% TCA. The nature of phospho-amino acid (s) was analyzed according to Nath and Majumder [24].

2.10. Raising the polyclonal antibody against the purified CIK and purified physiological substrate (MPS)

Antiserum against the purified CIK and purified substrate were raised in rabbit by four successive injections. The immunoglobulins of the immune serum were precipitated twice with 50% ammonium sulphate and IgG was isolated by passing through DEAE-Sepharose Column. The purified IgG was collected in 0.25 M PBS pH 8.0 and dialysed against the same buffer overnight. The titre of antibody was determined by using western blot as well as ELISA [25] using HRP conjugated goat anti rabbit IgG as second antibody.

Monovalent antibody was produced by digesting the raised polyclonal antibody with papain [26]. 1 mg of purified MPS antibody was treated with 0.001 mg of crystalline papain in 1 ml of 10 mM sodium phosphate buffered

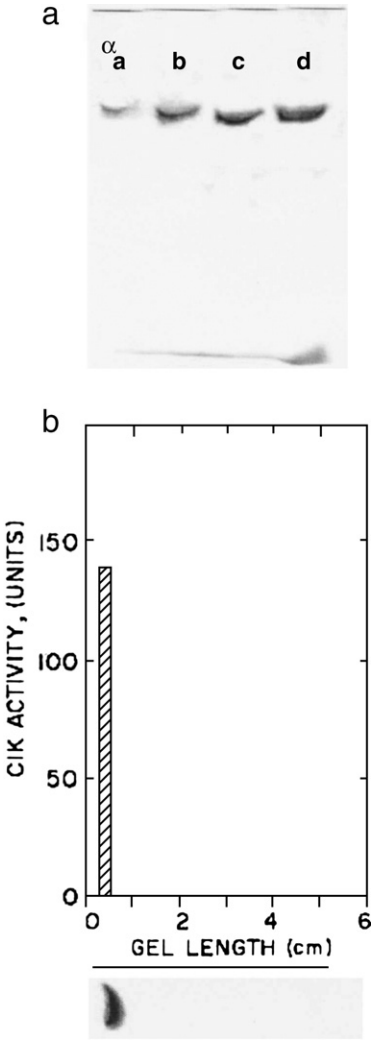

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of the purified CIK. CIK activity elution and staining of gel were carried out as described as Materials and methods. Standard assay method was used to measure CIK in each eluted fraction. Gel Electrophoresis pattern of CIK Lane a, 3 μg; Lane b, 6 μg; Lane c, 15 μg; Lane d, 30 μg. CIK activity measured in the gel slices.

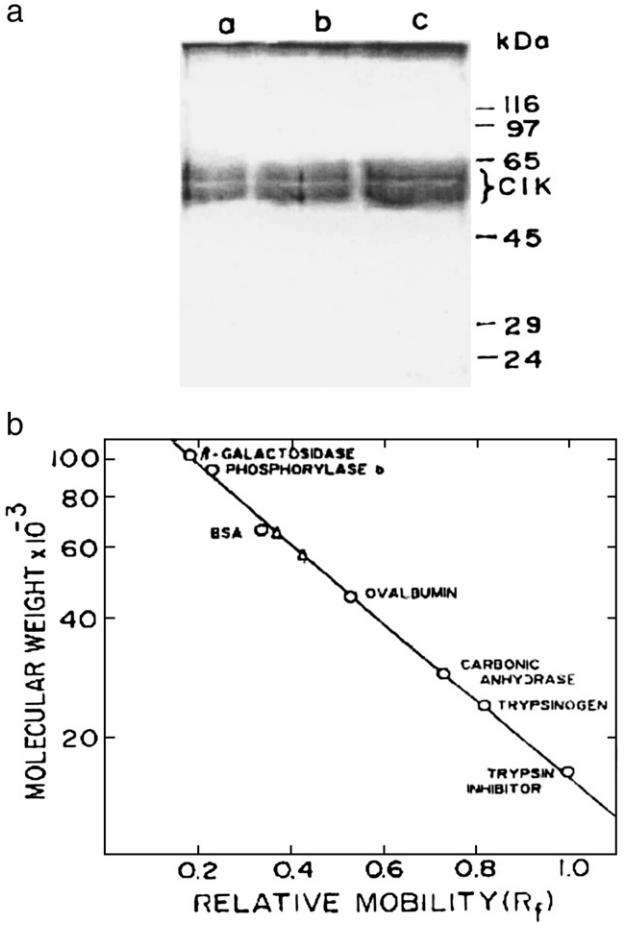

Fig. 2. (a) SDS-PAGE of CIK using 10% polyacrylamide gel. Markers were β-galactosidase (116 kDa), Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), Ovalbumin (45 kDa) Carbonic anhydrase (29 kDa), Trypsin inhibitor (20 kDa). Purified CIK 5 μg (lane a), 10 μg (lane b), 25 μg (lane c), were loaded in three successive lanes. (b) Determination of mol.wt. of CIK by SDS -PAGE.

0.15 M NaCl, pH 7.3 with 1 mM EDTA and 25 mM β-mercaptoethanol. The mixture was incubated at 37 °C for 1 h. Then 30 mM iodoacetamide was added and incubated at 37 °C for 15 min. The mixture was then applied on CM-cellulose column equilibrated previously with 10 mM acetate buffer, pH 5.5 after dialyzing the sample with the same buffer. The Fv fragment was eluted in the unbound fraction [27]. The purity of the Fv fragment of MPS antibody was confirmed by performing immuno-electrophoresis [28].

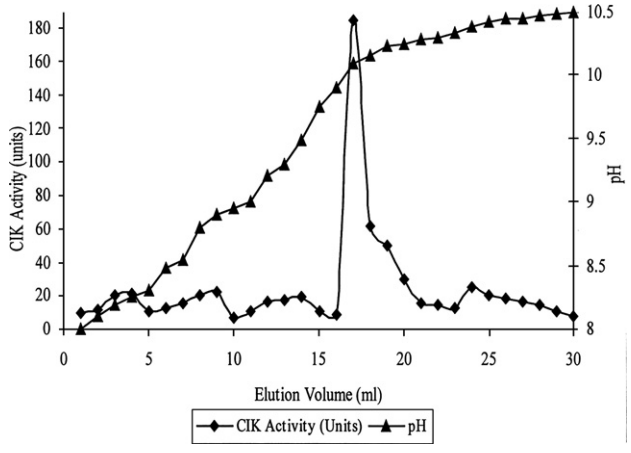

Fig. 3. Isoelectric focusing of CIK activity using sucrose density gradient technique. CIK activity obtained from step V was subjected to discontinuous sucrose gradient and focused as the procedure described in Materials and methods. The pH of each fractions was measured by microelectrode pH meter.

Table 2
Effect of different nucleotides and inorganic pyrophosphate on the phosphorylation of casein by isolated kinase

System	^{32}P-Incorporation in casein (count/min)
Control	1000
+ATP	91.2
+dATP	992
+GTP	901
+ADP	892
+AMP	871
+Na-pyrophosphate	850

Standard assay conditions were used with the alterations indicated. Each non-radioactive phosphate was used at 20 mM concentration. The data are representative of 5 such experiments.

2.11. Indirect immunofluorescence (IIF)

Goat sperm derived from corpus and cauda epididymis were collected in 50 mM PBS pH 7.4. Caput cells were isolated by using Ficoll gradient. The forward motile cells were isolated by layering cauda cell suspension containing 2% ficoll on the bottom of a 5-ml beaker having 4 ml RPS medium and after keeping the beaker at 37 °C for 15 min. Forward motile cells moved to the upper surface leaving the non forward motile cells on the bottom layer. In all cases the sperm suspension were centrifuged at 500×*g* for 5 min at 4 °C. The resulting sperm pellet containing approximately 5×10^6 sperm, were incubated in PBS containing 1% BSA for 30 min at 4 °C. After washing the sperm pellets were incubated in CIK antibody (1:50) in PBS containing 1% BSA at 4 °C for 1 h. The control experiment was run in the same way where the sperm cells were incubated with preimmune blood serum. After washing with PBS, FITC-conjugated anti rabbit IgG was added at a dilution of 1:40 and incubated again for 60 min at 4 °C. The cells were further washed in PBS and mounted in PBS pH 8.0 containing 90% glycerol, sodium azide

Table 3
General properties of the CIK

Assay system	Enzyme activity units	Enzyme activity %
Complete	210	100
$-Mg^{2+}$	4.2	0.47
$-Mg^{2+}$ +EDTA (5 mM)	0.12	0
$-Mg^{2+}$+Co (5 mM)	20	9.5
$-Mg^{2+}$ +Co (20 mM)	91	42.8
$-Mg^{2+}$+Mn (2 mM)	5.21	2.48
$-Mg^{2+}$+Mn (20 mM)	20.03	9.5
$+Zn^{2+}$ (5 mM)	184.0	87.6
$+Zn^{2+}$ (10 mM)	120.0	57.14
+NaF (10 mM)	205	97.61
Na3VO4 (100 μM)	200	97
EGTA (200 μM)	181	86.1
Ca^{2+} (100 μM)	199.25	94.8
Ca^{2+} (500 μM)	205.01	97.6
Ca^{2+} (1 mM)	200	95.2
Ca^{2+} (100 μM)+Calmodulin (5 μg)	192	91.4
+cAMP (5 μM)	190	88
+cAMP (10 μM)	182	86.1
+cGMP (5 μM)	200	97
+cGMP (10 μM)	205	97.61

Standard assay conditions were used except for the alterations indicated. The data are representative of 5 such experiments.

Table 4
Effect of different phospholipids on the activity of isolated protein kinase

System	Protein kinase activity (units) Meant±SEM
Control	200.7±0.1
+$CaCl_2$ (100 μM)	190.7±0.2
+$CaCl_2$+Phosphatidylserine (5 μg)	199±1
+$CaCl_2$ +Phosphatidylserine (5 μg)+Diolin (1 μg)	194±0.001
+$CaCl_2$ +Phosphatidylcholine (5 μg)	221±0.12
+$CaCl_2$ +Phosphatidylethanolamine (5 μg)	200.4±0.11
+$CaCl_2$ +Phosphatidylinositol (5 μg)	199.2±0.21
+Phosphatidylserine (5 μg)	210±0.4
(25 μg)	219±0.02
+Diolin (5 μg)	197±0.12
+Phosphatidylcholine (5 μg)	228±0.1
(25 μg)	202±1.01
+Phosphatidylethanolamine (5 μg)	201.21±0.05
(25 μg)	192±0.11

Standard assay conditions were used except for the additions indicated. These data are representative of 4 such experiments.

and 1 mg/ml p-Phenyldiamine to reduce photobleaching during observation. The fluorescence was visualized through the Leitz fluorescence microscope.

2.12. Microscopic and spectrophotometric assay of sperm flagellar motility

Percentage of forward motility was estimated by conventional microscopic method using a haemocytometer as a counting chamber. To estimate the possibility of artifact due to sperm adhesion to glass, motility assays were carried out in the presence of epididymal plasma to cause nearly 100% inhibition of sperm adhesion to glass [29]. Spermatozoa (0.5×10 cells) were incubated with epididymal plasma (EP) (0.6 mg protein/assay) in the presence of antibody of CIK or preimmune serum at RT for 10 min, 20 min, 30 min, 60 min and 2 h respectively in a total volume of 0.5 ml of RPS medium. A portion of the cell suspension was then injected into the haemocytometer. Immediately spermatozoa that showed well defined forward motility (FM cells) and total cell numbers were counted under a phase contrast microscope at 400× magnification. The percentage of FM cells was then calculated.

Forward motility of spermatozoa was quantitated using spectrophotometric method [30]. The microscopic method of motility assay described above takes into consideration the number of cells with forward progression but not their velocity, whereas the spectrophotometric method is based not only on the motile cell number but also on their velocity. The weakly motile cells are not detected by this method. The method consists of layering 50 μl of freshly extracted cauda epididymal spermatozoa (200×10^6/ml) mixed with 10% Ficoll in a total volume of 0.5 ml RPS medium with a Hamilton syringe at the bottom of a standard cuvette containing 1.3 ml of RPS medium which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa that moved upward in the light beam at any particular time were registered continuously as an increase of absorbance at 545 nm with a Gilford spectrophotometer equipped with the recorder. After reaching the maximum absorbance (Aeq) the content of the cuvette was mixed and the absorbance for all the cells was noted (At). The percentage of the cells that showed vigorous forward motility was calculated as Aeq/Atx100. The change of velocity after treatment with CIK antibody was measured according to change of forward motility activity. One unit of forward motility activity of the most vigorous group of spermatozoa (responsible for the first slope) was defined as an initial linear increase of absorbance of 0.01/min under standard assay condition. Specific activity of sperm forward motility was expressed as units of forward progression per 10^7spermatozoa.

Table 5
Substrate specificity of the purified CIK

Substrates	Protein kinase activity (units) Mean±SEM
Calf thymus histone (1.25 mg/ml)	20.6±0.5
Protamine (1.25 mg/ml)	30±0.1
Casein (1.25 mg/ml)	200±0.7
Phosvitin (1.25 mg/m)	180±1.1
Plasma membrane (Boiled) (0.25 mg/ml)	190±0.67
Poly (Glu Na:Tyr,4:1)(0.5 mg/ml)	0
(1 mg/ml)	0
Calmodulin (0.5 mg/ml)	0
(1 mg/ml)	0

Standard assay conditions were used except that casein was replaced by the specified concentration of different protein substrates. The data are representative of 3 experiments.

2.13. Protein estimation

Unless otherwise specified, the protein content of the samples were estimated according to Lowry et al. [31]. using bovine serum albumin as standard. Protein concentration of the samples containing glycerol and Triton were estimated by the method of Bensadoun and Weinstein [19] that involves precipitation of protein with TCA and Sodium-deoxycholate prior to the colorimetric assay of protein.

2.14. Statistical analysis

All experiments were repeated at least five times. The data were presented as the mean±SEM. Significance of difference between different treated and control groups were analyzed by paired Student's *t*-test.

3. Results

The summary of the purification of the CIK has been shown in Table 1. In the final step the isolated kinase showed approximately

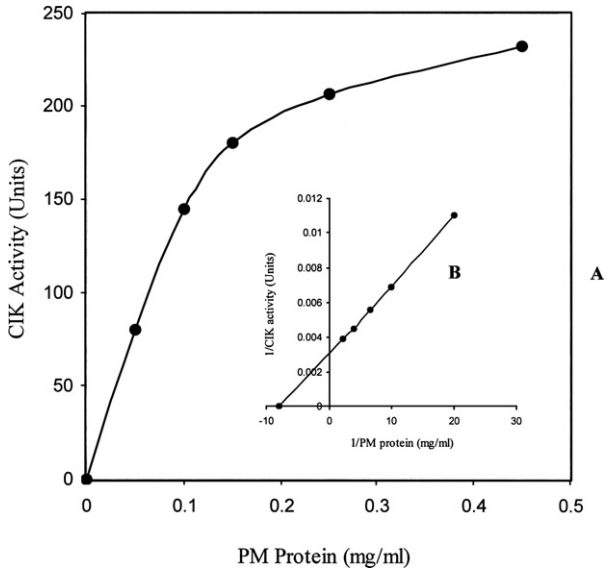

Fig. 4. (A) Effect of different conc. of MPS (Major plasma membrane protein substrate) on the activity of CIK. Standard assay conditions were used except boiled plasma membrane proteins used as substrate instead of casein. The insert shows the Lineweaver–Burk plot of these data. These data are representative of four such experiments. (B) Effect of different concentration of casein (approximately MW 23 kDa) on the activity of CIK. Standard assay conditions were followed using casein as the substrate. The insert shows the Lineweaver–Burk plot of these data. These data are representative of four such experiments.

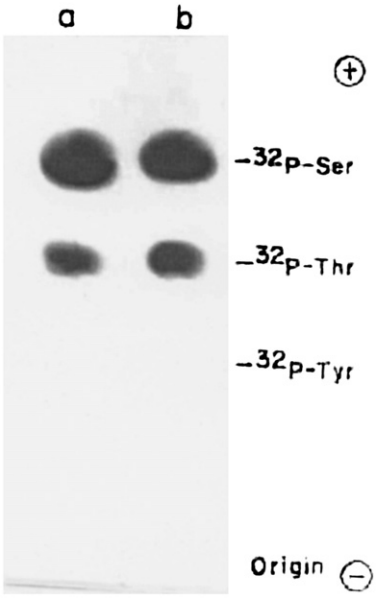

Fig. 5. Identification of the phosphorylated amino acid. Plasma membrane (boiled) lane a and casein (lane b) were phosphorylated under assay condition and ^{32}P-labeled products were analysed by cellulose thin layer chromatography, as described in Materials and methods. The position of authentic internal standards is indicated as p-Ser, phosphoserine, p-thr, phosphothreonine, p-Tyr, phosphotyrosine.

150 fold purification and 48% recovery of enzyme activity. A considerable loss of the total activity occurred during the purification procedure and this hindered a reliable determination of the specific activity increase of CIK. CIK as membrane kinase, is very difficult to handle and to maintain the membrane micro-environment we have added different preservatives including high concentration of glycerol and Triton X-100 in a cocktail (Buffer A) to overcome the possibility of loss of activity in isolated condition. The purified CIK (after step VI) showed a single protein band in all the three lanes indicating apparent homogeneity and comigration of activity peak with the band also confirms the homogeneity of the isolated enzyme (Fig. 1). The purity has also been suggested by the result of SDS gel electrophoresis, gel filtration chromatography, sucrose density gradient chromatography.

3.1. Physical properties of CIK

The molecular weight of the purified CIK as estimated by Sephacryl S-200 gel filtration was approximately 120±15 kDa (data not shown). SDS gel electrophoresis of purified CIK (5 μg, 10 μg, 25 μg) showed that the enzyme was made up of two subunits of about 63 kDa and 55 kDa (Fig. 2). Apparent stoichiometric analysis indicated that the subunits were in monomeric forms and the values were also consistent with the molecular weight obtained by the gel filtration chromatographic techniques. One activity peak was obtained when isolated CIK from step VI was subjected to sucrose density gradient according to Mitra et al. [10]. The fraction having pH 10.1 showed the activity (CIK) peak (Fig. 3).

3.2. Biochemical properties of CIK

The amount of casein phosphorylated by CIK increased linearly with time for a period of 5 min. A proportional increase in the activity of the enzyme was observed with at least as much as 250 units of CIK (data not shown). The enzyme showed maximal activity at pH 8.5–9.5. However further increase in pH up to 11.0 caused greater decrease in the activity of CIK.

The apparent Km value for ATP of CIK was approximately 33.3 μM (data not shown). Non radioactive ADP, AMP, dATP, GTP, Na-pyrophosphate (each 20 mM) had no effect on the

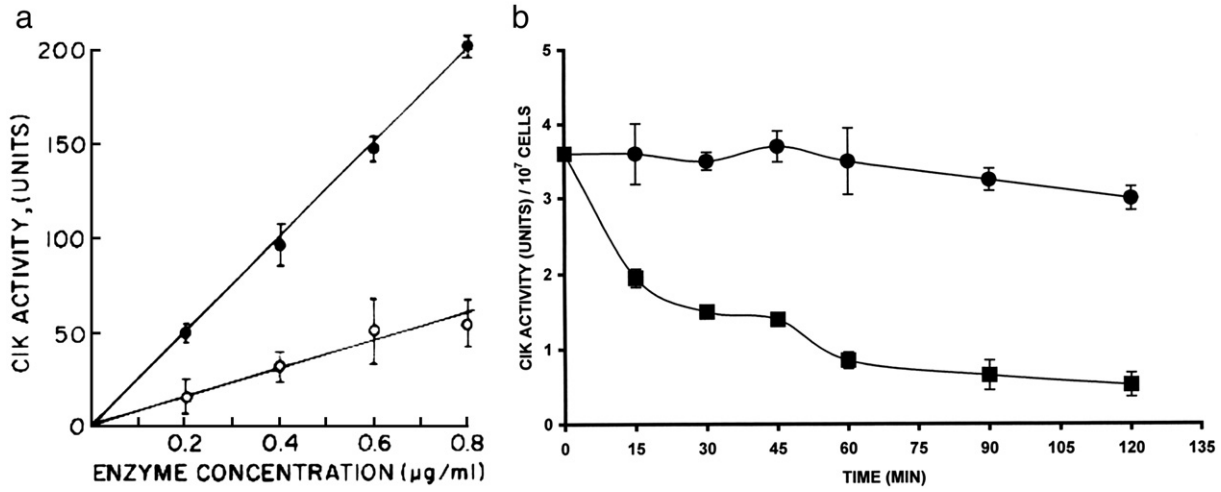

Fig. 6. Effect of CIK-antibody on kinase activity of (a) purified CIK and of (b) intact cauda epididymal sperm cells. CIK was incubated with antibody before estimation of kinase activity in standard assay conditions as described in Materials and methods. (○) Standard assay system for ecto-CIK activity in intact cells (■) contained 250 μg of casein, 20 nmol of ATP containing $50–80\times10^4$ cpm, 1 mol of magnesium chloride and intact spermatozoa ($7–8\times10^6$ cells) in a total vol of 0.2 ml RPS medium and incubation were carried out at 37 °C for 3 min. The reaction was stopped with addition of 0.1 ml of 1.5% casein containing 250 mM potassium phosphate and 10 mM ATP and 2 ml of 10% TCA. The resulting protein suspensions were then processed and enzyme activity was measured as mentioned in assay of 'Isolated CIK'. Preimmune rabbit serum incubated with CIK and intact cells were used as control preparation (●).

incorporation of ^{32}P from [γ^{32}-P] ATP to casein (Table 2) indicating that these reagents compete little with ATP as donor of phosphate for the kinase mediated protein phosphorylation reaction. As shown in Table 3, EDTA (5 mM) completely inhibited the activity of CIK indicating that the enzyme activity of isolated kinase is dependent on bivalent metal ions. CIK was activated maximally by Mg^{2+}. It could be activated by CO^{2+}, Mn^{2+} but could not replace Mg^{2+}. Ca^{2+} had no effect on enzymatic activity. Zn^{2+} (10 mM) inhibited about 45% of enzymatic activity in presence of Mg^{2+}. The apparent Km value for Mg^{2+} was approximately 5 mM . EDTA (10 mM), NaF (10 mM), VO_4 (50 μM, 100 μM) had no effect. We found that CIK preparation did not undergo autophosphorylation (data not shown).

3.3. Effect of different effectors

The kinase was not activated by cAMP (5 μM) or cGMP (5 μM), calmodulin (5 μg) and Ca^{2+} (100 μM) (Table 3). The kinase was also not activated in the presence or absence of $CaCl_2$ (100 μM) and/or phosphatidylserine (5 μg), phosphatidylcholine (5 μg) phosphatidylethanolamine (5 μg) phosphatidylinositol (5 μg) or in presence of phosphatidylserine plus 1–3 Diolein (1 μg) (Table 4). A criterion generally used for the classification of casein kinases is their responses to various effectors. Casein kinase I is generally inhibited by polyamine like spermine, spermidine or putrescine, polylysine, and high concentration of heparin. While casein kinase II is inhibited by high dose of heparin but greatly stimulated by spermine or polylysine. Casein kinase II requires monovalent cation for its activity [32]. We found here that the isolated CIK activity was not activated by spermine (8 mM), polylysine (100 μg/ml) or any other polyamines or inhibited by heparin (0.25 μg/ml). This enzyme also not inhibited by Quercetin, well-known inhibitor of casein kinase or by cAMP dependent protein kinase inhibitor or activated by monovalent cations NaCl (1 mM), KCl (1 mM) (data not shown).

3.4. Substrate specificity

The kinase showed high specificity in phosphorylating the acidic proteins and a much higher affinity for casein (Table 5). Whole casein, purified after being freed from lipids and other interfering substances rather than α-casein and other casein was most useful substrate for the CIK. The apparent Km for casein was approximately 1 mg/ml (data not shown). The apparent Km value for membrane proteins was approximately 35 μM (0.8 mg/ml) (Fig. 4B). The apparent Km value for MPS was approximately 1 μM (100 μg/ml) (Fig. 4A). The kinase was assayed with kemptide, calmodulin as the substrates, showed little enzyme activity. Investigation was carried out to know whether the isolated kinase can cause phosphorylation of the peptide poly (Glu Na : Tyr. 4:1), a known substrate of tyrosine protein kinase (Swarup et al.). But it did not show any phosphorylation.

3.5. Analysis of amino acids phosphorylated

All the radioactivity of ^{32}P labeled casein was associated with alkali–labile phosphoester bond (data not shown). Amino acid analysis of the ^{32}P-labeled casein as well as plasma membrane proteins demonstrated that the kinase catalyzed the phosphorylation of serine and threonine residues of the protein. The result (Fig. 5) showed that the kinase did not cause phosphorylation of tyrosine residues of casein. The enzyme when assayed for tyrosine kinase substrate, also indicating that it is not a tyrosine kinase. (Table 5).

3.6. Effect of CIK-antibody on specific activity of isolated CIK

To know immunogenicity of CIK, the titre of the antisera, developed against CIK, was tested by immunoblot and ELISA

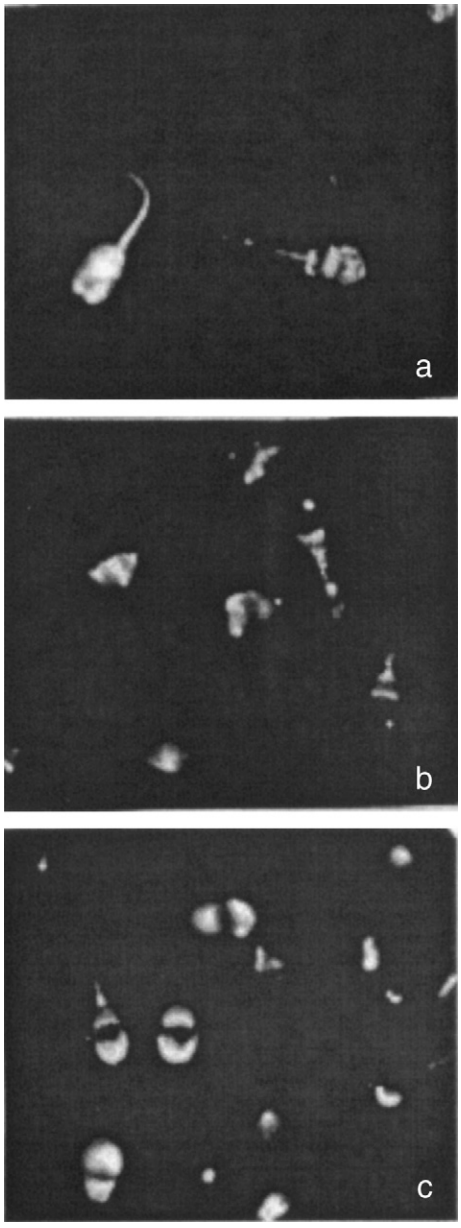

Fig. 7. Immunofluorescence of goat epididymal maturing spermatozoa. Sperm were isolated from (a) caput, (b) corpus (c) cauda part of epididymis. Cells were incubated with CIK-antibody, followed by FITC-labelled goat anti-rabbit IgG. Spermatozoa were examined by fluorescence microscope at 1000× magnification. Preimmune rabbit sera treated cells were used as the control cells.

tests. Different dilutions of antibody were used and the titre value was found to be 1:1000 dilution both by immunoblot and ELISA (data not shown). Moreover CIK antibody at a dilution of 1:500 did not agglutinate sperm cells. However the antibody at concentrations of 1:100 and above caused significant sperm agglutination. For this reason studies on the role of the antibody on sperm flagellar movement were carried out at the rate limiting concentration of the antibody (1:500).

CIK antibody drastically inhibited the purified enzyme activity when casein was used as the exogenous substrate for phosphorylation. About 80% activity was inhibited using 1:500 dilution of antibody in assay medium (Fig. 6a). The activity loss was about 85% in antibody treated intact cell CIK activity (Fig. 6b). The control rabbit serum from non-immunized rabbit did not have any significant effect on the kinase reaction. So this polyclonal antibody can be used to modulate the membrane CIK activity to monitor different physiological functions.

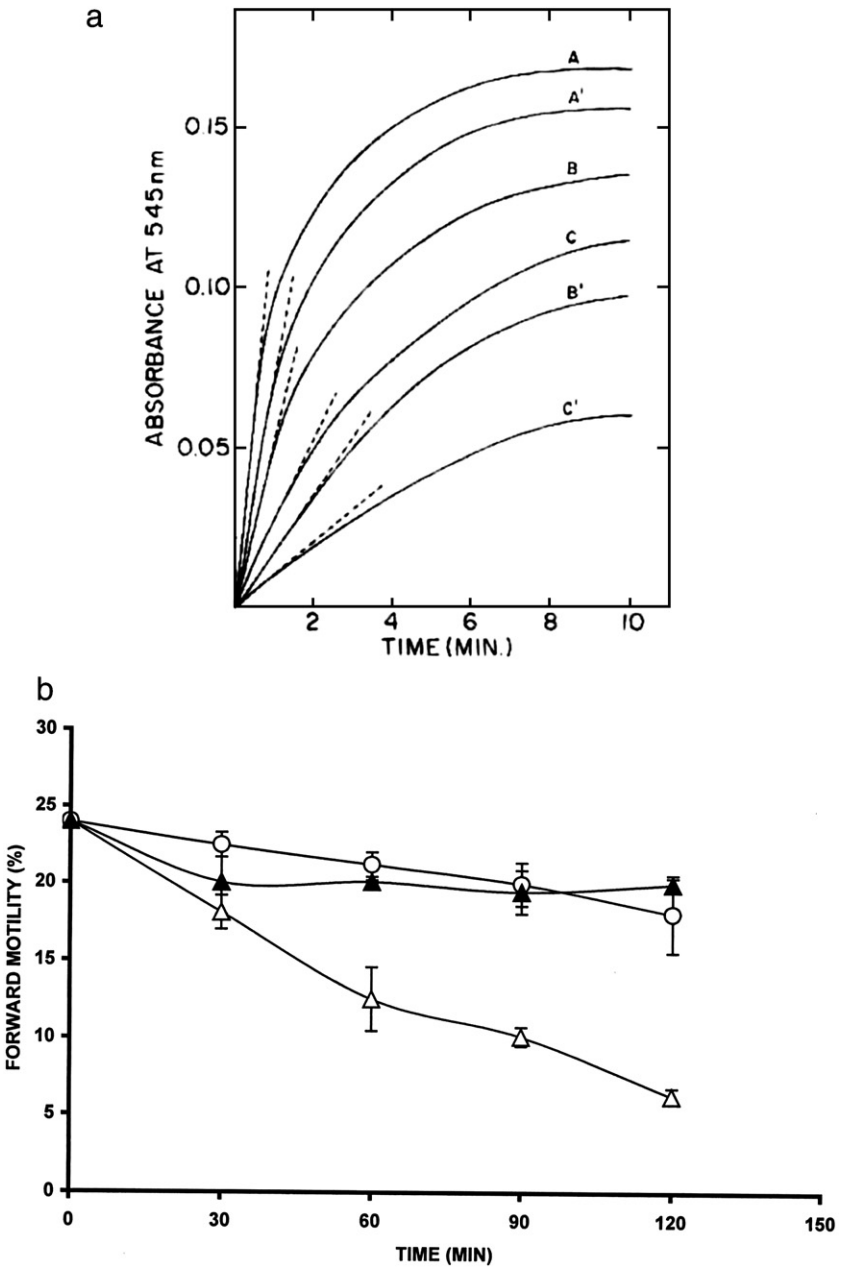

Fig. 8. (a) A representative spectrophotometric tracing showing the effect of CIK antibody on sperm forward motility percentage assayed spectrophotometrically. The experiment was carried out under standard assay conditions using 10^7cells/assay. The slope of the initial curve can be calculated from the broken line. Absorbance of all the cell suspension after mixing the content of the cuvette (At) was 0.810. A, B, C are control sere treated cells for 30 min, 1 h, 2 h respectively and A' B' C' are CIK-antibody treated cells respectively. (b) Effect of antibody on percentage of forward motility of mature cells as measured by spectrophotometric method. Sperm added with antibody was studied at different time scale (Δ) against preimmuned sera added cells (○) and preabsorbed antibody (▲) treated cells. Preabsorbed antibody was prepared by incubating antigen (CIK in excess) with antibody for 1 h at 37 °C and passing through casein-Sepharose 4B column to remove unbound CIK. The eluted fraction of preabsorbed antibody was then used in additional control system. These data showed the mean SEM of seven such experiments.

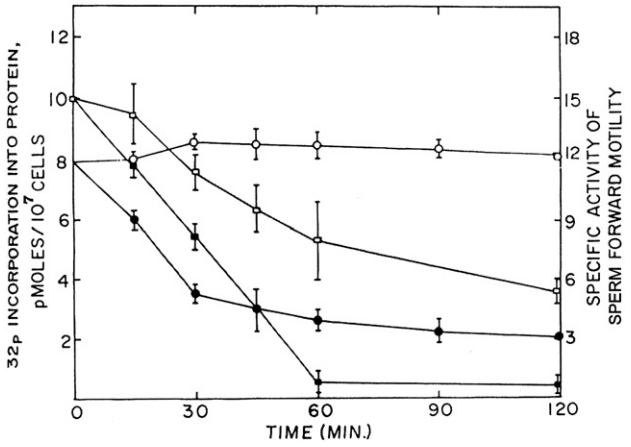

Fig. 9. Comparative analysis of inhibitory effect of CIK antibody on the mature cell surface protein phosphorylation as well as on specific activity of forward motility or velocity by spectrophotometric assay method depending on time. Protein phosphorylation in presence of CIK-antibody (●); phosphorylation in presence of preimmune rabbit sera (o); specific activity of forward motility in presence of antibody (■) and specific activity of forward motility in control sera treated cells (□). The standard assay system for phosphorylation of intact cell surface proteins contained intact spermatozoa ($1–2\times10^6$), 5 nmol of [γ^{32} P] ATP containing $1–8\times10^5$ cpm, 2 μmol of $MgCl_2$ and 0.2 μmol of EGTA in a total volume of 0.2 ml of RPS medium. Incubation was carried out at 37 °C for 1 min and the reaction was stopped by the addition of 0.2 ml of 10 mM ATP, 250 mM potassium phosphate and 5 ml of 10% TCA. After 60 min the resulting cell suspension was processed as protein suspension in assay of isolated CIK.

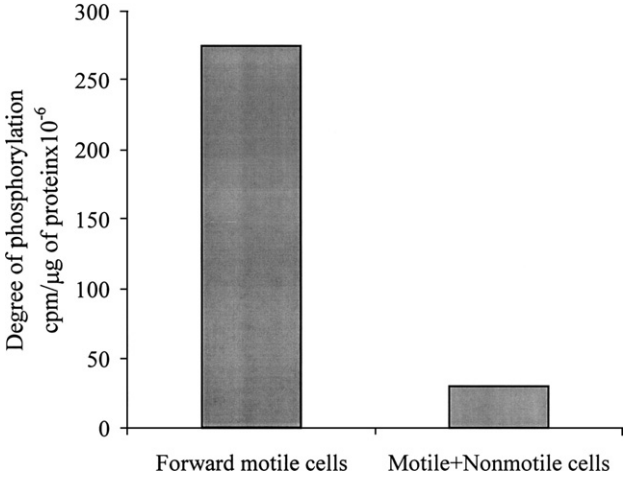

Fig. 10. Degree of surface protein-phosphorylation in forward motile cells and motile+nonmotile cells. Cells were separated by swim up technique and plasma membranes were isolated. The degree of phosphorylation was estimated under standard assay conditions. The data are representative of six such experiments.

3.7. Distribution of CIK on sperm surface

Distribution of CIK on the sperm surface as well as change of its localization during epididymal maturation was analyzed by the indirect immunofluorescence technique (Fig. 7). Binding of CIK antibody to the whole sperm was visualized by binding of second antibody (FITC-conjugated antirabbit IgG) to the first.

CIK antibody was found to bind intensely with the acrosomal region of the head in caput and cauda sperm cells but they showed a dot like arrangement on the tip of the head in the corpus sperm (Fig. 7b). Posterior fragment of head and the short neck were also intensely FITC labelled in caput, corpus, cauda cells (Fig. 7a–c). Only an anterior major portion of post acrosomal region was devoid of FITC staining. In the tail region intense fluorescence was found primarily on the middle piece and faintly on the principle piece in all cell preparation. Tail is completely devoid of CIK except the extreme tip (Fig. 7). Negative control using preimmune rabbit serum led to no detectable fluorescence on the head or tail region of maturing sperm cells confirming the specificity of the antibody. This observation gave support to the view that CIK was localized on the acrosomal region, posterior zone of post-acrosomal region, neck region middle piece and principal piece of the tail of epididymal sperm and epididymal maturation event is associated with a marked lateral movement of cell surface CIK.

3.8. Effect of CIK-antibody on forward motility of spermatozoa

Microscopic assay method as described in Materials and methods did not show any significant effect on the percentage of forward motility (%FM) in cauda mature sperm cells when treated with CIK antibody (1:500 dil) at different time scale (15 min, 30 min, 45 min, 1 h and 1.5 h) as compared to the control serum treated sperm. (data not shown).

However the spectrophotometric assay method showed a drastic retardation of forward motility with time when treated with CIK antibody as compared to control serum treated sperm (Fig. 8b). At 1:500 dilution of antibody forward motility percentage decreased about 30%, 50%, 75% after 30 min, 1 h, 2 h of incubation respectively (Fig. 8B) in comparison to control serum treated and preabsorbed antibody treated cells. The effect of preabsorbed antibody (with antigen CIK) on motility-percentage also indicate the specificity of antigen–antibody

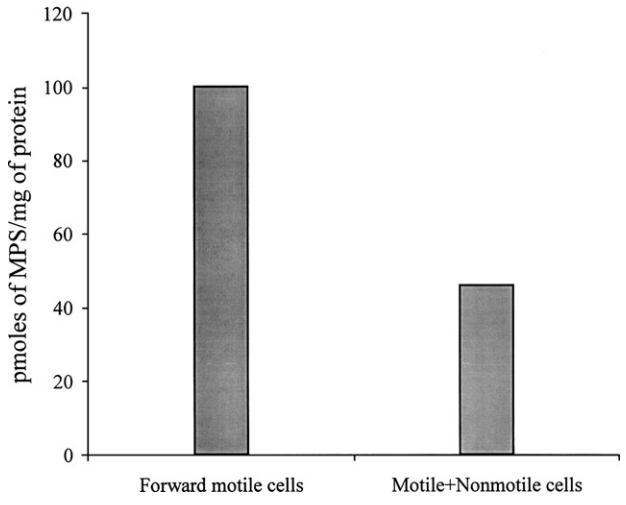

Fig. 11. Immunodetection of MPS in forward motile and other (motile+nonmotile) cells. Cells were separated by swim up technique and plasma membranes were isolated, solubilised in Triton X-100 and centrifuged. The supernatant was used in ELISA. Presence and concentration of MPS was detected with MPS antibody. The data are representative of three such experiments.

binding and presence of the antibody as the specific motility inhibitor in this system. Forward motility activity as measured by the initial slope of the curve also decreased to 12,8.5, 4.5, 7.5 and 7.0 units on 15, 30 and 45 min and 1 h and 2 h treated sperm respectively from 15, 14.5, 11.5, 9.5, 8.5 and 5.5 units (Fig. 8A). A comparative analysis of forward motility activity of spermatozoa and sperm surface protein phosphorylation profile showed a close relationship between CIK activity or surface protein phosphorylation and velocity of mature spermatozoa (Fig. 9).

The difference between these two methods of motility assessment is due to the fact that microscopic method takes into consideration only the numbers of cells with forward progression not the velocity whereas the spectrophotometric method shows not only the motile cell numbers but also their velocity and gives more accurate estimate of the forward motility index.

3.9. Association of membrane protein-phosphorylation and MPS with forward motile cells

To determine the role of membrane protein-phosphorylation on forward motility, the degree of phosphorylation was estimated in forward motile cells. Forward motile cells were separated by swim up technique from the cauda part of the epididymis. Plasma membrane was isolated from those cells. Membrane protein-phosphorylation profile was estimated in plasma membrane of forward motile and rest part (motile+nonmotile cells) of the cell population. It was found that the amount of MPS is approximately 9 times higher in forward motile cells compared to the rest part of spermatozoa (non-forward motile cell population) (Fig. 10).

The concentrations of MPS were estimated in vigorously motile sperm with vertical movement and the rest part of the cell population (low/non-motile cells) by ELISA using MPS antibody and HRP-conjugated anti-rabbit-IgG. It was found that concentration of MPS was approximately two-fold higher in the vigorously motile cell population as compared to the cells with low/no motility (Fig. 11). Concentration of MPS in high-motility cells was 100 pmol/μg of protein whereas in the weakly-motile cell population the MPS concentration was only 46 pmol/μg of protein. The data thus demonstrate that the outer cell-surface MPS plays an important role in regulation of sperm forward-motility.

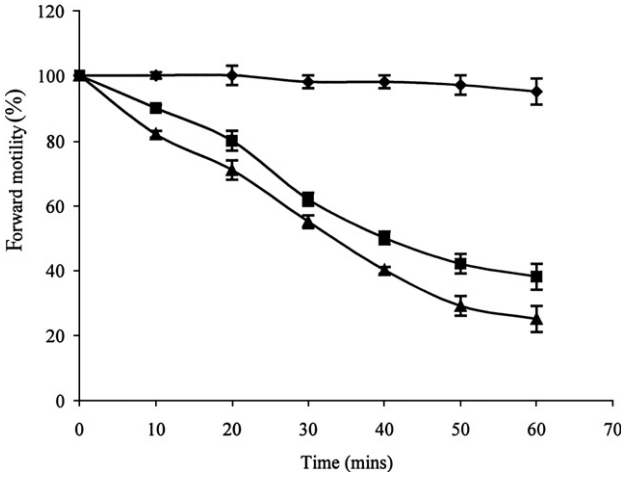

Fig. 12. Effect of MPS-antibody on forward motility of cauda epididymal spermatozoa. The sperm cells were incubated with MPS-antibody at different concentration MPS antibody 1:10 (■), MPS antibody 1:100 (▲). Preimmuned sera treated cells were used as control preparation (●). The forward motility was estimated at different intervals. The results shown are mean±SEM of four experiments.

3.10. Role of MPS on sperm forward motility

When the cauda-epididymal-sperm suspension (containing forward motile, non-motile and motile cells) was incubated with MPS-antibody, forward-motility was estimated in different time-intervals. Significant fall in forward-motility was noticed even after 10 min of incubation. After 60 min of incubation at 1:10 and 1:100 dilutions of MPS-antibody forward motility was inhibited 75% and 60% respectively (Fig. 12).

4. Discussion

The first two reports on the localization of a protein kinase (ecto-kinase) on the external surface of mammalian cells were published in the year 1976 [33,34]. Schlaeger and Kohler [33] demonstrated the presence of a cAMP-dependent ecto-protein kinase on the rat C-6 glioma cells whereas Mastro and Rozengurt [34] showed that the outer surface of the cultured 3T3 cells possess a protein kinase that causes phosphorylation of the membrane-bound proteins. Since then many investigators have provided various evidences for the occurrence of several types of protein kinase in a variety of cell types. Presence of cAMP-dependent ecto-protein kinases have been demonstrated on the external surface of spermatozoa of several species [2–6,35], 3T3 fibroblast cells [36,37], HeLa cells [34,38], rat adiposite cells [39]. Cyclic AMP-independent ecto-protein kinases (ecto-CIK) and their physiological endogenous substrates have been documented in many cell systems like goat spermatozoa [7–10,14,21]; PC12 neuronal cells[40], HeLa cells [41–44], rat myoblast cells[45], neutrophils [46], human monocytes [47], human platelets[48,49], human leukemic cells [50,51], 3T3 fibroblast cells [52], TB lymphoma cells [53] and RBL 2H3 cells [54]. The ecto-CIK group of enzymes is the most extensively studied enzymes from the stand point of the phosphorylation of the endogenous membrane proteins. These intact cell-bound enzymes showed high efficacy for the phosphorylation of the serine/threonine residues of phosphoproteins. Preliminary studies of several investigators using the cell-bound uncharacterized ecto-kinase models have implicated that these enzymes may participate in the regulation of a variety of cell functions such as cytokine functions [56], platelet activation and secretion [50], neural differentiation [40], myogenic differentiation [45], myogenesis [57], etc. However, precise biochemical identity of the ecto-kinases and their specific membrane-bound protein substrates is largely unknown as no study has yet been reported on the purification of these enzymes/substrates to apparent homogeneity. This study describes for the first time the purification of an ecto-CIK to apparent homogeneity, its characterization and functional significance using caprine sperm as the model.

The CIK solubilized from goat sperm plasma membrane with Triton X-100 was purified to apparent homogeneity as indicated by non-denaturing PAGE (Fig. 1), molecular sieving

and chromatofocussing (data not shown). The isolated kinase is a dimer possessing two subunits: 63 and 55 kDa (Fig. 2). It is a serine/threonine kinase (Fig. 5). The CIK is a strongly basic protein as indicated by its high isoelectric point: pI-9.95 (Fig. 3). The results (Table 3) demonstrate that the isolated CIK is distinct from cAMP- and cGMP-dependent protein kinases, Ca-calmodulin protein kinase, protein kinase C and tyrosine kinase. This membrane-associated CIK showed some similarities with casein kinases (I and II) [58], because like these kinases, CIK also showed higher specificity for the phosphorylation of casein as compared to the widely used basic proteins, histone and protamine. Unlike the casein kinases, CIK was neither activated by monovalent metal ions (data not shown) nor retained by DEAE-cellulose at pH 7.0 (Table 1) under the conditions of a low ionic strength buffer. Heparin is known to be a specific inhibitor of casein kinase II [58]. Unlike the casein kinase II, CIK is rather insensitive to the action of heparin. Polyamines had no appreciable effect on CIK activity indicating thereby that CIK is different from the polyamine-dependent protein kinase reported in bovine epididymal sperm [59,60] and ejaculated sperm [32]. A special feature of the sperm ecto-CIK is that it has high degree of substrate specificity for the membrane-bound proteins as compared to the other exogenous proteins (Table 4). This view is supported by the finding that 100 kDa phosphoprotein of the mature sperm membrane serve as the major substrate of CIK [21]. The data demonstrate that CIK is an unique cell surface kinase and it is also membrane protein-specific kinase which specialise in phosphorylating the serine and threonine residues of the outer cell-surface phosphoproteins.

It is of interest to note that CIK antibody markedly inhibits the intact sperm ecto CIK activity (VIB) as well as ecto-protein kinase-mediated membrane protein phosphorylation (Fig. 9). The CIK is primarily localized in the acrosomal cap area of the external surface of the mature sperm head as demonstrated by indirect immunofluorescence studies (Fig. 7). Although earlier investigators have provided several lines of evidences for the occurrence of ecto-protein kinases in a variety of cell types, the above-mentioned findings provide confirmatory evidence for the localization of an ecto-protein kinase on a mammalian cell surface. Several investigators reported the presence of membrane bound-protein kinase and phosphoproteins in many cell types that have been implicated to play an important role in cellular regulation [24,40–42,44,45,47,49,51,52,55,56,59,61–67]. However, little is known about the surface topography of these kinases and their protein substrates. The results of this study support the view that ecto-CIK or this group of kinase is responsible for the observed phosphorylation of the serine/threonine residues of plasma membrane proteins reported by the above investigators.

Rapid turn over of these cell surface phosphoproteins exhibit the characteristics of regulatory proteins [24,68]. The observation that the epididymal sperm maturation event is associated with a remarkable lateral movement of CIK particularly on the sperm head leading to its realignment on the cell surface (Fig. 8A–C), strengthens the above view. Our recent study has demonstrated that the major protein substrate of sperm ecto-kinase is a 100-kDa phosphoprotein, MPS [21]. Like kinase, the substrate protein is also located on the acrosomal cap region of sperm head. Antibody of ecto-CIK inhibits 75% forward motility in 1: 500 antibody dilution (Fig. 8B). Antibody of the major physiological protein substrate of ecto-CIK, MPS also inhibits 60% and 75% sperm motility and forward motility respectively at 1:10 antibody dilution [21]. It is of interest to note that like the ecto-CIK antibody (Fig. 9) antibody of MPS also severely inhibit membrane protein phosphorylation [21]. The rate of sperm surface protein phosphorylation is nine-fold higher in forward motile cells as compared to the rest part (nonforward motile + non-motile) of the cell population (Fig. 10) whereas the concentration of MPS was two-fold higher in forward motile cells as compared to the other cells (Fig. 11). The observations of spectrophotometric method show that treatment of the motile spermatozoa with ecto-CIK antibody causes marked fall in velocity of the cells because the no of spermatozoa that swim upwards against gravity to enter the light path decreases as time dependent absorbance indicating the lowering of forward motility index or velocity of the most vigorous motile population of spermatozoa (Fig. 8A). The MPS-antibody treatment also significantly reduces the number of forward-motile sperm cells (Fig. 12). The data demonstrate that ecto-CIK through its substrate protein: MPS plays a vital role in the regulation of sperm velocity although the biochemical events in between the phosphorylation process and the flagellar motility are yet to be elucidated.

Acknowledgements

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