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OCCURRENCE OF A CYCLIC AMP-DEPENDENT PROTEIN KINASE ON THE OUTER SURFACE OF RAT EPIDIDYMAL SPERMATOZOA

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SUMMARY

Endogenous protein kinase activity was detected on the outer surface of rat cauda epididymal spermatozoa. The kinase activity of the intact sperm cells catalyses the transfer of the terminal phosphate of exogenous [γ^{32}-P]ATP to the alkali labile phosphoester bonds of exogenous calf thymus histones. There was little uptake of [γ^{32}-P]ATP and phosphorylation of endogenous proteins by intact spermatozoa. The amount of histones phosphorylated by the peripherial kinase is directly proportional to the sperm numbers and the reaction is linear for approx. 5 min. Cyclic AMP (2.5 μM) activates the kinase (approx. 120%) and also causes the release of the enzyme from spermatozoa into the medium. Approx. 80% of the peripherial kinase activity is released after 30 seconds of incubation of spermatozoa.

Cyclic AMP-dependent protein kinases have been demonstrated in bovine epididymal and sea urchin spermatozoa (1-3). The levels of these kinases are high in cytosol fraction of sperm cells and it has been suggested that the stimulatory effect of cyclic AMP on sperm motility (4-6) is mediated through the sperm cyclic AMP-dependent protein kinases (1). More recently a cyclic AMP-independent protein kinase has been identified in human seminal plasma (7). However, the exact roles of these enzymes in sperm functions are largely unknown. The present studies provide evidence for the occurrence of a cyclic AMP-dependent protein kinase on the outer surface of rat cauda epididymal spermatozoa. Cyclic AMP can cause the release of the enzymatic activity from cell surface into the medium.

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MATERIALS AND METHODS

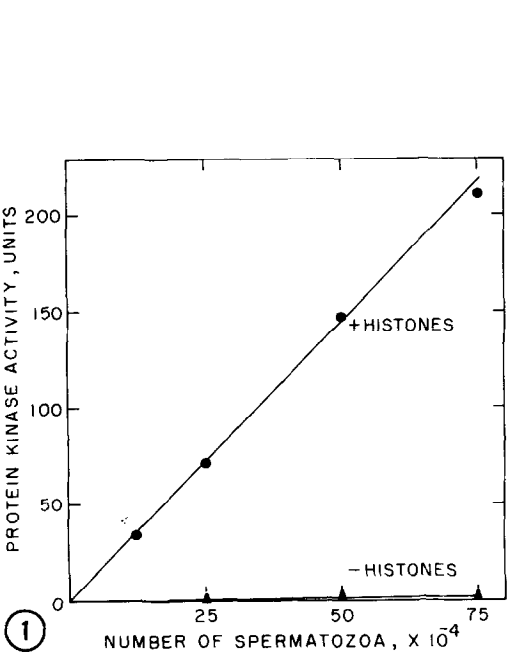
Chemicals - Cyclic AMP, ATP and calf thymus whole histones were obtained from Sigma. [^{32}P] orthophosphate (carrier free) was a product of Bhaba Atomic Research Centre, Bombay. [γ^{32}-P]ATP was prepared according to Glynn and Chappell(8) with some modifications (R.Biswas and G.C. Majumder, unpublished data).

Isolation of epididymal spermatozoa - Spermatozoa were obtained from cauda (tail) epididymides of adult albino rats by a slight modification of the procedure described earlier(9). The tissue was not chilled and extraction of spermatozoa with 0.25 M sucrose and subsequent centrifugations were carried out at room temperature. Each epididymis was cut to 3-4 pieces with a sharp razor blade and then suspended in 0.25 M sucrose (5 ml/epididymis) with gentle stirring. After 5 min the suspension was filtered through four layers of cheese cloth and the preparations of highly motile sperm as judged by light microscopy were used for these studies. Spermatozoa were sedimented by centrifugation at 800 x g for 1 min and the pellet was washed two times with 0.25 M sucrose. Finally the washed spermatozoa were suspended in 0.25 M sucrose and left at room temperature (10 to 30 min) until assayed for protein kinase activity. Sperm numbers in the samples were determined with a haemocytometer and the preparations of spermatozoa were observed to be highly pure by light microscopy.

Assay of protein kinase - The activity of protein kinase was measured by a slight modification of the method described previously (10). The standard assay medium contained 10 µmoles of sodium β-glycerophosphate-HCl (pH 6.5), 200 µg of calf thymus histones, 6 nmoles of [γ^{32}-P]ATP containing 20 to 7x10^4 cpm, 0.6 µmole of theophylline, 3 µmoles of magnesium chloride, 0.5 nmole of cyclic AMP, 25 µmoles of sucrose and intact spermatozoa (20 to 30x10^4 cells) in a total volume of 0.2 ml. The incubation was carried out at 37°C for 4 min and the reaction was stopped with the addition of 0.2 ml of 1% casein containing 2.5 mM potassium phosphate and 2 mM ATP and 2 ml of 10% trichloroacetic acid (TCA). The resulting precipitate was processed for the assay of radioactivity in protein (7). Samples were counted in an end window G.M. Counter (Bhaba Atomic Research Centre, Bombay). Systems lacking spermatozoa served as blanks. One unit of enzyme activity was defined as the amount of enzyme which catalyses the transfer of one pmole of ^{32}P from [γ^{32}-P] ATP to the recovered protein during 4 min under the standard assay conditions.

RESULTS AND DISCUSSIONS

As shown in Fig.1 rat epididymal intact spermatozoa possess endogenous protein kinase activity to cause phosphorylation of exogenous calf thymus histones. There was no detectable amount of phosphorylation of the endogenous cell proteins by the sperm protein kinase in presence (2.5 µM) or absence of cyclic AMP. The activity of the sperm kinase increased proportionately with



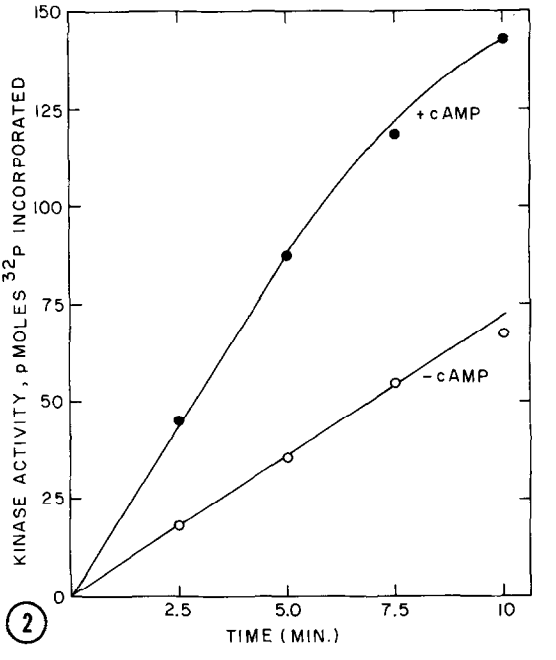


Fig.1. Phosphorylation of histones as a function of the number of intact epididymal spermatozoa in the standard assay procedure.

▲, without histones; ●, with histones.

Fig.2. Time course of the phosphorylation of histones by the endogenous protein kinase activity of the intact epididymal spermatozoa (3×10^5 cells) in the standard assay.

○, without cyclic AMP; ●, with cyclic AMP.

at least upto 200 units of the enzyme (approx. 75×10^4 spermatozoa). The amount of histones phosphorylated by the protein kinase of intact spermatozoa increased linearly with time for approx. 5 min and cyclic AMP (2.5 μM) markedly stimulates (approx. 120%) the sperm kinase activity.

^{32}P has not been incorporated into RNA and DNA since there was only a small amount of loss (approx. 10%) of radioactivity following heat treatment of the suspension of ^{32}P-labeled histones in 6% TCA at 90°C for 15 min. The data shown in Table I indicate that the observed radioactivity in protein is not due

Table I

Alkali lability of the phosphoester bonds of [^{32}P]histones

Histones were phosphorylated with intact sperm protein kinase and TCA-insoluble [^{32}P]histones were subjected to the various treatments as specified. ^{32}P-labeled histones from the untreated "control" tubes were washed with 5% TCA as described under "Materials and methods". The labeled histones were treated with methanol:chloroform(1:2) and hot sodium hydroxide (1 N) according to Kinzel and Mueller(12) and with 0.8 M hydroxylamine according to Hokin et al(11).

Treatments	[^{32}P]Histones(cpm)
Control	1560
Methanol:chloroform(1:2)	1500
Hydroxylamine(0.8 M)	1530
Sodium chloride(0.64 M)	1540
Sodium hydroxide (1 N)	0

to incorporation of ^{32}P into phospholipids. The insensitivity of the incorporated ^{32}P to hydroxylamine largely rules out an acyl-phosphate linkage as described for the (Na^+-K^+)-dependent ATPase(11), a well characterized phosphoprotein of the plasma membrane. The data indicate that nearly 100% of the radioactivity of the ^{32}P-labeled histones is associated with alkali-labile phosphoester bonds.

There was little uptake of [γ^{32}-P]ATP by spermatozoa under the standard protein kinase assay conditions indicating that the phosphorylation of histones by intact spermatozoa takes place on the outer cell surface. Observation under a light microscope revealed that there was no detectable broken or damaged cells in the fresh or the incubated (for protein kinase assay) spermatozoa. These results indicate that the sperm kinase activity is not due to the presence of damaged cells in the sperm

preparations or damage caused to the cells during the incubation.

There was no appreciable loss of protein kinase activity of spermatozoa following repeated (3 times) washings of the cells with 0.25M sucrose indicating that the observed protein kinase activity is of sperm origin and not due to contamination from the epididymal fluid. It was further observed that spermatozoa do not lose any appreciable amount of kinase activity when washed with normal (0.9% NaCl), hypotonic (0.6% NaCl) or hypertonic (1.3% NaCl) saline, indicating that the kinase activity is not loosely bound to sperm cell surface by electrostatic interactions. Time course of the reaction (Fig.2) strongly suggests that the protein kinase activity of intact sperm cells is not due to leakage of the enzyme from the cytoplasmic fraction of these cells. The data are consistent with the view that the sperm kinase activity is tightly bound to the outer cell surface.

Results in the Table II show the effect of cyclic AMP on the release of protein kinase activity from spermatozoa into the medium. Sperm cells lost approx. 95% of protein kinase activity as a result of incubation for 4 min with cyclic AMP and histones and nearly 100% of the released enzyme activity was recovered in the cell-free supernate (Exp.I). In presence of cyclic AMP alone (without histones) the cells lost similar amount (approx.87%) of kinase activity but the amount of activity recovered in the cell-free supernate was much lower (approx. 47%). Spermatozoa lost approx.60% of the kinase activity when preincubated with cyclic AMP (2.5 μM) for 4 min whereas there was little loss of the enzyme activity when the preincubation was performed with cyclic AMP and histones (results not shown). It thus appears that the lower recovery of the kinase activity in the supernate (without histones) may be due to instability of the released enzyme which is stabi-

Table II

Cyclic AMP-mediated release of protein kinase activity from intact epididymal spermatozoa into the medium

Rat cauda epididymal spermatozoa were incubated at 37°C for the specified periods under the standard protein kinase assay conditions except that cyclic AMP, histones and $[\gamma^{32}\text{-P}]$ATP were omitted (control). Final concentrations of cyclic AMP and histones when present during the incubations were 2.5 μM and 1 mg/ml respectively. At the end of the incubation periods the reaction mixtures were quickly chilled in ice-water and immediately centrifuged in cold at 1500xg for 2 min. The clear cell-free supernates were removed, the sperm-pellets were washed with 0.25 M sucrose and the washed cells were finally suspended in 0.25M sucrose. The activities of protein kinase in the resulting cell-free supernates and sperm-pellets obtained from 3.7×10^5 spermatozoa were estimated under the standard assay conditions with cyclic AMP.

Experiment	Period of incubation	Incubated system	Protein kinase activity (units) Sperm-pellet	Cell-free supernate
I	4 min	Control	96	21
		+Cyclic AMP	11	60
		+ " +Histones	7	114
II	0.5 min	Control	90	17
		+Cyclic AMP	18	77

lized markedly by the histones. It is thus clear that cyclic AMP mediates the release of protein kinase activity from the sperm cell surface. Data from Exp.II confirms this view and demonstrates further the rapidity of the action of cyclic AMP since approx 80% of cell-bound protein kinase activity was released into the medium in only 30 seconds of incubation of the cells. It thus appears unlikely that cyclic AMP enters the cells to cause release of kinase activity from the cytoplasmic fraction of spermatozoa. These data thus adds further support to the view that the observed cyclic AMP-dependent protein kinase activity of spermatozoa is located on the outer cell surface.

Studies from a large number of tissues indicate that cyclic

AMP-dependent protein kinase consists of a catalytic (C) and a regulatory (R) subunits (10,13-15). Cyclic AMP activates the enzyme by binding to R and thereby causes the dissociation of C which is the active form of the enzyme. At present the molecular nature of the sperm cell surface cyclic AMP-dependent protein kinase is not known. It is possible that the peripherial sperm kinase may be a complex of C and R subunits and R may have anchored into the lipid bilayer of the plasma membrane leaving C outside the cell surface. On the basis of this postulation the site of binding of C with R is located outside the cell surface and hence cyclic AMP will have an easy access (without entering the cells) to interact with R, thereby causing the release of only C from the cell surface leaving R still bound to the plasma membrane. This postulation is consistent with the rapid action of cyclic AMP to cause release of protein kinase activity from intact spermatozoa.

Recently a cyclic AMP-dependent protein kinase has been demonstrated on the outer surface of rat C-6 glioma cells (16). Cultured 3T3 cells also possess a cell surface protein kinase which causes the phosphorylation of the endogenous membrane-bound proteins(17). The present studies provided evidence for the occurrence of a cyclic AMP-dependent protein kinase on the periphery of rat epididymal spermatozoa. The sperm enzyme causes the phosphorylation of exogenous histones without causing any detectable phosphorylation of endogenous cell proteins. Motility can be induced and prolonged in the spermatozoa of several mammalian species by cyclic nucleotides(4-6). It is tempting to propose that an early action of exogenous cyclic AMP on sperm cells is to cause the release of cell surface protein kinase into the surrounding fluid. However, little is known about the functional significance of this novel action of cyclic AMP on intact spermatozoa. Studies

are in progress to characterise further the peripherial sperm protein kinase.

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REFERENCES

1. Hoskins, D.D., Casillas, E.R. and Stephens, D.T., Biochem.Biophys.Res. Commun., 48, 1331 (1972).
2. Garbers, D.L., First, N.L. and Lardy, H.A., J.Biol.Chem., 248, 875 (1973).
3. Lee, M.Y.W. and Iverson, R.M., Biochim.Biophys.Acta, 429, 123 (1976).
4. Garbers, D.L., Lust, W.D., First, N.L. and Lardy, H.A., Biochemistry, 10, 1825, (1971).
5. Hoskins, D.D., Hall, M.L. and Munsterman, D., Biol.Reprod., 13, 168 (1975).
6. Wyker, R. and Howards, S.S., Fert.Steril., 28, 108 (1977).
7. Majumder, G.C. Biochem.Biophys.Res.Commun., in press(1978).
8. Glynn, I.M. and Chappell, J.B., Biochem.J., 90, 147 (1964).
9. Majumder, G.C. and Turkington, R.W., Biochemistry, 13, 2857(1974).
10. Majumder, G.C. and Turkington, R.W. J.Biol.Chem., 247, 7207(1972).
11. Hokin, L.E., Sastry, P.S., Galsworthy, P.R. and Yoda,A. Proc.Natl.Acad.Sci.USA., 54, 177 (1965).
12. Kinzel, V. and Mueller, G.C., Biochim.Biophys.Acta, 322, 337(1973).
13. Langan, T.A., Adv.Cyclic Nucleotide Res., 3, 99 (1973).
14. Gill, G.N. and Garren, L.D., Proc.Natl.Acad.Sci.USA, 68, 786(1971).
15. Erlichman, J., Hirsch, A.H. and Rosen, O.M. Proc.Natl.Acad. Sci.USA, 68, 731(1971).
16. Schlaeger, E.J. and Kohler, G., Nature, 260, 705(1976).
17. Mastro, A.M. and Rozengurt, E., J.Biol.Chem., 251, 7899(1976).