Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 592-598

CASEIN KINASE I IN BOVINE SPERM: PURIFICATION AND CHARACTERIZATION

Prem S. Chaudhry, Peggy A. Newcomer and Edmund R. Casillas

Department of Chemistry, New Mexico State University
Las Cruces, New Mexico 88003

Received July 22, 1991

A highly purified preparation of sperm casein kinase I was obtained by sequential chromatography with phosphocellulose, gel filtration on sephacryl S-300, Affi-gel blue and DEAE-Cellulose. The chromatographic behavior and properties of the enzyme suggest that the sperm enzyme is similar to casein kinase I from other tissues. Antibodies against calf thymus casein kinase I cross-react with the sperm enzyme. A special feature of the sperm enzyme is that the activity is stimulated by spermine.

Casein Kinase I is a ubiquitous, cyclic AMP- and Ca^{2+}- independent serine/threonine protein kinase. The enzyme has been identified in all eukaryotes examined and has been isolated from cytosolic, membranous and nuclear fractions of mammalian cells. The mammalian enzyme is a monomeric protein with a molecular mass ranging from 30 to 42 kDa. It prefers acidic proteins such as casein and phosvitin as substrates, utilizes ATP as phosphate donor, and the activity is greatly stimulated by monovalent cations (see references 1-3 for review). No specific activators or inhibitors of casein kinase I are known and the enzyme is identified by its elution characteristics from DEAE-cellulose and phosphocellulose. The wide spread distribution of casein kinase I and the fact that it phosphorylates a large number of enzymes involved in the regulation of metabolic pathways and other cytosolic, membranous and nuclear proteins, suggest the importance of this multipotential protein kinase in cell function. In the course of our studies on sperm casein kinase II (4) we found that bovine sperm contain a highly active casein kinase I. Since the enzyme has not been previously identified in spermotozoa,we have purified and characterized this protein kinase from bovine epididymal spermatozoa.

MATERIALS AND METHODS

The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (5), and casein kinase II from bovine sperm (6). DEAE-cellulose (DE-32) and phosphocellulose (PII) were purchased from Whatman; sephacryl S-300 from pharmacia and Affi-gel blue from Bio-Rad. [γ-^{32}P] ATP was from New England Nuclear. Kemptide, wiptide and synthetic peptide substrates of casein kinase I and II were from Penninsula Laboratories. ATP, casein, heparin and polyamines were from Sigma. All other reagents and materials were of analytical grade.

0006-291X/91 $1.50

Purification of Casein Kinase I

Enzyme Solubilization. Spermatozoa removed from the caudal epididymis were washed twice with 0.9% NaCl and then suspended in 500 ml buffer A (20 mM Hepes, pH 7.4, 7 mM2-mercaptoethanol, 2 mM EDTA, 2mM EGTA, 0.5 M NaCl, 1 mM PMSF, 10 mg/1 trypsin inhibitor, 10 mg/1 TLCK, 5 mg/1 aprotinin and 2 mM benzamidine). The cell suspension was sitrred gently on ice for 1 h, and then centrifuged at 27,000 x g for 30 min. The sperm pellet was reextracted with 100 ml of buffer A and centrifuged as described above. The pellet thus obtained after the 2nd extraction was resuspended in 100 ml of fresh buffer A and was then sonicated for 5 min at 2°C with a Branson sonifer (output control setting 3). The supernatant fractions were pooled and centrifuged at 105,000 x g for 2 h.

Phosphocellulose Chromatography. The clear supernatant obtained above was dialyzed overnight against 4 l of buffer B (20 mM Hepes, pH 7.4, 7 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 0.3 M NaCl, 0.1 mM PMSF, 1 mg/1 TLCK, 1 mg/1 aprotinin and 2 mM benzamidine). The sample containing 750 mg protein, was applied to a phosphocellulose column (6 x 14 cm) which had been equilibrated with buffer B. After the column was washed with 2 1 of buffer B, the enzyme was eluted with 250 ml of buffer B, but containing 1.5 M NaCl. The eluate was dialyzed against 4 1 of buffer C (20 mM Hepes, pH 7.4, 7 mM 2-mercaptoethanol, 0.4 mM EDTA, 0.4 mM EGTA, 0.4 M NaCl, 0.04% sodium azide, 0.1 mM PMSF, 1mg/1 TLCK and 2 mM benzamidine). The sample containing 35 mg protein was applied to a second phosphocellulose column (4 x 6 cm) preequilibrated with buffer C. The column was washed with 500 ml of buffer C and the proteins were eluted with 600 ml of a linear salt gradient (0.3 to 1.5 M NaCl) in buffer C. Two peaks of casein kinase activity were detected by phosphocellulose chromatography of solubilized sperm proteins. The first peak, eluted with 0.7 M NaCl, contains casein kinase I, and the other eluted with 1.2 M NaCl, contains casein kinase II.

Gel Permeation Chromatograhy. The pooled enzyme fraction (peak1) from the phosphocellulose column was concentrated to 4 ml by ultrafiltration and was then applied to a sephacryl S-300 (2.5 x 110 cm) gel permeation column which was equilibrated and developed with buffer C which contained 0.5 M NaCl and 10% glycerol. Casein Kinase I elutes from the gel permeation column in a manner corresponding to a Mr of 35 - 40 kDa.

Affi-Gel Blue Chromatography. The pooled enzyme fraction from the gel filtration column was diluted with 20 mM Hepes, pH 7.4 buffer until the NaCl concentration was 0.25 M and then applied to a column (2.5 x 6 cm) of Affi-gel blue equilibrated with buffer C which contained 0.25 M NaCl and 50% glycerol. The column was washed with 50 ml of the buffer and then treated with 100 ml of a linear salt gradient (0.25 to 2.5 M NaCl) in the equilibration buffer. The enzyme elutes from the column with 1.5 M NaCl and fractions with high activity were pooled.

DEAE-Cellulose Chromatography. The kinase containing fraction from the Affi-gel blue column was dialyzed against buffer C without NaCl, but containing 50% glycerol. The dialyzed enzyme preparation was then applied to DEAE-Cellulose Column (1.5 x 5 cm) equilibrated with the dialysis buffer. The column was washed with 50 ml of the same buffer and proteins were eluted with 100 ml of a linear salt gradient (0 to 0.5 M NaCl). Fractions with high activity were pooled, concentrated and stored in 50% glycerol at -20°C.

Protein kinase assay

The reaction mixture contained 10 mM Hepes, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 150 mM NaCl, 400 µg casein, 10 mM magnesium acetate, 0.3 mM [γ-^{32}P]ATP (0.5 µCi) and 50 µl of enzyme in a final volume of 340 µl. After incubating the reaction mixture for 30 min at 30°C, the reaction was terminated by adding 4 ml of 5% TCA -0.25% Na tungstate. The precipitate was collected by centrifugation, washed three times with TCA -Na tungstate, and analyzed for radioactivity. When specific peptides were used as substrates, the reactions were stopped by adding 10 µl of 100 mM phosphoric acid. Aliquots from the reaction mixture were spotted on squares of Whatman P81 phosphocellulose filters and the filters were then washed four times (15 min per wash) in 75 mM phosphoric acid, dried and counted for radioactivity.

Immunoidentification of Casein Kinase I

After separation on SDS-PAGE, the proteins were transferred for 3 h at 300 mA to a nitrocellulose membrane. The membrane was incubated successively with 3% gelatin in TBS (20 mM Tris-Cl,

pH 7.5, containing 500 mM NaCl) for 1 h, with monospecific antibodies against calf thymus casein kinase I in TBS containing 1% gelatin for 2h, and with ^{125}I-Protein A for 1 h. Following each incubation, the membrane was washed extensively with TTBS (TBS containing 0.05% Tween 20). ^{125}I-labelled protein bands were located by autoradiography.

Miscellaneous methods

Protein was determined by Schaffner and Weissmann procedure (7). SDS-PAGE was performed in 10% gels (8); proteins were electrophoretically transferred to a nitrocellulose membrane (9).

RESULTS AND DISCUSSION

This report demonstrates for the first time the existence of casein kinase I in bovine sperm. Since no activators or inhibitors of the enzyme are known, and also since casein kinase II (4,6) and cAMP-dependent protein kinase (10,11) utilize casein as a substrate, the activity in the crude cytosolic and particulate fractions was determined with Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg, a specific peptide substrate of casein kinase I (12). Most (90%) of the casein kinase I activity in spermatozoa is particulate and is associated with sperm structures. A small portion (10%) of the activity was solubilized by sonication without the addition of detergent or salt to the medium. However, more than 90% of the particulate activity was solubilized by repeated extraction with 0.5 M NaCl. Two peaks of casein kinase activity were detected by phosphocellulose chromatography of solubilized sperm proteins. Peak 1 which eluted at 0.7 M NaCl contains casein kinase I, and Peak II, eluted at 1.2 M NaCl contains casein kinase II. These two enzyme activities from other cells (13), including cytosolic sperm enzymes (4), are normally eluted from phosphocellulose with 0.5 and 0.7 M NaCl. The reason for the tight-binding of the salt extracted enzymes to phosphocellulose is not apparent. Peak 1 containing casein kinase I was further purified by sequential chromatography on sephacryl S-300, Affi-gel blue and DEAE-cellulose. After phosphocellulose chromatography, casein kinase I is separated from other sperm protein phosphotransferases such as cyclic AMP-dependent protein kinase, protein kinase C and casein kinase II. A typical purification procedure is summarized in Table 1. The specific activity of 30 n mol min^{-1} mg^{-1} of purified enzyme is comparable to casein kinase I purified from testis (14).

Table 1. Purification of casein kinase I from bovine sperm[a]

Step	Protein mg	Specific Activity n mol min^{-1} mg^{-1}	Recovery %
Phosphocellulose (Peak 1)	14.5	4.0	100
Sephacryl S-300	4.6	10.9	87
Affi-gel blue	1.5	24.9	65
DEAE-Cellulose	0.6	30.2	31

[a] 750 mg of sperm proteins solubilized with 0.5 M NaCl were used for enzyme fractionation.

Table 2. Effect of various kinase effectors on purified sperm casein kinase I

Addition	Protein kinase activity p mol^{32}P/min
200 μM EGTA	12.9
200 μM EGTA, 300 μM Ca^{2+}	7.6
200 μM EGTA, 300 μM Ca^{2+}, 5 μg CaM	7.6
200 μM EGTA, 300 μM Ca^{2+}, 2 μg phosphatidylserine	7.7
200 μM EGTA, 10 μM cAMP	12.4

Assay mixtures contained 400 μg casein, 0.6 μg enzyme from Affi-gel blue and various protein kinase effectors as indicated.

Since the recovery of the enzyme after DEAE-Cellulose was low, the fraction from Affi-gel blue chromatography was utilized for most of the studies described below. The enzyme is not homogeneous at this stage, but is free of other major protein phosphotransferases such as Ca^{2+}-dependent and cAMP-dependent protein kinases. Added Ca^{2+}, calmodulin, phosphatidylserine or cAMP did not stimulate the enzyme activity (Table 2). The activity was stimulated by the addition of NaCl and optimal activity was observed with 300 mM NaCl (Fig. 1). The enzyme phosphorylated casein and the specific peptide substrate of casein kinase I but Kemptide, a

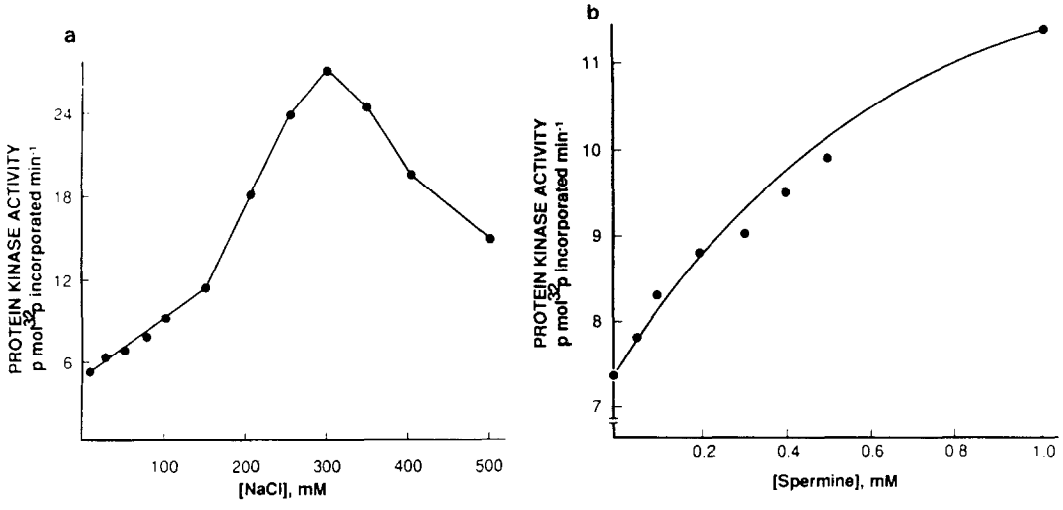

Figure 1. a) **Effect of NaCl on purified sperm casein kinase I.** Assay mixtures contained 400 μg casein, 0.6 μg enzyme from Affi-gel blue, and varying amounts of NaCl. b) **Effect of spermine on purified sperm casein kinase I.** Assay mixtures contained 400 μg casein, 0.6 μg enzyme from Affi-gel blue and varying amounts of spermine.

Table 3. Substrate specificity of purified sperm casein kinase I

Addition	Sperm casein kinase I activity p mol^{32}P/min	Sperm casein kinase II activity p mol^{32}P/min	cAMP-dependent kinase activity p mol^{32}P/mn
Casein	9.16	16.90	3.60
Casein + 50 μM wiptide	9.10	16.70	0
Casein + 1 μg/ml heparin	8.90	1.30	--
Kemptide *	0.05	0.08	96.70
Kemptide + 50 μM wiptide	--	--	0.78
Casein kinase I peptide substrate**	1.30	0.03	0.02
Casein kinase I peptide substrate + 1 μg/ml heparin	1.20	--	--
Casein kinase II peptide substrate ***	0.05	2.70	0.03
Casein kinase II peptide substrate + 1 μg/ml heparin	--	0.05	--

* Kemptide - a specific peptide substrate of cAMP-dependent protein kinase (15).
** Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg, a specific substrate of casein kinase I (12).
***Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu, a specific substrate of casein kinase II (16).
Assay mixtures contained either 0.6 μg purified sperm casein kinase I from Affi-gel blue or 20 ng pure sperm casein kinase II or 70 ng pure catalytic subunit of cAMP-dependent protein kinase from bovine heart, 400 μg casein or 1 mM peptide substrates, 10 mM magnesium acetate, 150 mM NaCl, and 0.15 mM AT^{32}P in a total volume of 50 μl. Some assays contained 50 μM wiptide, a specific inhibitor of cAMP-dependent protein kinase or 1 μg/ml heparin, a specific inhibitor of casein kinase II.

specific substrate of cAMP-dependent protein kinase was not phosphorylated. Also, the activity was not affected by wiptide, a selective inhibitor of cAMP-dependent kinase (Table 3). These results suggest that this kinase is not the free catalytic subunit of cAMP-dependent protein kinase.

Autophosphorylation is a common characteristic of most protein kinases. To determine if the sperm enzyme is autophosphorylated, the purified enzyme preparation from DEAE-Cellulose was incubated with AT^{32}P. Analysis of the products of this reaction with SDS-PAGE and autoradiography showed that 34 and 37 kDa peptides are progressively phosphorylated as a function of time (Fig. 2). A mobility shift from 34 to 37 kDa protein band after phosphorylation was not evident. In this regard, Dahmus reported that calf thymus casein kinase I undergoes a mobility shift after being autophosphorylated which results in the production of species that migrate with Mr's corresponding to 34.4, 35.5, 36.4, 37.7 and 38.9 kDa (17). In the case of the purified sperm enzyme, both the 34 and 37 kDa peptides are recognized by antibodies against calf thymus casein kinase I. However, the antibodies recognized only the 37 kDa peptide in the whole sperm cells lysed in Laemmli buffer immediately after collection. If the cells were incubated at room temperature for 2 h before lysis, both the 37 and 34 kDa peptides are recognized by the antibodies (Fig. 3). These results suggest that the 34 kDa peptide observed in the purified preparation is a proteolytic product of the 37 kDa species and that the proteolysis readily occurs in spermatozoa maintained for a short period at room temperature.

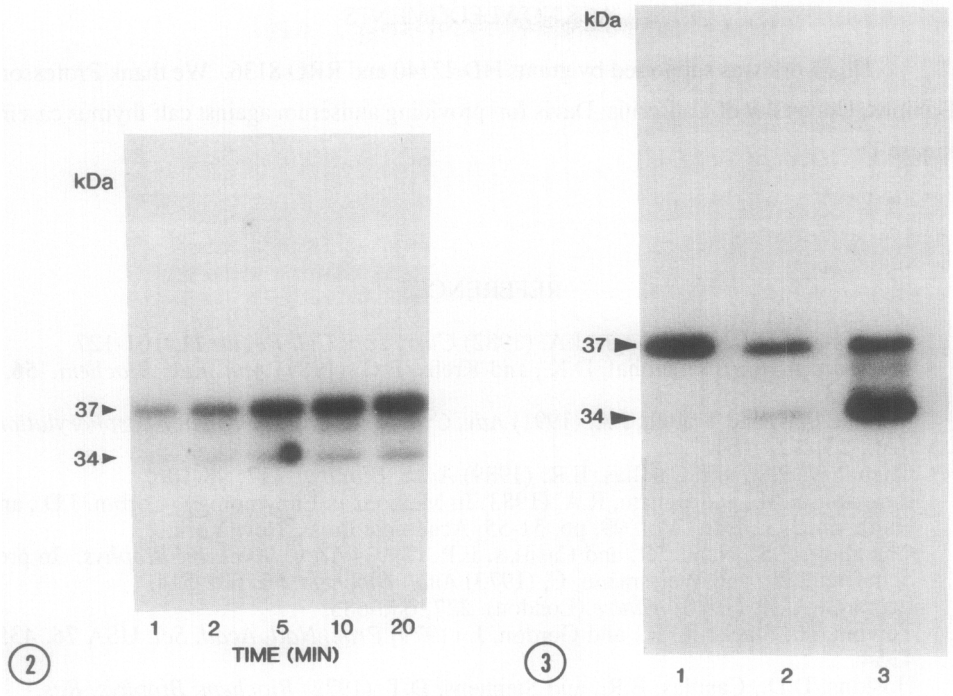

Figure 2. **Autophosphorylation of sperm casein kinase I.** Purified sperm casein kinase I from DEAE-Cellulose chromatography was incubated with 1 μM [γ-^{32}P] ATP (2 μci) for various times and the products were analyzed by SDS-PAGE and autoradiography.

Figure 3. **Immunoblot analysis of bovine sperm casein kinase I.** The blots were probed with antibodies against calf thymus casein kinase I and ^{125}I-Protein A. 1) Freshly collected epididymal sperm lysed and solubilized in Laemmli buffer. 2) Epididymal sperm incubated for 2 h at room temperature before lysis. 3) purified sperm casein kinase I. Lanes 1 and 2 contain 75μg each of sperm proteins and lane 3 contains 2 μg of purified sperm casein kinase I from DEAE-Cellulose.

Type II casein kinases from most sources are stimulated by polyamines, while type I casein kinases are not affected by these compounds (13). Sperm casein kinase I however, is unusual in that the activity is stimulated by spermine (Fig. 1). Since spermidine, cadaverine, putrescine and Mg^{2+} did not stimulate the enzyme, we believe that spermine may be a physiological effector of the enzyme and does not act as a nonspecific cation in stimulating the activity. It is possible that the stimulation by spermine may have resulted from the presence of a small amount of casein kinase II in the preparation; however, this seems unlikely because sperm casein kinase II elutes from phosphocellulose at a much higher salt concentration. Also, casein kinase II (Mr 130 kDa) is well separated from casein kinase I (~37 kDa) on gel filtration, and the casein phosphorylating activity in our casein kinase I preparation was not inhibited by heparin, a selective inhibitor of casein kinase II. In addition, the purified casein kinase II was stimulated 12-fold by 0.5 mM spermine, while casein kinase I was only stimulated 1.6-fold under similar conditions.

In order to understand the functional role of casein kinase I in spermatozoa, the physiological substrates of the enzyme must be identified. The availability of the purified enzyme now should help identify its endogenous substrates.

ACKNOWLEDGMENTS

This work was supported by grants HD 22140 and RRO 8136. We thank Professor Dahmus, University of California, Davis for providing antiserum against calf thymus casein kinase I.

REFERENCES

1. Hathaway, G.M., and Traugh, J.A. (1982) *Curr. Top. Cell Regul.* **21**, 101-127.
2. Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. (1987) *Ann. Rev. Biochem.* **56**, 567-613.
3. Tuazon, P.T., and Traugh, J.A. (1991) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.*, **23**, 123-164.
4. Chaudhry, P.S., and Casillas, E.R. (1989) *Arch. Biophys.* **217**, 98-106.
5. Reimann, E.M., and Beham, R.A. (1983) In Methods in Enzymology (Corbin, J.D., and Hardman, J.G., Eds.) Vol. 99, pp. 51-55, Academic Press, New York.
6. Chaudhry, P.S., Nanez, R. and Casillas, E.R. (1991) *Arch. Biochem. Biophys.* In press.
7. Schaffner, W., and Weissmann, C. (1973) *Anal. Biochem.* **50**, 502-514.
8. Laemmli, U.K. (1970) *Nature* (London). **227**, 680-685.
9. Towbin, H., Staehelin, R., and Gordon, J. (1979) *Proc. Natl. Acad. Sci.* USA **76**, 4350-4354.
10. Hoskins, D.D., Casillas, E.R., and Stephens, D.T. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1331-1338.
11. Garbers, D.L., First, N.L., and Lardy, H.A. (1973) *J. Biol. Chem.* **248**, 875-879.
12. Agostinis, P., Pinna, L.A., Meggio, F., Marin, O., Goris, J., Vandenheede, J.R., and Merlevede, W. (1989) FEBS Lett. **259**, 75-78.
13. Hathaway, G.M., and Traugh, J.A. (1983) In Methods in Enzymology (Corbin, J.D., and Hardman, J.G. Eds.) Vol. 99, pp. 317-331. Academic Press, New York.
14. Chijiwa, T., Hagiwara, M., and Hidaka, H. (1987) *J. Biol. Chem.* **264**, 4924-4927.
15. Kemp, B.E., Graves, D.J., Benjamin, E., Krebs, E.G. (1977) *J. Biol. Chem.* **252**, 4888-4894.
16. Kuenzel, E.A., and Krebs, E.G. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 737 -741.
17. Dahmus, M.E. (1981) *J. Biol. Chem.* **256**, 3319-3325.