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Purification and Characterization of Two Casein Kinases from Ejaculated Bovine Spermatozoa[1]

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Two protein kinases active on casein and phosvitin were partially purified from the soluble fraction of ejaculated bovine spermatozoa. They were operationally termed casein kinase A and B based on the order of their elution from a phosphocellulose column. CK-A showed an approximate molecular mass of 38 kDa, and it phosphorylated serine residues of casein and phosvitin utilizing ATP as a phosphate donor (K_m 19 μM). Enzyme activity was maximal in the presence of 10 mM $MgCl_2$, whereas it decreased in the presence of spermine, polylysine, quercetin, and NaCl (20–250 mM). CK-B seemed to have a monomeric structure of about 41 kDa; it underwent autophosphorylation and cross-reacted with polyclonal antibodies raised against recombinant α, but not β, subunit of human type 2 casein kinase. It phosphorylated both serine and threonine residues of casein and phosvitin, utilizing ATP (K_m 12 μM) but not GTP as a phosphate donor. Threonine was more affected in the phosphorylated phosvitin than in the partially dephosphorylated substrate. CK-B was active toward the synthetic peptide Ser-$(Glu)_5$ and calmodulin (in the latter case, in the presence of polylysine), and it was activated by spermine, polylysine, $MgCl_2$ (30 mM), and NaCl (20–400 mM). The activity of the enzymes was not affected by cAMP, or the heat-stable inhibitor of the cAMP-dependent protein kinase, or calcium.

Protein kinases are ubiquitous enzymes whose nomenclature is not unequivocally established; often they are conveniently identified according to specific substrates phosphorylated (see Refs. *1-3* for reviews). Casein kinases [EC 2.7.1.37] are multipotential cyclic nucleotide- and Ca^{2+}-independent protein kinases which phosphorylate acidic model-substrates such as casein and phosvitin. These enzymes have been found in various tissues and they are probably involved in the regulation of metabolic pathways, transduction of extracellular signals and cellular proliferation (*4, 5*). Two distinct casein kinases have generally been identified in different cell types. They have been designated type 1 and type 2 casein kinases according to the elution order from a DEAE-cellulose column (*6, 7*). They have different structural and catalytic properties, *e.g.* casein kinase type 1 is a monomeric enzyme, of about 40 kDa, which utilizes only ATP as a phosphate donor and phosphorylates only serine residues of native casein, whereas type 2 casein kinase has an oligomeric structure (generally heterotetrameric, composed of two α and two β subunits of molecular mass around 40 and 25 kDa, respectively), modifies both serine and threonine residues and can utilize ATP as well as GTP as a phosphate donor.

The spermatozoa of both invertebrate species, such as sea urchin and starfish (*8, 9*), and vertebrate species, such as rainbow trout (*10*), rat (*11*), goat (*12*), bull (*13*), and man (*14*) are very rich in protein kinase activities.

Cyclic AMP-dependent protein kinase, that has been shown to be involved in the regulation of spermatozoal motility, accounts for by far the bulk of the protein kinase activity of these cells (*13-16*). Little attention has up to now been devoted to cAMP-independent protein kinases of spermatozoa, and little is known about the physiological functions and enzyme properties of spermatozoal casein kinases.

We present here a partial purification and characterization of two casein kinases from ejaculated bovine spermatozoa. The two kinases are distinct from each other and, with some discrepancies, they show characteristic properties of type 1 and type 2 casein kinases, respectively.

MATERIALS AND METHODS

Materials—Whole casein, and α_{s1}- and β-subunits of casein were prepared according to Mercier *et al.* (*17*), and α_{s2}- and k-subunits according to Brignon *et al.* (*18*) and Zittle and Custer (*19*), respectively. Phosvitin was prepared according to Mecham and Olcott (*20*); it was partially dephosphorylated by incubation with potato acid phosphatase (Sigma), and the inorganic phosphate released, evaluated according to Martin and Doty (*21*), was compared with that obtained by total alkaline hydrolysis of phosvitin. Calmodulin was purified to homogeneity from calf brain as in Ref. *22*. The synthetic peptides Ser-$(Glu)_5$ and $(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr were kindly provided by Dr. F. Marchiori, Dept. of Organic Chemistry, University of Padova. The inhibitor of cAMP-dependent protein kinase

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Abbreviations: CK, casein kinase; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

purified according to Walsh *et al.* (*23*) was kindly donated by Dr. P. Parker, Ludwing Institute for Cancer Research, London. Heparin, spermine, PMSF, histone II-AS, poly-L-glutamic acid (M_r 50,000-100,000), poly-L-lysine (M_r 3,000-70,000), and cellulose phosphate were purchased from Sigma. [γ-^{32}P]ATP and [γ-^{32}P]GTP were from the Radiochemical Center, Amersham; Ultrogel AcA 44 from LKB, and heparin-Sepharose from Pharmacia. Polyclonal antibodies raised against recombinant human α and β subunits of casein kinase 2 were a generous gift from Dr. O.-G. Hissinger, Hamburg, Germany.

Preparation of Spermatozoal Soluble Fraction—Bovine semen (about 30 ml containing $0.9\text{-}1.2\times10^9$ spermatozoa/ml) was collected by means of an artificial vagina from bulls housed at the Center for Artificial Insemination in Bassano del Grappa (Vicenza, Italy). Spermatozoa were isolated by 6 min centrifugation at $850\times g$ of 2 ml aliquots of seminal fluid layered onto 2 ml of medium consisting of 135 mM NaCl, 6 mM KCl, 1.5 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 3 mM $KHCO_3$, 2 mM glucose, and 10 mM Na-HEPES, pH 7.4 (isolation medium), supplemented with 30% (v/v) Percoll. The cellular pellet was resuspended in the isolation medium, centrifuged again at $650\times g$ for 7 min, and finally resuspended in 30 ml of medium containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, 100 μM PMSF, 20 μg/ml pepstatin, and 20 μg/ml leupeptin (Buffer A). The cells were disrupted by the French press technique (3,800 psi). The cellular lysate was centrifuged at $1,200\times g$ for 10 min, and the soluble fraction (generally containing about 2 mg protein/ml), was obtained by further centrifugation of the supernatant at $100,000\times g$ for 60 min.

Purification of Enzymes—The soluble fraction was concentrated by ultrafiltration through a Diaflo UM 10 membrane and gel filtered through an Ultrogel AcA-44 column (1.6×86 cm) equilibrated and operated with buffer A containing 0.5 M NaCl, at a flow rate of 7 ml/h. Fractions (1 ml) containing casein kinase activity were pooled, concentrated by Diaflo ultrafiltration, dialyzed overnight against buffer A, and loaded on a phosphocellulose column (1.4×6 cm), equilibrated with the same buffer. The column was eluted by applying a linear gradient of 0.01-0.8 M NaCl and fractions of 1 ml were collected and tested for enzyme activity. Two casein kinase activity peaks, eluted at 0.34 and 0.54 M NaCl and operationally termed casein kinase A (CK-A) and casein kinase B (CK-B), respectively, were collected, dialyzed against buffer A, and separately chromatographed over a heparin-Sepharose column (1.4×3 cm), eluted with a linear gradient of 0.01-1.0 M NaCl. Pooled fractions of CK-A, eluted at 0.31 M NaCl, and CK-B, eluted at 0.55 M NaCl, were concentrated to about 1 ml by Diaflo ultrafiltration, dialyzed against buffer A, and supplemented with 50% glycerol and the protease inhibitors leupeptin (40 μg/ml), pepstatin (20 μg/ml), and trypsin inhibitor (20 μg/ml).

Assay of Protein Kinase Activity—CK-A and CK-B activity was assayed by incubation at 37°C of 10-20 μl of either chromatographic fractions or enzyme preparations in the presence of 10 μM [γ-^{32}P]ATP with a specific radioactivity of about 0.5 μCi/nmol. The incubation medium (0.1 ml) contained 50 mM Tris-HCl, pH 7.0, and 10 mM $MgCl_2$, for the activity assay of CK-A, or 50 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, and 100 mM NaCl for the CK-B assay.

When whole casein (2 mg/ml), casein subunits and phosvitin were used as phosphorylatable substrates, the reaction was stopped after 15-60 min by adding 3 ml of 10% (w/v) trichloroacetic acid and 0.2 mg of phosvitin was then added as a carrier protein. The radiolabeled proteins were recovered by centrifugation, washed three times with cold 10% (w/v) trichloroacetic acid, dissolved in Instagel (Packard) and counted for radioactivity.

The phosphorylation of the synthetic peptide Ser-$(Glu)_5$ was evaluated by isolating ^{32}P-Ser after 4 h hydrolysis at 110°C with 6 N HCl followed by high-voltage paper electrophoresis, as in Ref. *24*. In the case of calmodulin, the reaction was carried out in the presence of 0.06 mg/ml polylysine, and stopped by addition of 2% SDS, 5 mM EDTA, and 1% 2-mercaptoethanol (final concentrations). The sample was boiled for 3 min and subjected to gel electrophoresis on vertical plates of SDS-containing polyacrylamide (10%) (SDS/PAGE), essentially according to Laemmli (*25*). The slabs were stained with Coomassie Blue, dried and autoradiographed, and the labeled proteic band was cut out and counted for radioactivity. The phosphorylation of histone II-AS was performed according to Beavo *et al.* (*26*).

Analysis of Phosphoaminoacids—Isolation and determination of ^{32}P-Ser and ^{32}P-Thr from phosphorylated protein substrates were performed by pH 1.9 high-voltage paper electrophoresis after hydrolysis in 6 N HCl, as described in Ref. *27*. The experimental values were corrected for hydrolytic losses of 48 and 13% for ^{32}P-Ser and ^{32}P-Thr, respectively.

Autophosphorylation of the Enzymes—Autophosphorylation of CK-A and CK-B was performed by incubating the enzymes (0.1-0.3 μg) at 37°C for 30-90 min in the presence of 10 μM [γ-^{32}P]ATP (5 μCi/nmol), in the same incubation medium used for the enzyme activity assay but without the protein substrate. The reaction was stopped by addition of 2% SDS, 5 mM EDTA, and 1% 2-mercaptoethanol (final concentrations). The samples were boiled, and subjected to polyacrylamide gel electrophoresis and autoradiography as described above. The apparent molecular mass of the proteins was calculated by calibration with the following marker proteins: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Assay of Immunoreactivity with Anti-Casein Kinase 2 Subunit Antibodies—Western blots experiments were performed with enzyme fractions which had been subjected to 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters, in a Hoefer apparatus at 400 mA for 2 h. The filters were blocked for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. Rabbit antisera raised against recombinant human α and β subunits of casein kinase 2 (*28*), were used at dilutions of 1:500 and 1:200, respectively. Immunoreactive proteins were incubated with donkey anti-rabbit Ig, biotinylated and detected by incubation with streptavidin-conjugated alkaline phosphatase.

Protein Determination—The protein concentration of chromatographic fractions was measured by optical absor-

bance at 280 nm, whereas that of the final enzyme preparation was determined according to Bradford (*29*).

RESULTS

Partial Purification of Two Casein Kinases—A peak of casein kinase activity, corresponding to an apparent M_r of about 40,000 emerged from an Ultrogel AcA 44 column loaded with the soluble fraction of ejaculated bovine spermatozoa (Fig. 1A). The fractions containing enzyme activity were pooled, concentrated, dialyzed, and chromatographed on a phosphocellulose column, from which two peaks of casein kinase emerged (Fig. 1B). One peak, operationally termed casein kinase A (CK-A) eluted at 0.34 M NaCl, whereas a second peak, termed CK-B, eluted at 0.54 M NaCl.

The two fractions obtained from phosphocellulose were further purified through heparin-Sepharose. The elution profiles are reported in Fig. 2 and show that CK-A emerged as a single peak whereas CK-B was resolved into two peaks. The first small peak was due to contaminating CK-A (see later) and was discarded. The second peak was used for the CK-B characterization.

The heparin-Sepharose fractions of CK-A and CK-B were concentrated and used within a few days, since they rapidly lost their activity even in the presence of glycerol and the protease inhibitors PMSF, leupeptin, pepstatin, and trypsin inhibitor.

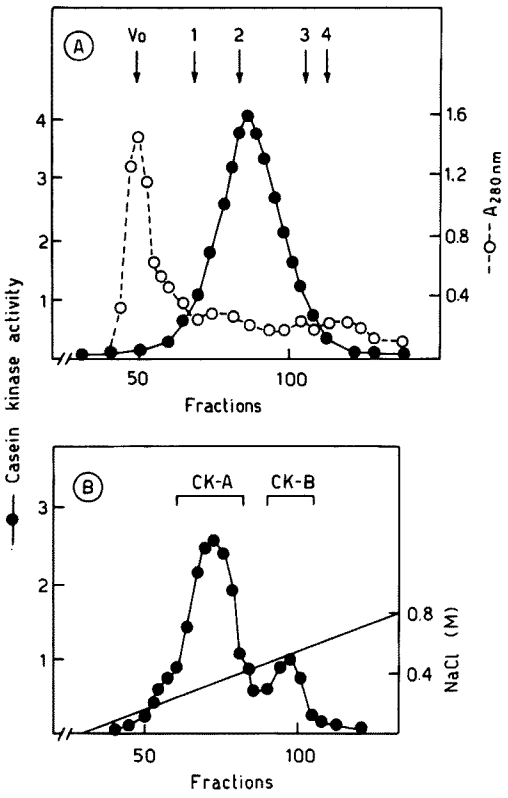

Fig. 1. **Ultrogel AcA 44 filtration (A) and subsequent phosphocellulose chromatography (B) of soluble fraction of ejaculated bovine spermatozoa.** Arrows indicate elution of: bovine serum albumin (1), ovalbumin (2), carbonic anhydrase (3), and trypsinogen (4). Fractions 71-102 were collected from the Ultrogel eluate and loaded onto the phosphocellulose column.

A considerable loss of the total activity also occurred during the purification procedure, and this hindered a reliable determination of the specific activity increase. The final CK-A preparation (containing an average of 2.2 μg protein/ml) showed a specific activity approximately 680-fold higher than that of the crude soluble fraction with a 0.23% yield of the total activity. The specific activity of the purified CK-B preparation (0.8 μg protein/ml) was approximately 950-fold higher than that of crude cytosolic fraction (yield 0.14%).

Characterization of the Two Casein Kinases—Attempts to determine the M_r of the two fractions of casein kinase by SDS/PAGE were not completely satisfactory because, together with major bands of about 38 and 41 kDa for CK-A and CK-B, respectively, a few other faint bands were present in the gel (Fig. 6B). These values were however consistent with those obtained by a second gel filtration through the Ultrogel AcA 44 column of the enzymatic peaks obtained after the phosphocellulose chromatography. Confirmation of the CK-B M_r was obtained by the autophosphorylation and immunoreactivity experiments (see later).

The dependence of the enzyme activity on the buffer pH was investigated and the results (Fig. 3A) show that CK-A had an optimum pH around 7 whereas that of CK-B was between 7.5 and 8. The two enzyme preparations were differently affected by NaCl addition to the assay medium: CK-A activity was progressively decreased by increasing the NaCl concentration, being completely inhibited by 0.25 M NaCl, whereas CK-B was activated by addition of NaCl

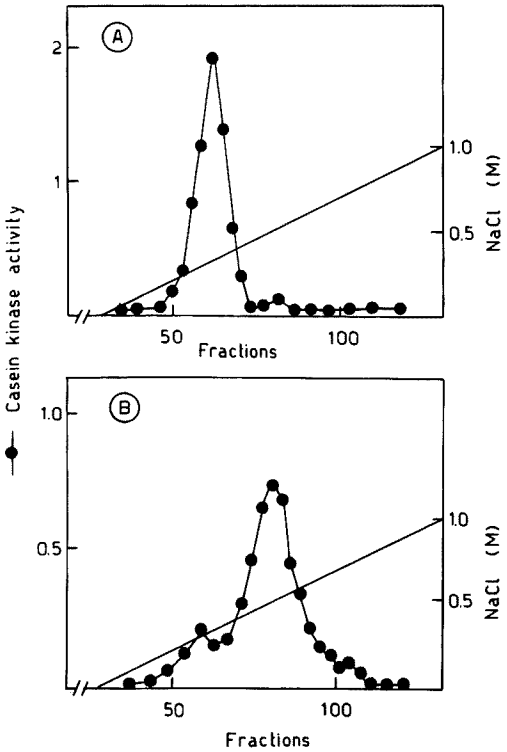

Fig. 2. **Heparin-Sepharose chromatography of the two casein kinase fractions resolved by phosphocellulose chromatography.** Chromatographic profiles of the conventionally termed CK-A and CK-B are shown in Panels A and B, respectively.

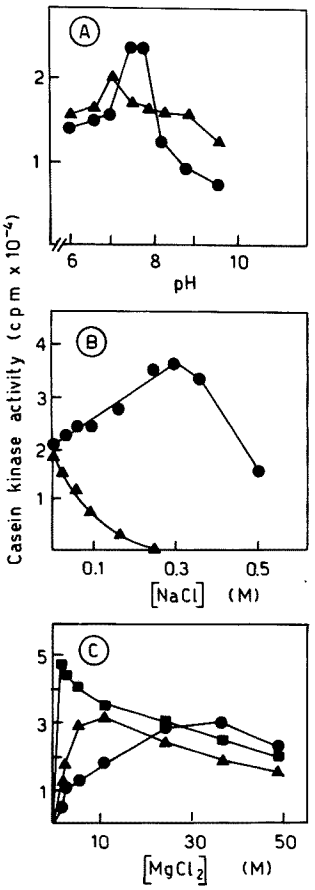

Fig. 3. **Effect of pH (A) and increasing concentration of NaCl (B) and $MgCl_2$ (C) on the activities of CK-A (▲) and CK-B (●, ■).** Experimental conditions are described under "MATERIALS AND METHODS." Values are means of at least four determinations and the standard error was always less than 11%. Points (■) express CK-B activity measured in the presence of 1.3 mM spermine.

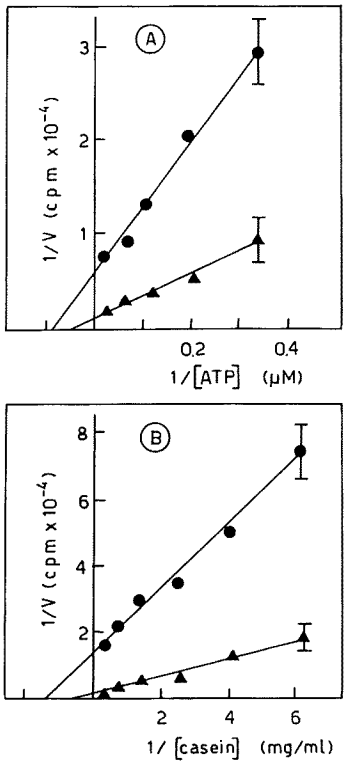

Fig. 4. **Double reciprocal plots of CK-A (▲) and CK-B (●) activities against ATP (A) or casein (B) concentrations.** Values are means of at least four determinations with bars indicating the SD.

TABLE I. **Substrate specificity of the two casein kinases.** Enzyme activities are expressed (means ± SD, $n=4$) as pmol of ^{32}P incorporated per min of incubation carried out as described in "MATERIALS AND METHODS" in the presence of 2 mg/ml casein, casein subunits, and phosvitin, 0.6 mg/ml Ser-$(Glu)_5$ and $(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr or 0.06 mg/ml calmodulin.

Substrate	CK-A activity	CK-B activity
Whole casein	2.75±0.3	2.45±0.4
α_{s1}-subunit	1.05±0.1	2.82±0.4
α_{s2}-subunit	1.61±0.1	4.53±0.4
β-subunit	1.93±0.3	5.82±0.7
k-subunit	0.31±0.02	0.21±0.01
Phosvitin	0.85±0.1	3.94±0.4
Dephospho-phosvitin	1.36±0.2	3.23±0.3
Calmodulin	0	4.96±0.6
Ser-$(Glu)_5$	0	1.50±0.2
$(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr	0	0

TABLE II. **Protein kinase activities on casein in the presence of various effectors.** Enzyme activities, expressed as % of controls, are means (±SD) of at least four determinations.

Addition	CK-A activity	CK-B activity
–	100	100
Heparin (0.1 mg/ml)	62±13	38±6
Quercetin (100 μM)	44±6	48±5
Spermine (1.3 mM)	55±7	230±46
Polylysine (80 μg/ml)	60±7	194±21
Polyglutamate (0.6 mg/ml)	77±8	93±9
cAMP (2 mM)	98±5	95±7
Inhibitor of cAMP-dependent protein kinase (0.4 mg/ml)	90±8	96±4

in the range of 0.02-0.4 M with the highest activity at 0.3 M NaCl (Fig. 3B).

Both enzyme preparations were stimulated by $MgCl_2$, but CK-A was maximally activated by about 10 mM and CK-B by about 30 mM $MgCl_2$ (Fig. 3C). In the presence of 1.3 mM spermine the highest CK-B activity was observed at 1.5 mM $MgCl_2$.

CK-B showed a higher affinity than CK-A for both ATP and casein: the calculated K_m values for ATP were 12±2 μM (mean±SD, $n=4$) for CK-B and 20 ±3 μM (mean± SD, $n=4$) for CK-A and those for whole casein were 0.7± 0.1 and 1.5±0.1 mg/ml for CK-B and CK-A, respectively (Fig. 4).

The enzymes did not label casein when [γ-^{32}P]GTP was used instead of [γ-^{32}P]ATP as the phosphate donor, at variance with the result obtained with casein kinase 2 purified from rat liver (*30*) and assayed under the same experimental conditions (not shown).

Substrate Specificity—The efficiency in phosphorylating the casein subunits and phosvitin was then tested and it was found that CK-A was more active on whole casein than on casein subunits, whereas the opposite was the case for CK-B, with the exception of k-subunit (Table I). Since the activity on whole casein was similar for both kinase fractions, CK-B was much more active than CK-A on α_{s1}, α_{s2}, and β casein subunits. Also phosvitin and dephospho-phosvitin were phosphorylated more efficiently by CK-B, which, at variance with CK-A, was also active on the synthetic peptide Ser-$(Glu)_5$, which is considered to be a specific peptide substrate for CK-2 (*31*), and calmodulin [in the presence of polylysine, which is required for its phosphorylation (*32*)]. The enzymes showed no activity on the

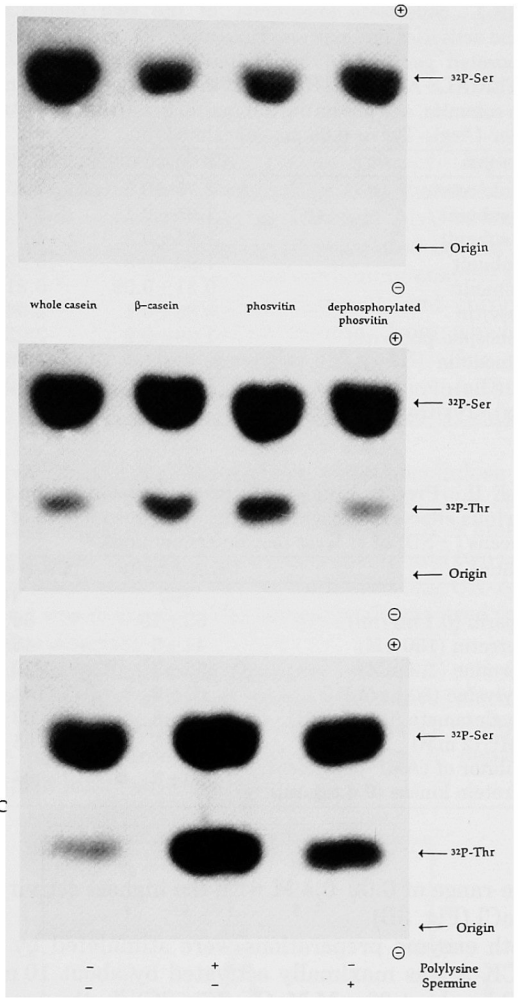

Fig. 5. **Isolation of phosphoamino acids from different substrates phosphorylated by CK-A (panel A) and CK-B (panel B); effect of polylysine (80 μg/ml) and spermine (1.3 mM) on amino acids phosphorylated in casein by CK-B (panel C).** Phosvitin was 30% dephosphorylated as described in "MATERIALS AND METHODS." Autoradiograms are representative of experiments performed in triplicate.

peptide $(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr, a specific substrate for protein kinase C (*33*).

Response to Various Effectors—A criterion generally employed for the classification of casein kinases is their response to various effectors. We found that CK-A was inhibited by a number of compounds including spermine, polylysine, and high concentrations of heparin, while CK-B was also inhibited by high doses of heparin but greatly stimulated by polylysine and spermine, the effect of the latter being particularly evident at low $MgCl_2$ concentrations (Fig. 3C). Both enzymes were about 50% inhibited by 100 μM quercetin, a well known inhibitor of casein kinases (*7*), while they were slightly affected by polyglutamate, cAMP, inhibitor of the cAMP-dependent protein kinase (Table II), $CaCl_2$ (1 mM), or EGTA (5 mM) (not shown) added to the incubation mixture.

Analysis of Amino Acids Phosphorylated—In order to determine the amino acid targets of CK-A and CK-B the two enzymes were incubated, in the presence of ATP, with either whole casein or its β-subunit or phosvitin. The autoradiograms of the phosphorylated substrates hydrolyzed and subjected to paper electrophoresis (Fig. 5) show that CK-A phosphorylated only serine residues, whereas CK-B preferred serine but was also active on threonine. Figure 5 also shows that CK-A more efficiently phosphorylated whole casein than its β-subunit (see also Table I), and partially dephosphorylated than phosphorylated phosvitin. CK-B phosphorylated almost equally the serine residues of both whole casein and its β-subunit but affected threonines more efficiently in the latter substrate (where the $^{32}P_i$ bound to this amino acid was about 24% of that incorporated by serine) than in whole casein (where ^{32}P-threonine was about 13% of ^{32}P-serine). The degree of threonine phosphorylation was about 3-fold higher in phosphorylated phosvitin than in phosvitin subjected to a dephosphorylating pretreatment.

As expected from the finding that CK-A affected only serine residues, the inhibitory effect of either spermine or polylysine (Table II) resulted in decreased phosphorylation of this amino acid (not shown). On the contrary, these effectors caused a marked increase and a slight increase in phosphorylation of threonine and serine residues of casein, respectively (Fig. 5C). In the presence of polylysine the $^{32}P_i$ bound to threonine was about 61%, whereas in the presence of spermine, it was 42% of the total radioactive phosphate incorporated into casein.

Autophosphorylation of the Enzymes—Since autophosphorylation is a general feature of casein kinases, we performed some experiments in order to establish whether this process also occurred in the spermatozoal enzymes. We found that CK-A preparations did not undergo autophosphorylation, whereas a band of labeled phosphoprotein of approximate molecular weight 41,000 was found in experiments carried out with CK-B preparations (Fig. 6).

Immunoreactivity of Spermatozoal Casein Kinases with Anti-Human Casein Kinase 2 Subunit Antibodies—In order to assess possible homologies between casein kinases from bovine spermatozoa and typical casein kinase 2, immunoblot analysis was performed with rabbit antisera raised against recombinant α or β subunits of human CK-2. As shown in Fig. 6, anti-α antisera cross-react with a proteic band of about 41 kDa present both in the crude soluble fraction and in the purified CK-B, but not in the CK-A preparations. As expected, these antibodies also react with CK-2 purified from rat liver (*30*).

As was predictable on the basis of the low molecular weight of spermatozoal casein kinases in comparison with typical heterotetrameric CK-2, no interaction was observed between anti-β recombinant subunit antisera and purified CK-B (and CK-A) preparations.

DISCUSSION

The present results unequivocally show that two distinct casein kinases, operationally termed CK-A and CK-B, are present in the soluble fraction of ejaculated bovine spermatozoa. CK-A elutes first from the phosphocellulose column, modifies only serine residues in casein and phosvitin, utilizes only ATP as a phosphate donor with an apparent K_m of 19 μM, is only slightly sensitive to heparin inhibition, is inhibited by spermine and does not phosphorylate Ser-$(Glu)_5$, which is considered a specific substrate for casein kinases of type 2. These properties meet the

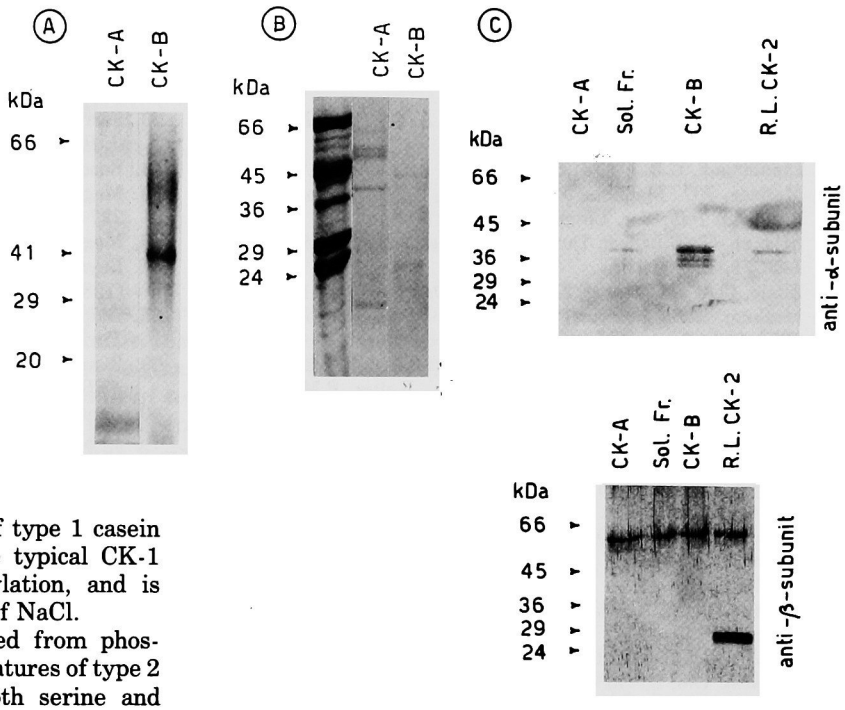

Fig. 6. Autophosphorylation (panel A), SDS/PAGE (panel B), and immunoblots (panel C) of CK-A and CK-B preparations. (A) Representative autoradiogram of purified CK-A and CK-B preparations incubated in the presence of [γ-^{32}P]ATP; (B) electrophoretic gels of purified enzymes stained with Coomassie Blue; (C) immunoblots obtained with 50 μl of crude soluble fraction, purified CK-A and CK-B preparations probed with antibodies raised against recombinant α and β subunits of human casein kinase 2. Immunoblot of casein kinase 2 purified from rat liver (R.L. CK-2) (*30*) and added at a concentration giving 5-fold higher enzyme activity, is also shown for a comparison. Other experimental details are described in "MATERIALS AND METHODS."

criteria for assigning CK-A to the group of type 1 casein kinases. However, CK-A differs from the typical CK-1 since it does not undergo autophosphorylation, and is inhibited by relatively low concentrations of NaCl.

The second casein kinase fraction eluted from phosphocellulose displays some characteristic features of type 2 casein kinases, *i.e.*, it phosphorylates both serine and threonine residues of casein and phosvitin, is activated by spermine and polylysine, and is active on Ser-$(Glu)_5$. CK-B however differs from the typical CK-2 since it does not utilize GTP as a phosphate donor, it is inhibited only by high heparin concentration (*34*), it is less active, particularly toward threonine residues, on dephosphorylated than native phosvitin (*35*), and it appears to consist of only one subunit-type. A heterotetrameric structure composed of two larger α subunits (molecular mass 36-44 kDa) and two smaller β subunits (24-28 kDa) is shared by most animal CK-2. The β subunits undergo fast autophosphorylation through an intramolecular mechanism (*36*). We have unsuccessfully searched for a β autophosphorylatable subunit at each step of the purification procedure. Negative results were also obtained from the immunoblot analysis carried out with antibodies raised against human β-subunit of CK-2 and tested on both crude soluble fraction and purified CK-B from ejaculated spermatozoa. This should rule out the occurrence of the dissociation and loss of the β subunit during the enzyme purification procedure, but we cannot exclude a possible rapid and extensive proteolysis during the cellular disruption, though this was performed in the presence of a number of protease inhibitors.

The absense of the non catalytic β subunits has also been noted for nuclear CK-2 from porcine and rat liver (*37, 38*) as well as in lower eukaryotes and plants (*39-42*), where autophosphorylation seems to occur on the catalytic enzyme protein (*39, 42*).

Interestingly however CK-B immunoreacted with antibodies raised against the α-subunit of human CK-2, showing an immunological relationship with the classical mammalian type 2 casein kinases.

Recently, Chaudhry and Casillas purified from epididymal bovine spermatozoa a casein kinase which is activated by spermine and polylysine, utilizes both ATP and GTP as phosphate donors, and is inhibited by micromolar concentrations of heparin, thus showing typical features of CK-2 (*43*). Whether these differences are due to different sources of spermatozoa, *i.e.*, epididymal and seminal fluid, and therefore to different stages of maturation, remains to be established. It might be worth mentioning that on the one hand, it has been found that changes of properties of casein kinases occur in parallel with cellular differentiation and maturation (*44-46*), while on the other hand, protein kinase C activity was found in the epididymal but not in ejaculated spermatozoa (*47*) (and R. Deana, unpublished observations).

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