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Major 56,000-Dalton, Soluble Phosphoprotein Present in Bovine Sperm Is the Regulatory Subunit of a Type II cAMP-Dependent Protein Kinase

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It has been shown that cAMP-dependent phosphorylation of a soluble sperm protein is important for the initiation of flagellar motion. The suggestion has been made that this motility initiation protein, named axokinin, is the major 56,000-dalton phosphoprotein present in both dog sperm and in other cells containing axokinin-like activity. Since the regulatory subunit of a type II cAMP-dependent protein kinase is a ubiquitous cAMP-dependent phosphoprotein of similar subunit molecular weight as reported for axokinin, we have addressed the question of how many soluble 56,000-dalton cAMP-dependent phosphoproteins are present in mammalian sperm. We report that in bovine sperm cytosol, the ratio of the type I to type II cAMP-dependent protein kinase is approximately 1:1. The type II regulatory subunit is related to the non-neural form of the enzyme and undergoes a phosphorylation-dependent electrophoretic mobility shift. The apparent subunit molecular weights of the phospho and dephospho forms are 56,000 and 54,000 daltons, respectively. When bovine sperm cytosol or detergent extracts are phosphorylated in the presence of catalytic subunits, two major proteins are phosphorylated and have subunit molecular weights of 56,000 and 40,000 daltons. If, however, the type II regulatory subunit (RII) is quantitatively removed from these extracts using either immobilized cAMP or an anti-RII monoclonal affinity column, the ability to phosphorylate the 56,000- but not 40,000-dalton polypeptide is lost. These data suggest that the major 56,000 dalton cAMP-dependent phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase and not the motility initiator protein, axokinin.

Key words: sperm, phosphorylation, cAMP-dependent protein kinase, motility, axokinin

Abbreviations used: RI, regulatory subunit of a type I cAMP-dependent protein kinase; RII, regulatory subunit of a type II cAMP-dependent protein kinase; mAB, monoclonal antibody; 8-N_3-[^{32}P]cAMP, 8-azido-[^{32}P]cAMP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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cAMP and Ca^{2+} have been shown to be important regulators of flagellar motion [1–7]. We have previously demonstrated that a type II cAMP-dependent protein kinase [8,9] and a Ca^{2+}/calmodulin-regulated cyclic nucleotide phosphodiesterase [10] are integral components of demembranated rat sperm flagella, suggesting that both enzymes are involved in the regulation of motion. It is generally believed that cAMP plays a primary role in the initiation of motion. Several results indicate that the cAMP-dependent phosphorylation of a soluble protein present in detergent extracts of both mammalian and sea urchin sperm is the signal for the initiation of motion. Tash and coworkers [11] have suggested that the initiation of motion in modeled dog sperm is dependent on the cAMP-dependent phosphorylation of a 56,000 dalton polypeptide. The identification of this motility initiator protein, called axokinin, was based on the observation that this was the only major cAMP-dependent phosphoprotein common to all cells and tissues which displayed axokinin-like activity. In addition, Brandt and Hoskins [12] also identified a 55,000-dalton cAMP-dependent protein in bovine sperm extracts. They showed that the ability to phosphorylate this protein was related inversely to the motility state of the sperm prior to extraction.

It is known, however, that mammalian sperm contains significant amounts of cAMP-dependent protein kinases [13–15]. In other cells and tissues, it has been shown that the regulatory subunit of the type II protein kinase is phosphorylated by the catalytic subunit [16]. RII also has an apparent subunit molecular weight similar to that reported for the cAMP-dependent motility initiator protein mentioned in the above studies [16]. The purpose of this present investigation was to characterize the nonflagellar type II cAMP-dependent protein kinase in bovine sperm and to determine whether multiple 56,000-dalton cAMP-dependent phosphoproteins exist in the cytosolic and detergent extracts of these cells.

MATERIALS AND METHODS

Materials

cAMP, ATP, leupeptin, pepstatin, aprotinin, and benzamidine were purchased from Sigma Chemical Co. [^{3}H]cAMP and [γ-^{32}P]ATP were purchased from Amersham; 8-N_3-[^{32}P]cAMP was from ICN. Immobilized protein A and cAMP coupled to agarose (hexane spacer arm) were obtained from Pharmacia. The nitrocellulose paper (0.45 μm) was purchased from Schleicher and Schuell, IgG-free serum albumin from United States Biochemical Corporation, and peroxidase-conjugated goat antimouse IgG was purchased from Cooper Biomedical. All other chemicals were of reagent grade. Bovine heart RII and catalytic subunit were a generous gift from Dr. C. S. Rubin (Albert Einstein College of Medicine).

Preparation of Sperm Extracts

Bovine caudal epididymii were obtained from a local slaughterhouse and processed within 2 hr of removal from the animal. Sperm was obtained by mincing the caudal epididymii and releasing the sperm into buffer A (50 mM Tris HCl, pH 8.1, containing 2 mM $MgCl_2$; 150 mM NaCl; 0.1 mM EDTA; 5 mM benzamidine; 1 mM EGTA; and 1 μg/ml aprotinin, leupeptin, and pepstatin) for 15 min at room temperature. Sperm cells were then washed twice in buffer A and centrifuged for 10 min (3,000 g) at 4°C. Sperm cells were then processed for the preparation of either the lubrol or the cytosolic extract.

Preparation of Lubrol Extract

Washed sperm cells were resuspended in 2 vol of buffer A containing 0.2% Lubrol. After gentle mixing, the suspension was incubated for 15 min on ice, then centrifuged at 17,000 g for 40 min at 4°C. The supernatant was collected and, if not used immediately, stored at −70°C until use.

Preparation of Cytosol

Washed sperm cells from two caudal epididymii were suspended in 10 ml of buffer A, rapidly frozen in liquid nitrogen, and thawed. The cells were then centrifuged at 31,000 g for 20 min. The cytosolic extract was collected and, if not used immediately, stored at −70°C until use.

Affinity Purification of Monoclonal Antibodies

For these studies, we used three monoclonal antibodies—622, 526, and 294. They were obtained by injecting hybridomas into pristane-primed Balb/c mice and collecting the ascites fluid. mAB 622 and mAB 294 cross-react with both bovine heart and bovine brain RII. mAB 526 cross-reacts specifically with bovine brain RII. Antibodies were purified from ascites fluid by the method of Ey et al. [17]. The ascites fluid containing monoclonal antibodies was applied to a 5 ml protein A Sepharose column which had been equilibrated with 0.1 M sodium phosphate, pH 8.0. The column was washed with this buffer until protein was no longer eluted from the column. The monoclonal antibodies were specifically eluted with 0.1 M sodium-citrate, pH 3.5. One-milliliter samples were eluted directly into 200 μl of 1 M Tris HCl, pH 9.0 and were then aliquoted and stored at −70°C.

Preparation of Immobilized Monoclonal Antibody

mAB 622 was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. At room temperature, 1.4 mg of mAB 622 was coupled to 1.5 ml activated Sepharose 4B for 2 h.

Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out in 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate, according to the method of Laemmli [18]. The gels were run at a constant current of 25 mA, stained with Coomassie brilliant blue (0.02%) and destained prior to drying. When immunoblotting was necessary, the resolved polypeptides were transferred to nitrocellulose paper (0.45 μm) by the Western blotting procedure described by Burnette [19]. Electroblotting was performed at room temperature for 8–16 hr at 150 mA constant amperage, in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM glycine, and 20% methanol. Immunoblots were blocked with 10% milk for 2 hr at room temperature and then treated sequentially with the appropriate monoclonal antibody and peroxidase conjugated goat antimouse IgG (1:1,000 dilution).

Phosphorylation Procedures

Two methods were used:

1. Phosphorylation of proteins blotted onto nitrocellulose as described by Valtorta et al. [20]: Nitrocellulose blots containing the proteins of interest were incubated for 1

hr in a blocking solution, (50 mM Tris HCl [pH 7.4], containing 200 mM NaCl, 0.4% (wt/v) Ficoll 400, 0.1% Triton X-100) to reduce the background radioactivity. Nitrocellulose strips were then incubated for 90 min at room temperature in 50 mM HEPES (pH 7.4), containing 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM 2-mercaptoethanol, 0.01–0.1 μM catalytic subunit of cAMP-dependent protein kinase, 50 nM [γ-^{32}P]ATP (500 μCi/nmol), and 0.1% Triton X-100. The reaction was terminated by washing the strips (3 hr to overnight) in 50 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 0.1% Tween 20 (v/v). The dried nitrocellulose blots were exposed either to Kodak 5XAR film in the presence of an intensifying screen (Quanta III, DuPont) at −70°C or to Kodak SB5 or BB5 x-ray film at room temperature.

2. Direct phosphorylation of cytosolic and detergent extracts: Reaction mixtures (final volume 100 μl) contained 40 mM Tris HCl, pH 7.8, 5 mM $MgCl_2$, 0.1–0.2 μM of catalytic subunit, 10 μM cAMP, and 10 μM [γ-^{32}P]ATP. The reaction was initiated by the addition of [γ-^{32}P]ATP and performed at room temperature for the times indicated in the legend. Phosphorylation was terminated by the addition of sample application buffer, and the samples were processed for SDS-PAGE and autoradiographed as described previously.

Detection of RII Binding Proteins in Cytosol

The detection of RII binding proteins was performed by indirect visualization using nonradiolabeled RII, followed by mAB 622 as described by Leiser et al. [21].

Precipitation of the Regulatory Subunit of Type II Protein Kinase With mAB 622 Coupled to Sepharose 4b

Immobilized mAB 622 was washed twice in 20 mM Tris HCl, pH 8.1, and resuspended in 1 vol of the same buffer. This suspension was added to either cytosol extract or Lubrol extract (1:2 dilution) and gently agitated overnight at 4°C. The reaction mixtures were then centrifuged for 10 min at 900g, room temperature. The pellets were washed twice in 20 mM Tris-HCl, pH 7.4. The initial supernatant was submitted to a second incubation with immobilized mAB 622 for 1 hr at 4°C and then centrifuged. This final supernatant plus the two pellets were then submitted to phosphorylation and to immunoblotting with mAB 622 as previously described.

Photoaffinity Labeling of Sperm Preparations

The incorporation of 8-N_3-[^{32}P]cAMP was performed according to a modification of the general procedure described by Walter et al. [22]. The reactions were performed in 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.5 mM EDTA, 0.1 mM 1-methyl-3-isobutylxanthine, containing 0.15 μM 8-N_3-[^{32}P]cAMP, in a final volume of 120 μl. Incubations were carried out in a U-shaped lucite microtiter plate (Cooke) for 45 min at room temperature in the dark. Wells were then irradiated for 10 min at a distance of 10 cm on ice, with a Mineralight ultraviolet lamp. The control contained 0.1 mM cAMP. The samples were then processed for electrophoresis as described previously.

RESULTS

RI:RII Ratio in Bovine Epididymal Sperm Cytosol

The subunit molecular weights of the cAMP-binding protein present in bovine sperm cytosol were determined using the photoaffinity probe 8-N_3-[^{32}P]cAMP. We

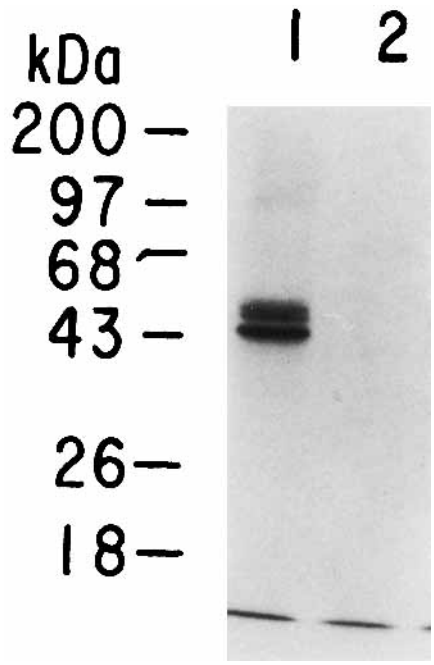

Fig. 1. Photoaffinity labeling of bovine sperm cytosol with 8-N_3-[^{32}P]cAMP. Photoaffinity labeling was performed as described in Materials and Methods. **Lanes 1** and **2** represent labeling in the presence and absence of 1 mM cAMP, respectively.

observe three major radiolabeled bands of Mr 48,000, 54,000, and 56,000 daltons that bind the photoprobe in a specific manner—ie, labeling is blocked in the presence of excess cAMP (Fig. 1, lanes 1 and 2). When this experiment is performed with the detergent extract, a similar pattern is observed (Fig. 10, lanes 1 and 2). In some experiments, an additional band at 40,000 dalton is found (data not shown). The 56,000 and 54,000 polypeptides represent the phosphorylated and dephosphorylated forms of RII (see below). The 48,000-dalton polypeptide is presumably the RI regulatory subunit, while the 40,000-dalton radiolabeled polypeptide is the major proteolytic cleavage product of the regulatory subunit as reported previously [24]. Similar findings have been reported for rat sperm by Horowitz et al. [5] and for bull sperm by Nolan et al. [15].

Due to the different efficiencies in the labeling of RI and RII by 8-N_3-[^{32}P]cAMP, this approach only gives qualitative information concerning RI:RII ratios in cell and tissue preparations. We have determined this ratio more accurately by immunoprecipitation of RII following the formation of the [^{3}H]cAMP:R complexes under equilibrium binding conditions and saturating levels of [^{3}H]cAMP. Following immunoprecipitation of RII with mAB 622 immobilized to Sepharose 4B, the [^{3}H]cAMP binding activity (ie, [^{3}H]cAMP:RI) remaining in the supernatant was measured. In bovine sperm cytosol, approximately 50% of the total cAMP binding activity is represented by RII (Fig. 2). In rat sperm cytosol, we have found that 75% of the total [^{3}H]cAMP binding activity is RII [8].

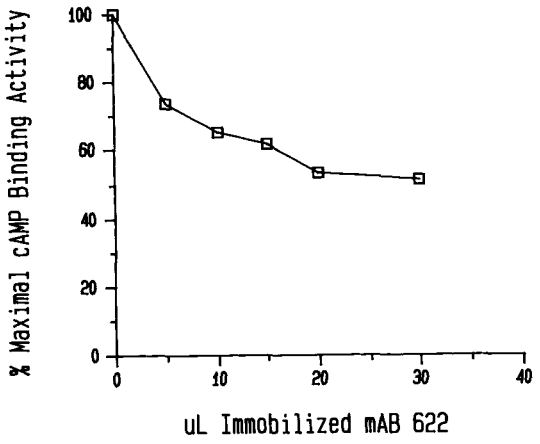

Fig. 2. Immunoprecipitation of RII:[^{3}H] cAMP binding activity in sperm cytosol using immobilized mAB 622. Cytosolic extracts, in a final volume of 100 µl, were incubated with 1 mM [^{3}H]cAMP for 45 min on ice in 20 mM HEPES (pH 7.4), 150 mM KCl, 0.05 mM EDTA, 0.01 mM 1-methyl-3-isobutylxanthine, and 1 mg/ml BSA. The indicated amounts of immobilized mAB 622 were added. After overnight incubation at 4°C, the samples were centrifuged at 900g for 10 min. The supernatant was collected and centrifuged again to ensure all immobilized mAB 622 was pelleted. The [^{3}H]cAMP:R complexes remaining in solution were measured using a Millipore filtration assay as previously described [23].

Immunological Cross-Reactivity of the Bovine Sperm Cytosolic RII With Neural and Non-Neural Forms of RII

The regulatory subunit of a type II kinase has been shown to exist in neural and non-neural specific isoforms. Monoclonal antibodies against bovine type II regulatory subunits exist which specifically cross-react only with the neural isoform. Figure 3 shows an immunoblot analysis of bovine sperm cytosol with a collection of anti-RII mAB's. Panel A demonstrates that mAB 526, a neural specific antibody, does not cross-react with the sperm RII even at high antibody concentrations (20 nM). Panels B and C show that mAB 294 and 622 do cross-react with the sperm RII. Both of these monoclonal antibodies cross-react with both non-neural—ie, heart—and neural forms of RII, albeit more strongly with the former. This data suggests that the sperm RII is not of the neural subtype. On close examination of these immunoblots, it can be seen that both mAB 294 and 622 recognize two polypeptides of similar electrophoretic mobilities. That these are the phospho and dephospho forms of RII was demonstrated by phosphorylation of sperm cytosol with nonradioactive ATP (1 mM) and exogenous catalytic subunit prior to SDS-PAGE/immunoblot analysis. As can be seen from Figure 4, prephosphorylation of the sperm cytosol caused a shift in the electrophoretic mobility of all of the RII recognized by mAB 622 to the slower migrating species.

Detection of RII Binding Proteins in Bovine Sperm Cytosol

RII has been shown to form specific complexes with a limited subset of cellular proteins. These include the microtubule-associated protein, MAP 2 and a 75,000-dalton calmodulin binding protein [25–27]. Lohman et al. developed an RII overlay system for the detection of RII-binding proteins [26]. Using this approach, Horowitz et al. [6]

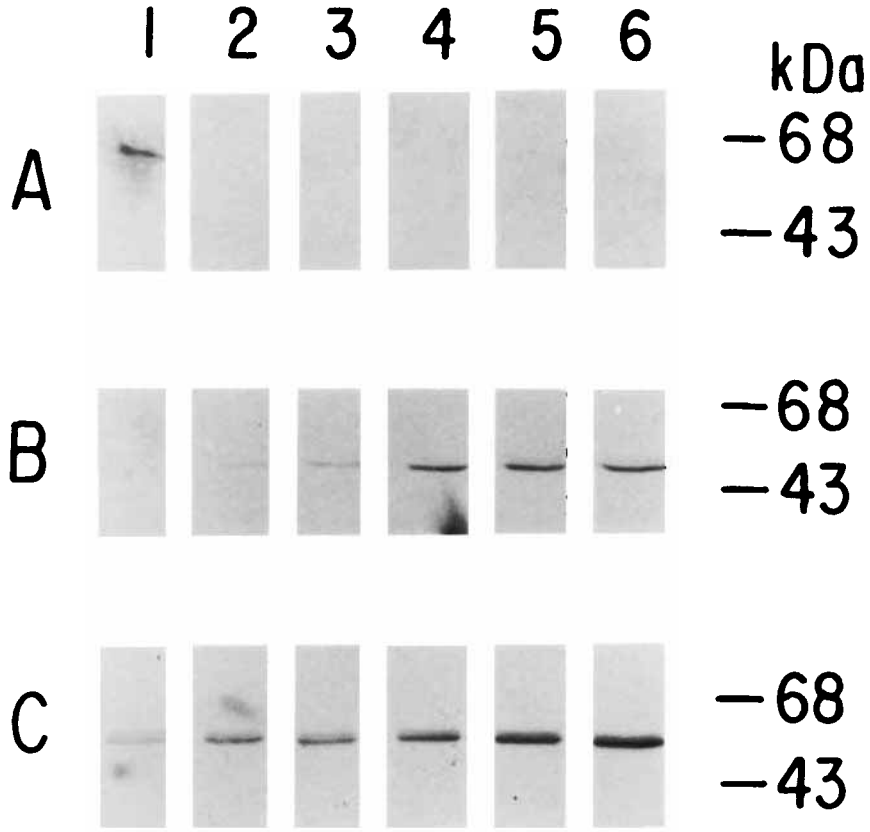

Fig. 3. Immunoblots of bovine sperm cytosolic RII with increasing amounts of purified anti-RII monoclonal antibodies. SDS-PAGE and immunoblotting was performed as described in Materials and Methods. Each lane received a constant amount of sperm cytosol. **A**: mAB 526. **B**: mAB 294. **C**: mAB 622. In each panel, **lane 1**, 0.2 nM; **lane 2**, 0.5 nM; **lane 3**, 1 nM; **lane 4**, 5 nM; **lane 5**, 10 nM; **lane 6**, 20 nM of each monoclonal.

demonstrated that RII could bind to proteins of subunit Mr 120,000 and 80,000 in both rat and bovine flagella. When a similar experiment was repeated with bovine sperm cytosol, no RII binding proteins could be identified (data not shown).

Are There Phosphoproteins, Other than RII, With Subunit Molecular Weights of 56,000 in Bovine Sperm Extracts?

It is known that the cAMP-dependent phosphorylation of a cytosolic protein is important for the initiation of flagellar motion in both mammalian and sea urchin sperm. Tash et al. [11] have suggested that this protein, termed axokinin, has a subunit molecular weight of 56,000 daltons. On close inspection of their two-dimensional gels of phosphorylated dog sperm extracts, phosphorylation of one major 56,000-dalton polypeptide is observed. The experiments described above document that RII is the major isoform present in both bovine and rat cytosol. In addition, we have shown that the sperm cytosolic RII can be phosphorylated by the catalytic subunit of cAMP-dependent protein

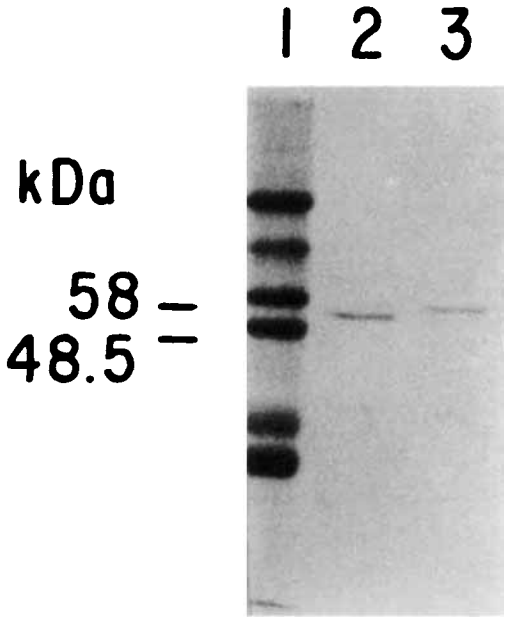

Fig. 4. Phosphorylation-dependent mobility shift of the cytosolic RII from bull sperm. Cytosolic extracts were phosphorylated prior to SDS-PAGE/immunoblotting as described in Materials and Methods. **Lane 1**: Molecular weight standards. **Lane 2**: Unphosphorylated cytosol. **Lane 3**: Prephosphorylated cytosol.

kinase. In light of these facts,it is important to establish how many phosphoproteins of this subunit molecular weight exist in sperm extracts. To approach this problem, we elected to remove quantitatively all RII from bovine cytosolic or detergent extracts, using either immobilized cAMP or mAB 622 coupled to Sepharose 4B, prior to phosphorylation. Figure 5A demonstrates that it is possible to remove all the RII from bovine sperm cytosol using either immobilized cAMP (lanes 6 and 7) or immobilized mAB 622 (lanes 8 and 9). Incubation of cytosol with Sepharose 4B alone did not result in the removal of RII. Figure 5B shows the immunoblot analysis of the pellets from the above precipitation experiments. Unexpectedly, we were unable to retrieve any RII from the immobilized cAMP after boiling in sample application buffer. RII could, however, be recovered from the immobilized mAB 622 by this treatment. Similar experiments with the Lubrol extracts demonstrated that immobilized mAB 622 could quantitatively remove RII from these extracts (Fig. 5C). To examine whether bovine cytosolic and detergent extracts contain 56,000 phosphoprotein(s) in addition to RII, the above precipitation experiments were repeated and the resulting supernatants subjected to phosphorylation in the presence and absence of exogenous bovine heart catalytic subunits for 30 sec or 5 min (Fig. 6 A,B). Untreated cytosolic extracts show a simple phosphorylation profile irrespective of whether they are phosphorylated in the presence (lane 1) or absence (lane 2) of catalytic subunit. In both cases, two major phosphoproteins of 56,000 and 40,000 daltons are observed. Tash et al. [11] also report that the two major cAMP-dependent phosphoproteins in dog sperm extracts have molecular weights of 56,000 and 40,000.

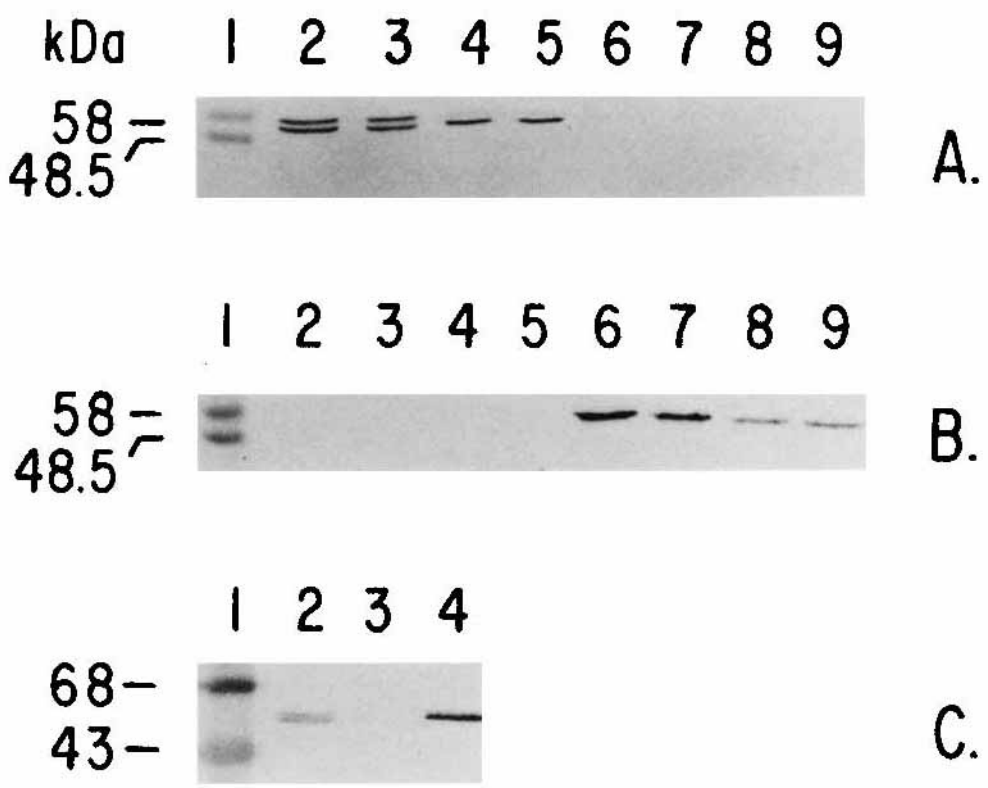

Fig. 5. Precipitation of the regulatory subunit of type II cAMP-dependent protein kinase by immobilized cAMP or anti-RII monoclonal antibody. The conditions for this experiment are described in Materials and Methods. In this experiment, RII was detected by SDS-PAGE followed by immunoblotting. **A** represents the supernatants obtained after incubation with Sepharose 4B (**lanes 4** and **5**), cAMP coupled to Sepharose 4B (**lanes 6** and **7**) and mAB 622 coupled to Sepharose 4B (**lanes 8** and **9**). Lanes 2 and 3 are untreated cytosolic extracts. **B** represents the pellets obtained after incubation with Sepharose 4B (**lanes 2** and **3**), cAMP coupled to Sepharose 4B (**lanes 4** and **5**) and mAB 622 coupled to Sepharose 4B (**lanes 6–9**). Lanes 6 and 7 are the pellets obtained from the first incubation while lanes 8 and 9 are the pellets obtained after the second incubation. **C—Lane 2**: Lubrol extract. **Lane 3**: Supernatant after incubation with mAB 622 coupled to Sepharose 4B. **Lane 4**: Pellet after incubation with mAB 622 coupled to Sepharose 4B. In all panels, lane 1 represents molecular weight standards.

Since the bovine cytosol contains endogenous catalytic subunits, the enhancement by exogenous catalytic subunits is not so apparent in these experiments. The inclusion of protein kinase inhibitor in the incubation mixture demonstrates that these two polypeptides are phosphorylated in a cAMP-dependent manner (Fig. 7).

It is apparent from Fig. 6A,B that treatment of bovine cytosol with either immobilized cAMP or mAB 622 results in the quantitative removal of all the 56,000 dalton phosphoprotein. Treatment with Sepharose 4B alone did not alter the phosphorylation profile. Similarly, we have shown that treatment of a Lubrol extract of bovine sperm with immobilized mAB 622 results in the removal of all the 56,000 dalton phosphoprotein (Fig. 7).

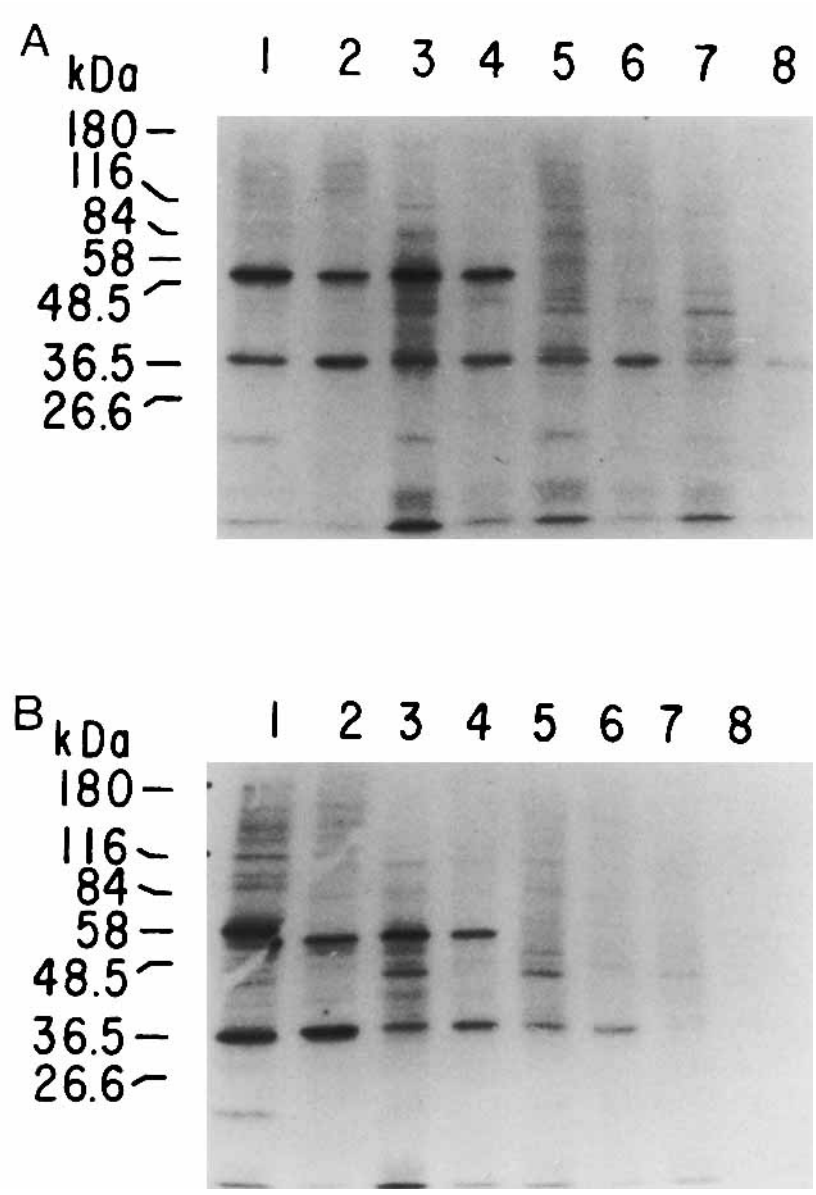

Fig. 6. Phosphorylation profile of RII-depleted bovine sperm cytosol. Phosphorylation reactions in the presence and absence of exogenous cAMP/catalytic subunit were performed as described in Materials and Methods. **A**: 30-sec incubation. **B**: 5-min incubation. In each panel, **lanes 1** and **2**, untreated cytosol; **lanes 3** and **4**, cytosol extracted with Sepharose 4B; **lanes 5** and **6**, cytosol extracted with immobilized cAMP; **lanes 7** and **8**, cytosol extracted with immobilized mAB 622. Lane 1, 3, 5, and 7 contained exogenous cAMP/catalytic subunit.

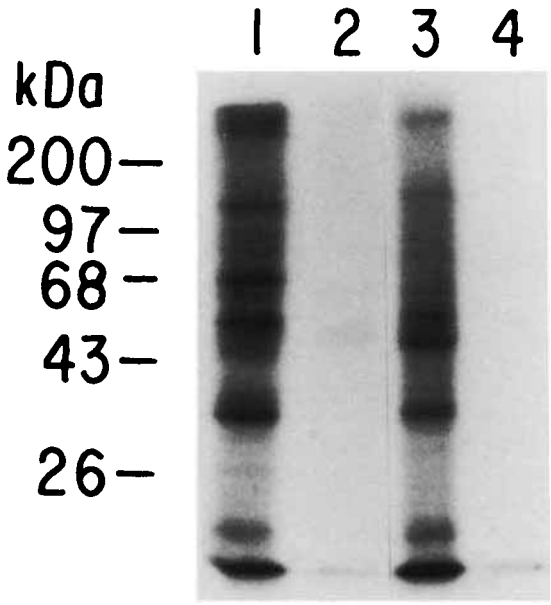

Fig. 7. Phosphorylation profile of RII-depleted bovine sperm Lubrol extract. Phosphorylation reactions in the presence and absence of protein kinase inhibitor were performed as described in Materials and Methods. **Lanes 1** and **2**, Lubrol extract incubated with Sepharose 4B; **lanes 3** and **4**, Lubrol extract incubated with immobilized mAB 622. Lanes 2 and 4 contain protein kinase inhibitor.

Effect of Heat Treatment on Phosphorylation Profile of Lubrol and Cytosolic Extracts of Bovine Epididymal Sperm

Tash et al. [11] have shown that axokinin, the reported initiator protein for flagellar motion, is heat stable. After heat treatment, a 56,000-dalton phosphoprotein is still present in their detergent extract of dog sperm, but all 8-N_3-[^{32}P]cAMP binding activity is lost. When bovine cytosolic or Lubrol sperm extracts are heat treated at 90°C for 5 min, centrifuged, and then subjected to SDS-PAGE/immunoblotting, it can be seen that RII has been removed from the cytosolic but not the detergent extracts (Fig. 8). If, after heat treatment, both extracts are resolved by SDS-PAGE, transferred to nitrocellulose, and then subjected to cAMP-dependent phosphorylation directly on the nitrocellulose, all potential phosphoprotein substrates have been removed from the cytosolic but not Lubrol extract (Fig. 9). Moreover, if the heat-treated Lubrol extract was subjected to photoaffinity labeling with 8-N_3-[^{32}P]cAMP, no specific labeling was observed (Fig. 10). It is apparent, therefore, that the presence of detergent stabilized RII to thermal aggregation and RII still remains a substrate for cAMP-dependent phosphorylation, but is no longer labeled by 8-N_3-[^{32}P]cAMP.

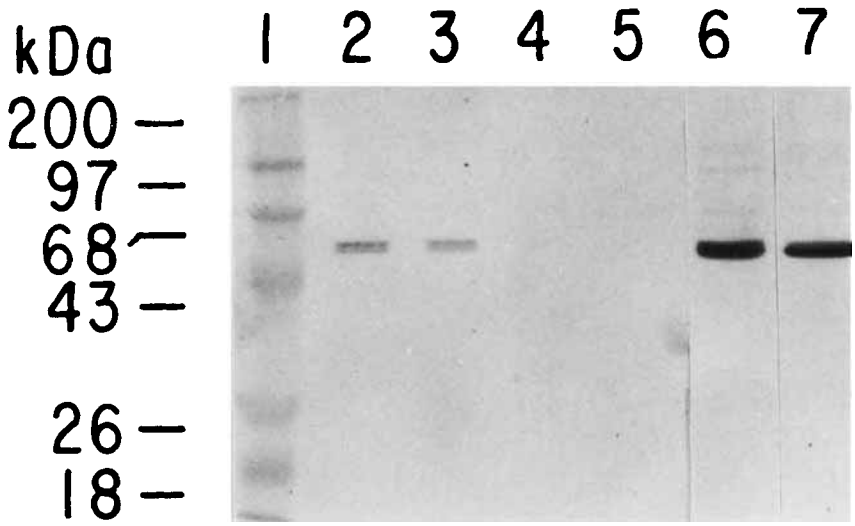

Fig. 8. Effect of heat treatment on immunoblot profile of Lubrol and cytosolic extracts of bovine epididymal sperm. Lubrol and cytosolic extracts were heated at 90°C for 5 min and centrifuged at 900g. Supernatants for heat-treated and control samples were resolved by SDS-PAGE and transferred to nitrocellulose. RII was visualized as described in Materials and Methods. **Lane 1**: Molecular weight standards. **Lanes 2** and **3**: Heat-treated Lubrol extract. **Lanes 4** and **5**: Heat-treated cytosolic extract. **Lane 6**: Lubrol extract. **Lane 7**: Cytosolic extract.

DISCUSSION

Previous investigations have shown that the cAMP-dependent phosphorylation is important for the initiation of flagellar motion in both mammalian and sea urchin sperm [2,3,5,6,12]. A direct involvement of a cAMP-dependent protein kinase in the initiation of motion was ruled out by the fact that the initiator activity could be resolved from the kinase activity by anion-exchange chromatography [5]. Moreover, purified kinase could not initiate motion in modeled sperm without the addition of some soluble protein [5]. Tash and coworkers have suggested that flagellar motion requires the cAMP-dependent phosphorylation of a 56,000-dalton, heat-stable, polypeptide present in dog sperm and a wide variety of other flagella [11]. They termed the motility initiator protein axokinin. The identification of the 56,000-dalton protein as axokinin was based on the observation that this was the only common major cAMP-dependent phosphoprotein present in extracts containing axokinin-like activity. However, since RII, a ubiquitous cAMP-dependent phosphoprotein, has a subunit molecular weight in the same range as reported for axokinin, we decided to characterize this major cAMP-dependent phosphoprotein present in bovine sperm extracts.

We have reported previously that a type II cAMP-dependent protein kinase is an integral component of the flagellum of both rat and bovine sperm [8,9]. The holoenzyme is associated with the flagellum via its regulatory subunit; the addition of cAMP dissociates the catalytic subunit from the flagellum. In this present paper, we show that the major cAMP-dependent protein kinase found in both the bovine and rat cytosol is also

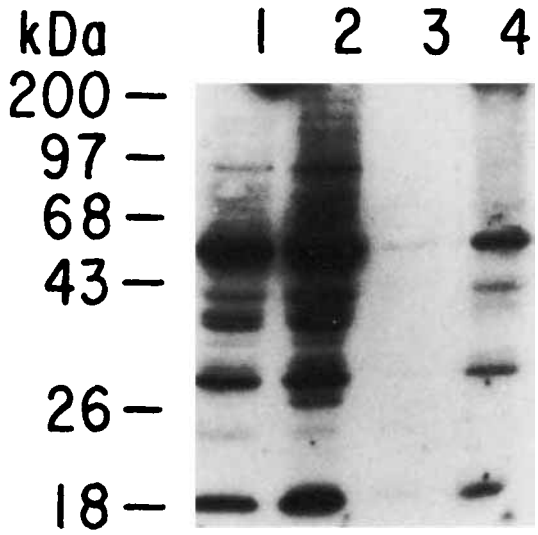

Fig. 9. Effect of heat-treatment on phosphorylation profile of Lubrol and cytosolic extracts of bovine sperm. Lubrol and cytosolic extracts of bovine sperm were heated at 90°C for 5 min and centrifuged at 900g. Supernatants from heat-treated and control samples were resolved by SDS-PAGE and transferred to nitrocellulose. Phosphorylation of polypeptide bound to nitrocellulose was carried out as described in Materials and Methods. **Lane 1**: Cytosolic extract. **Lane 2**: Lubrol extract. **Lane 3**: Heat-treated cytosolic extract. **Lane 4**: Heat-treated Lubrol extract.

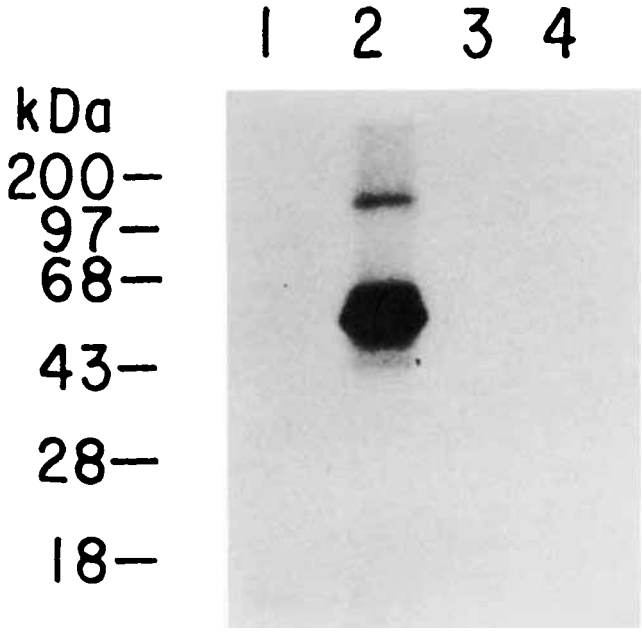

Fig. 10. Photoaffinity labeling of heat-treated Lubrol extracts of bovine sperm with 8-N_3-[^{32}P]cAMP. Lubrol extracts were heated at 90°C for 5 min and centrifuged at 900g. 8-N_3-[^{32}P]cAMP labeling was performed as described in Materials and Methods. **Lanes 1** and **2** represent unheated Lubrol extract. **Lanes 3** and **4** represent heat-treated Lubrol extract. Lanes 1 and 3 contain 0.1 mM cAMP.

of the type II subclass. Immunoblotting experiments reveal that the cytosolic RII is of the non-neural type. In addition, the sperm cytosolic RII, like its heart counterpart, undergoes a phosphorylation-dependent electrophoretic mobility shift on SDS-PAGE. The most important observations from this work are the findings that the treatment of Lubrol or cytosolic extracts of bovine sperm with either immobilized cAMP or an anti-RII monoclonal affinity column results in the quantitative removal of both RII and all of the 56,000-dalton cAMP-dependent phosphoprotein from solution. These results suggest that in bovine sperm, the major 56,000 phosphoprotein present in either cytosolic or detergent extracts is RII. The reason for the discrepancy with the data of Tash and coworkers who suggest that the major 56,000-dalton phosphoprotein in dog sperm is axokinin is unclear. Axokinin and RII, although distinct polypeptides, may share a common epitope for mAB 622. The finding, however, that immobilized cAMP also removes all of the 56,000-dalton phosphoprotein makes this possibility unlikely. Axokinin could be a special form of RII. This is also unlikely for several reasons. Tash et al. [11] showed that heat treatment destroyed all 8-N_3-[^{32}P]cAMP photoincorporation but not the ability to stimulate motion. Moreover, it has been reported that the polypeptide which initiated motion could be resolved from sperm RII by anion-exchange chromatography [5,6]. Other explanations are that in bull sperm, axokinin may be a relatively minor cellular component in comparison to RII or it may not have a subunit molecular weight of 56,000 dalton. These possibilities are currently under investigation.

In conclusion, there appears to be no doubt that the cAMP-dependent phosphorylation of a soluble protein is important for the initiation of flagellar motion in mammalian sperm. In bull sperm, however, it appears unlikely that this motility initiator protein/axokinin is the major 56,000-dalton cAMP-dependent phosphoprotein.

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REFERENCES

1. Acott TS, Hoskins DD: J Biol Chem 253:674, 1978.
2. Tash JS, Means AR: Biol Reprod 26:745, 1982.
3. Tash JS, Means AR: Biol Reprod 28:75, 1983.
4. Lindemann CB: Cell 13:9, 1978.
5. Ishiguro K, Murofushi H, Sakai H: J Cell Biol 92:777, 1982.
6. Murofushi H, Ishiguro K, Takahashi D, Ikeda J, Sakai H: Cell Motil Cytoskel 6:83, 1986.

7. Garbers DL,Kopf GS: Adv Cyclic Nucleotide Protein Phosphorylation Res 13:251, 1980.
8. Horowitz JA, Toeg H, Orr GA: J Biol Chem 259:832, 1984.
9. Horowitz JA, Wasco WM, Orr GA: J Biol Chem 263:2098, 1988.
10. Wasco WM, Orr GA: Biochem Biophys Res Commun 118:636, 1984.
11. Tash JS, Kakar SS, Means AR: Cell 38:551, 1984.
12. Brandt H, Hoskins DD: J Biol Chem 255:982, 1980.
13. Hoskins DD, Casillis ER, Stephens DT: Biochem Biophys Res Commun 48:1331, 1972.
14. Garbers DL, First NL, Lardy HA: J Biol Chem 248:875, 1973.
15. Noland TD, Corbin JD, Garbers DL: Biol Reprod 34:681, 1986.
16. Rangel-Aldao R, Kupiec JW, Rosen OM: J Biol Chem 254:2499, 1979.
17. Ey PY, Prowse SJ, Jenkin D: Immunochemistry 15:429, 1978.
18. Laemmli UK: Nature 227:680, 1970.
19. Burnette WN: Anal Biochem 112:195, 1981.
20. Valtorta F, Schiebler W, Jahn R, Ceccarelli B, Greengard P: Anal Biochem 158:130, 1986.
21. Leiser M, Rubin CS, Erlichman J: J Biol Chem 261:1904, 1986.
22. Walter U, Uno AYC, Greengard P: J Biol Chem 252:6494, 1977.
23. Gill GN, Walton GM: Adv Cyclic Nucleotide Protein Phosphorylation Res 10:93, 1979.
24. Owens JR, Haley BE: J Supramol Struct 5:91, 1976.
25. Valee RB, Bloom GS: Mod Cell Biol 3:21, 1986.
26. Lohmann SM, De Camilli P, Einig I, Walter U: Proc Natl Acad Sci USA 81:6723, 1986.
27. Sarkar D, Erlichman J, Rubin CS: J Biol Chem 259:9680, 1985.