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Association of Protein Kinase A Type I With Detergent-Resistant Structures of Mammalian Sperm Cells

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ABSTRACT The finding that flagellar movement in detergent-permeabilized sperm cells is restored when Mg ATP and cAMP are added implicated detergent-resistant protein kinase A (PKA) in the regulation of sperm motility. It is widely believed that only the PKA regulatory subunit RII can associate with the cytoskeleton and/or organelles. In this paper we used monoclonal antibodies against the PKA catalytic subunit and RI subunit and demonstrated that PKA type I is also associated with the sperm cytoskeleton. To our knowledge, this is the first report showing anchored PKA type I. This association was found in sperm of nonrodent mammalian species and, to a lesser extent, also in mouse sperm. The PKA catalytic subunit is bound to the cytoskeleton secondarily via its complex with the regulatory subunit. The detergent-resistant complexes of RI and catalytic subunits localize predominantly to the flagellum. Ultrastructural immunogold labeling revealed the association of detergent-resistant PKA type I with outer dense fibers (ODF) and the fibrous sheath (FS) but not with microtubules. This location is consistent with a proposed role of PKA in regulation of FS sliding on underlying ODF. *Mol. Reprod. Dev. 50:79–85, 1998.*

Key Words: PKA; mammalian sperm; sperm cytoskeleton

INTRODUCTION

Spermatozoa are terminally differentiated cells for which the only physiologic function is to fertilize eggs. To successfully fertilize an egg, sperm must possess an active and vigorous movement, which makes sperm unique cells and requires highly specialized structures of the sperm flagellum (Eddy and O'Brien, 1994, and references therein). How sperm movement is regulated remains unclear, although at least two second messengers, calcium and cyclic adenosine monophosphate (cAMP), are undoubtedly implicated in regulation of this process (Brokaw, 1987; Lindeman and Kanous, 1989). When spermatozoa are demembranated by using nonionogenic detergent (sperm models), they immediately lose their motility, but the movement is restored when Mg adenosine triphosphate (ATP) and cAMP are added (Lindemann, 1978). Mg ATP is a necessary substrate for dynein ATPase, an enzyme likely associated with the flagellar microtubules and the component believed to facilitate flagellar microtubule sliding (Gibbons and Fronk, 1972), but this enzyme does not require cAMP for its function. The only known intracellular target for cAMP is the regulatory subunit of protein kinase A (PKA). Following cAMP binding to the PKA regulatory subunit, the components of the inactive tetramer of two regulatory and two catalytic subunits dissociate, and the free catalytic subunits become active. cAMP is not likely directly involved in microtubule sliding but was shown to be important for sliding of the fibrous sheath (FS), another specialized structure of sperm flagella (Si and Okuno, 1993). The restoration of sperm movement in sperm models has several interesting implications.One is that PKA must be somehow resistant to extraction by nonionogenic detergents, typically Triton X-100, a feature interpreted as an association with cytoskeleton. Horowitz et al. (1984) reported that a significant portion of the cAMP binding activity and also cAMP-dependent kinase activity remains associated with the detergent-insoluble material following extraction of rat epididymal sperm with Triton X-100. Moreover, most of this cAMP binding activity have been found to be associated with the flagellum-enriched fraction. In addition, the association of PKA with the sperm cytoskeleton also has been documented in nonmammalian sperm (Yokota and Mabuchi, 1990).

The association of PKA with the cytoskeleton is not unique for sperm cells. Recently, it has been shown that

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PKA regulatory subunit type II (RII) may be anchored to organelles or cytoskeleton via A kinase anchoring proteins (AKAP) (Carr et al., 1992, 1993; Glantz et al., 1992). This association likely represents a new regulatory mechanism of enzyme signal transduction and is used, for instance, in transducing signals of receptor tyrosine kinases via proteins bearing SH2 domains (Zhou and Cantley, 1995). The principle of this mechanism is to bring enzymes close together with their substrates, allowing substrates to be phosphorylated more efficiently on transient kinase activation. A protein with a sequence homology to the AKAP family has been demonstrated recently in mouse sperm (Carrera et al., 1994). This protein is a major structural component of sperm tail FS, and although it binds RII subunit in a gel overlay assay, whether it acts as a PKA binding protein in vivo should be still clarified. Another protein with homology to the AKAP family has been shown to associate with mitochondrial membranes in spermatids (Lin et al., 1995). It is interesting to note that only PKA type II has been reported to interact with the anchoring proteins. This is consistent with the report of Horowitz et al. (1984), in which the authors suggested that only PKA type II (RII subunit) is associated with the detergent-resistant fraction of rat sperm, while PKA I was associated with the plasma membrane fraction. These authors, however, did not have specific probes to distinguish between RI and RII, and so the precise localization of PKA I and PKA II in mammalian spermatozoa remains to be clarified.

In this paper we used monoclonal antibodies against the PKA catalytic subunit and RI subunit and demonstrated that PKA I is also associated with the sperm cytoskeleton. To our knowledge, this is the first report showing anchored PKA type I. This association was found in nonrodent mammalian species and, to a lesser extent, also in mouse sperm. The catalytic subunit is bound to the cytoskeleton via the regulatory subunit, and the detergent-resistant RI and PKA catalytic subunits colocalize predominantly to the flagellum.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies against protein kinase A catalytic and regulatory subunit I (RI) (catalog no. P19920) were purchased from Transduction Laboratories (Lexington, KY). These antibodies do not crossreact with the PKA regulatory subunit type II (RII), as assessed by immunoblotting using antibodies against RII (Upstate Biotechnology, Lake Placid, NY) or antibody kindly donated by Stuart Moss (University of Pennsylvania). Antibodies against RII do not recognize porcine and bovine RII and were thus not used in this study. As a control of extraction, the distribution of other proteins into Triton-soluble and Triton-insoluble fractions was monitored using antiserum against hexokinase (Kaláb et al., 1994) and monoclonal antibody against boar spermadhesins (ACR. 3) (Moos et al., 1992; Pěknicová and Moos, 1990).

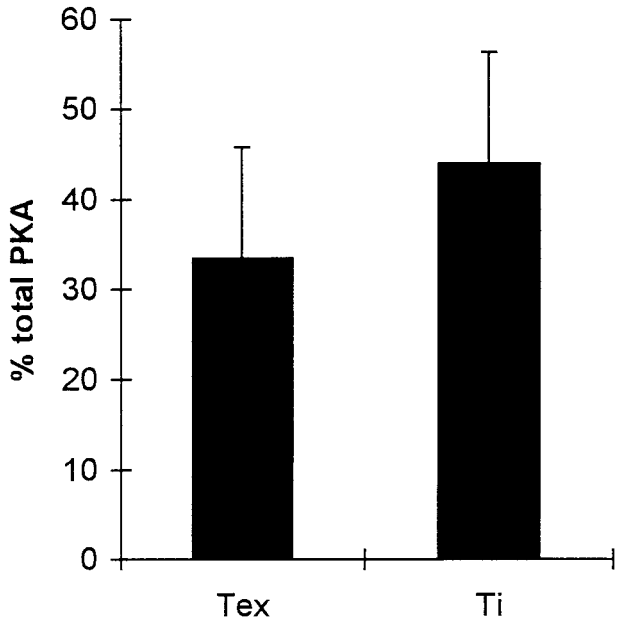

Fig. 1. Distribution of PKA activity into Triton-soluble and Triton-insoluble fractions. Boar spermatozoa were permeabilized with 1% Triton (Total), spun, and the supernatant saved (Tex). The pellets were washed twice with the same solution and resuspended in the original volume of 1% Triton X-100 buffer (Ti). All three fractions were then assayed for the presence of PKA activity. Means of four experiments and standard deviations are shown. PKA activity in Triton-permeabilized sperm (Total) was considered 100%. Extracts and suspensions corresponding to 5×10^4 cells were used in each assay.

Sperm Preparation and Fractionation

Porcine, bovine, and human ejaculated spermatozoa were purified from seminal plasma on 40-80% Percoll gradients, washed twice (10 min, 350*g*), extracted in 150 mM NaCl, 20 mM Tris (pH 7.4), 10 mM NaF, 1 mM EGTA, 10 mM leupeptine, and 1% Triton X-100 for 20 min at 4°C and spun at 10,000*g* for 5 min. The resulting supernatant was saved as a Triton extract (Tex). The pellets were then washed twice in the same solution and either resuspended in the original volume and saved as a Triton-insoluble fraction (Ti) for PKA activity analysis or reextracted in 2.5% SDS and the extracted material saved as a Ti fraction for immunoblotting analysis.

Immunocytochemistry

Washed sperm cells were attached to polybrene-coated glass slides, permeabilized with 0.1% Triton as described above, and fixed with 0.37% formaldehyde and 0.25% glutaraldehyde in 1 mM $MgCl_2$, 10 mM EGTA, 60 mM PIPES, and 25 mM HEPES (pH 6.9) for 1 hr at room temperature and then with methanol for 10 min at -20°C. The cells were then stained with antibody against PKA catalytic subunit (0.25 mg/ml), with anti-

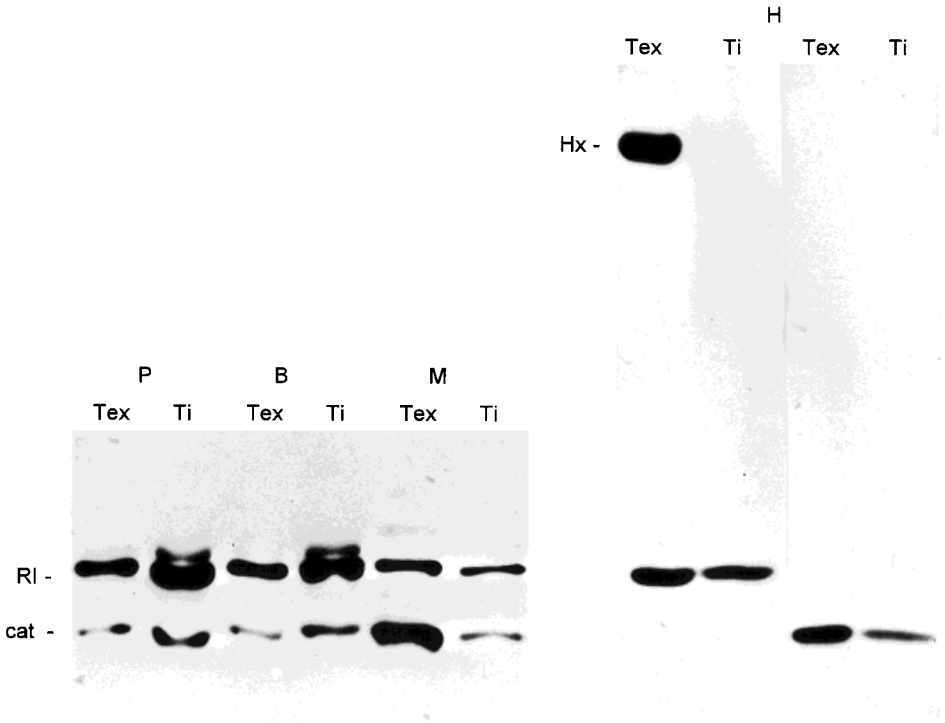

Fig. 2. Distribution of PKA catalytic and RI subunits in mammalian sperm. Porcine (P), bovine (B), mouse (M), and human (H) sperm were fractionated as described in the Materials and Methods section and in Fig. 1. The Triton extracts (Tex) and Triton-insoluble (Ti) fractions were assayed by immunoblotting with antibodies against PKA catalytic subunit (PKAc), regulatory subunit RI (RI), and antiserum against hexokinase (Hx). In boar, bovine, and mouse spermatozoa, PKA catalytic and RI subunits were detected at the same time on the same membrane. In human spermatozoa, PKA RI and hexokinase were detected on the same membrane, whereas PKA catalytic subunit was detected on another membrane using the same samples run in parallel in SDS-PAGE. Material corresponding to 5–8 $\times$ 10^4 cells was loaded per lane. Note that antibody against RI yields stronger signal in immunoblotting than antibody against PKAc, and so this assay cannot be used to assess a ratio of PKAc/PKA RI. The experiment was repeated three times; a typical result of a single experiment is shown.

body against PKA RI subunit (0.5 mg/ml), or with nonspecific monoclonal antibody against progesterone (PROG-13) (Pěknicová et al., unpublished findings) and the bound antibody visualized using FITC-conjugated antimouse immunoglobulins (Sigma, Prague, Czech Republic) and epifluorescence microscopy.

Immunoelectron Microscopy

Sperm cells were pelleted (centrifuged 350*g* for 10 min at room temperature), fixed (40 min at 0°C) in 3% paraformaldehyde plus 0.1% glutaraldehyde in Sörensen buffer (SB; 0.1 M Na/K phosphate buffer, pH 7.3), and washed twice in SB (350*g* for 10 min at room temperature). After centrifugation (350*g* for 10 min) into 1% agarose, blocks were incubated in 0.02 M glycine in SB (20 min), washed in SB, and dehydrated in ethanol. Ethanol was replaced in two steps by LR White resin, and blocks were polymerized by ultraviolet light (20 hr at 4°C). Then 80-nm sections were prepared, and nonspecific labeling was blocked by 10% normal goat serum in PBTB (phosphate-buffered saline supplemented with 0.1% Tween 20 and 1% bovine serum albumin; 30 min). Next, sections were incubated 45 min with anti-PKA RI antibodies (10 µg/ml) diluted in PBTB, washed three times with PBT (phosphate-buffered saline supplemented with 0.005% Tween 20), and incubated with 10 nm gold-conjugated goat antimouse antibody (1:30 in PBT, 30 min; British BioCell, Cardiff, U.K.). After washing in PBT and water, sections were contrasted 5 min with a saturated solution of uranyl acetate in water and observed using a Philips CM100 electron microscope. Control samples were incubated as above, but the primary antibody was omitted. For a statistical evaluation of the immunolabeling, 100 random sections through sperm cells were captured using a CCD camera attached to the electron microscope, and the densities of gold particles over various cell structures were determined using Lucia image-processing software (Laboratory Imaging, Prague, Czech Republic). The statistical significance of labeling density over various cell structures was evaluated using a one-sided *t* test. The variances were calculated from the original data (total number of gold particles over each structure in question and the total measured area of each structure) assuming a Poisson distribution of particle number and normal approximation.

Electrophoresis and Immunoblotting

Extracted proteins were subjected to SDS-PAGE (10% gel) and immunoblotting as described previously

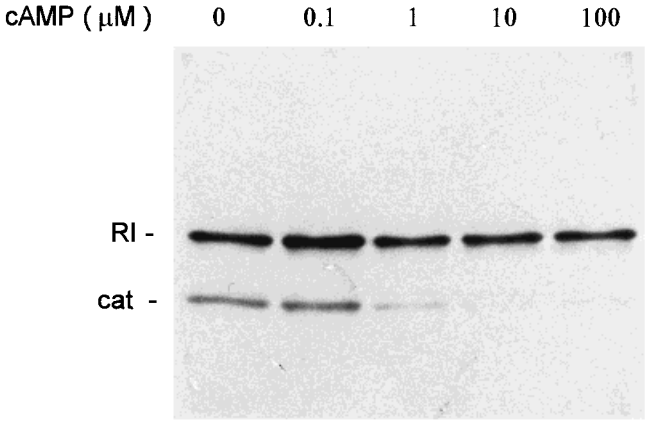

Fig. 3. Effect of cAMP on the association of PKA catalytic and RI subunits with the Triton-insoluble fraction. Boar sperm was extracted with 1% Triton X-100 in the presence of various concentrations of cAMP as indicated, washed, and the Triton-insoluble fraction subjected to SDS-PAGE and immunoblotting. PKA catalytic and RI subunits were detected at the same time on the same membrane. Material corresponding to 5–8 × 10^4 cells was loaded per lane. The experiment was repeated three times with similar results; result of single experiment is shown.

(Laemmli, 1970; Towbin et al., 1979) and detected using the ECL detection procedure (Amersham, M.G.P. Zlín, Czech Republic).

Protein Kinase Activity

The activity of protein kinase A in Triton-permeablized sperm and Triton-soluble and Triton-insoluble fractions was assessed by using PepTag (Promega, East Port, Prague, Czech Republic) nonradioactive PKA activity kit and the manufacturer's instructions. The activity in whole permeabilized sperm was set as 100% (total), and the activity in corresponding Triton-soluble (first sperm wash in 1% Triton; for composition of this buffer, see above) and Triton-insoluble (the pellet after three consecutive extractions with Triton) fractions were expressed as relative to total.

RESULTS

Since only a few reports demonstrated detergent-resistant PKA activity in rat and ram sperm cells, we wanted to know whether PKA activity is also associated with detergent-resistant sperm structures in other mammalian species. Washed boar spermatozoa were extracted with 1% Triton X-100 (for detailed composition, see the Materials and Methods), washed twice with the same solution, and the relative PKA activity was measured in individual fractions (Fig. 1). PKA activity was distributed nearly equally between Triton-soluble extract (material solubilized in the first of the three consecutive extractions in 1% Triton X-100) and the Triton-insoluble fraction. Moreover, both activities together represent most of the total PKA activity in permeabilized sperm; very low activity was detected in the second and third washes. This finding suggests that

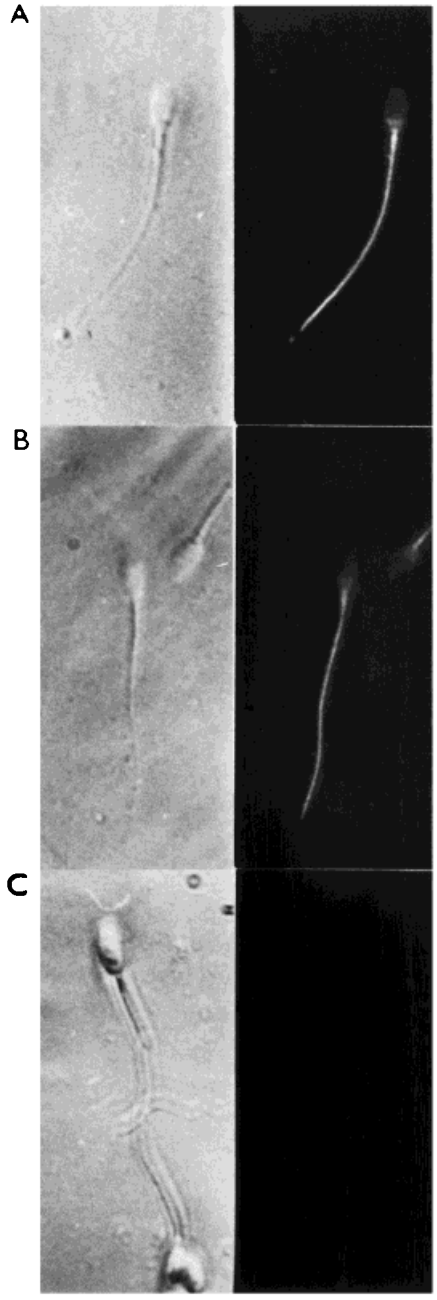

Fig. 4. Immunocytochemical localization of Triton-insoluble PKA subunits. Boar sperm were permeabilized with Triton X-100, processed as described in the Materials and Methods section, and stained with antibody against the PKA catalytic subunit (**A**), RI subunit (**B**), and control antibody (**C**). The bound antibodies were then visualized using FITC-labeled secondary antibody, and the cells were observed under a fluorescence microscope. The experiment was repeated four times with similar results; paired views of typical cells are shown.

association of PKA activity with the Triton-insoluble pellet is specific and not due to the inefficient extraction.

The distribution of PKA activity in mammalian sperm was well correlated with the distribution of PKA catalytic subunit (PKAc) (Fig. 2). In all species studied, the

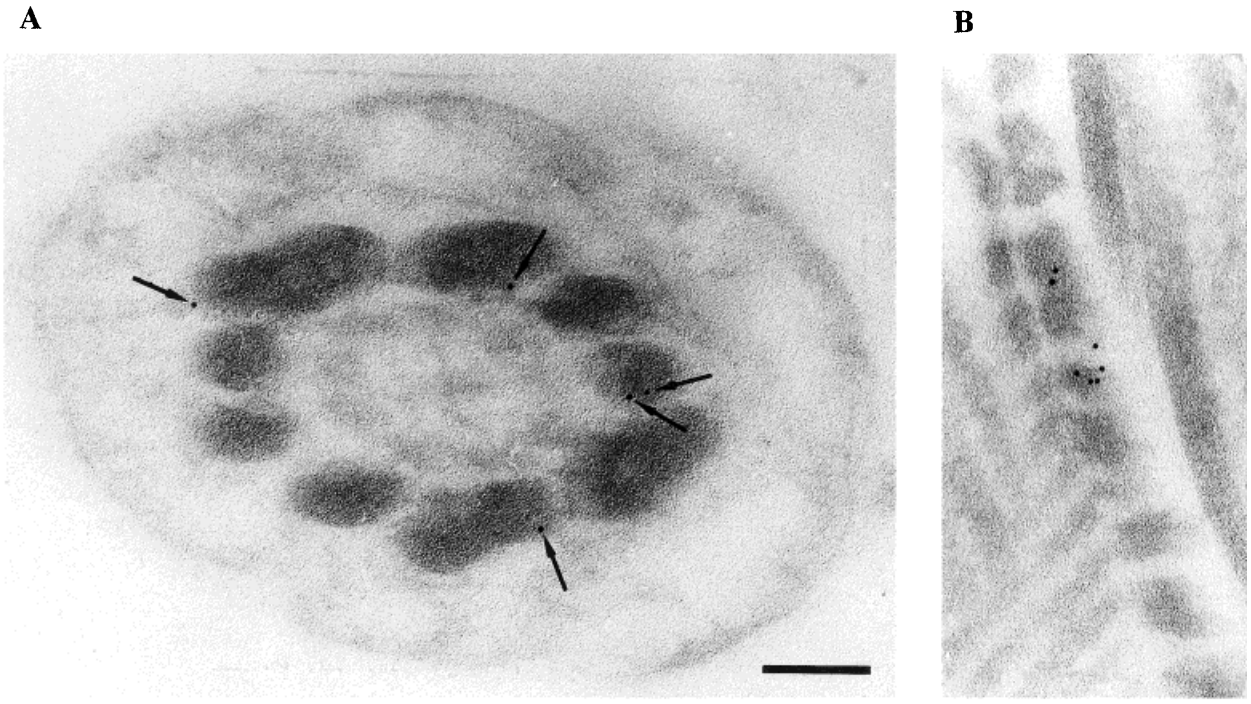

Fig. 5. Electron micrographs showing distribution of Triton-insoluble PKA RI subunits in sperm cells. Boar sperm was permeabilized with Triton X-100 and treated as described in the Materials and Methods section. The resulting ultrathin sections were decorated with antibody against the PKA RI subunit, followed by a secondary antibody labeled with 10-nm gold particles. A cross section of a sperm tail with labeled ODF (**A**) and a tangential section with labeled FS structures (**B**) are shown. Bar = 100 nm. We have tested different sperm from three boars, and the character of labeling was identical. For the figure presented, only one of this preparation was used.

PKAc was present also in a detergent-resistant fraction, although there were differences among the species in a ratio of Triton-extractable and Triton-insoluble PKAc. Surprisingly, the distribution of PKA RI, which is believed to be cytoplasmic or plasma membrane associated protein, followed the distribution of PKAc. This was in a sharp contrast to the distribution of another sperm cytoplasmic protein—hexokinase. Hexokinase, although very abundant in sperm cells, was completely washed out by three consecutive extractions in Triton X-100 (see Fig. 2). Similar results were obtained when the distribution of ACR.3 antigen, a plasma membrane-associated protein (Moos et al., 1992), was observed (results not shown).

It was still not clear which of the PKA subunits is anchored to the cytoskeleton directly and which of them, if any, is associated secondarily. To answer this question, we dissociated PKA catalytic and regulatory subunits completely before detergent fractionation. Sperm were extracted three times with 1% Triton in the presence of various concentrations of cAMP as indicated (Fig. 3). While the presence of 1 mM cAMP released PKAc presumably to the detergent-soluble fraction completely, even a 100 mM concentration of cAMP only slightly affected RI subunit extractability. This is direct evidence that only the regulatory subunits are bound to the cytoskeleton directly, while PKAc is associated only as a result of its complex with the regulatory subunits.

It was interesting to know where the detergent-resistant PKA type I is located. We extracted sperm cells with Triton X-100 and observed the location of PKAc and RI subunits by immunofluorescence (Fig. 4). We found that both subunits located to the entire flagellar region, including the connecting piece, and more moderate staining also was found in the postacrosomal region of sperm heads. In contrast, we never observed staining of the acrosomal region in Triton-extracted sperm cells.

To find more about the precise subcellular location of the detergent-resistant PKA I, we used immunogold labeling and electron microscopy (Figs. 5 and 6). Sections of detergent-treated boar sperm were labeled with an antibody against PKAc and PKA RI, and the density of gold grains per square micrometer was calculated for the FS (fibrous sheath), ODFs (outer dense fibers), microtubular region, and sperm nucleus. This quantitative analysis revealed the association of PKA I predominantly with ODFs and FS. In contrast, no significant association was found with either the microtubular region of sperm tail or the nucleus and associated structures.

DISCUSSION

In this paper we demonstrated that PKA type I is associated with the sperm cytoskeleton in several mam-

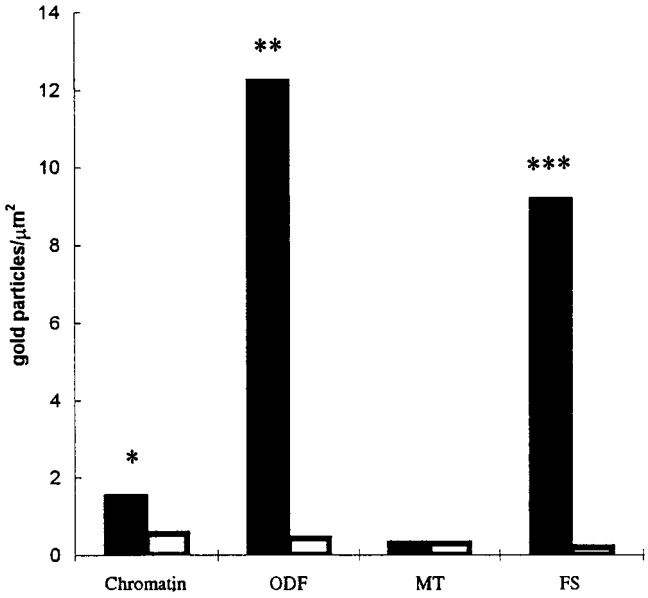

Fig. 6. Distribution of detergent-resistant PKA RI subunits in boar sperm. Boar sperm was treated as described in Fig. 5 and decorated with either antibody against RI subunit (black) or with control antibody (white). Average densities of gold particles associated with chromatin, ODFs, axonemal microtubules (MTs), and FS were calculated for both antibodies. At least 80 sections were scored for each structure. The statistical significance of labeling density over various cell structures was evaluated using a one-sided *t* test. The outer dense fibers and the fibrous sheath were significantly labeled with anti-PKA RI antibodies when compared with control section with secondary gold-labeled antibodies only (**$P \leq 0.005$ and ***$P \leq 0.001$, respectively). Chromatin area was weakly labeled (*$P \leq 0.05$). Labeling density over axoneme was not significantly different from the control incubations.

malian species. The complex is bound to the cytoskeleton via its regulatory subunit, and the detergent-resistant RI and catalytic subunits of PKA colocalize predominantly to the flagellum. This is not the first report showing PKA associated with sperm flagella, since the presence of detergent-resistant PKA activity has been reported in mouse (Horowitz et al., 1984), ram (San-Agustin and Witman, 1994), and sea urchin (Yokota and Mabuchi, 1990) sperm. All the reports, however, proposed that PKA type II and not PKA type I is associated with the sperm cytoskeleton. The discrepancy may be partially explained by the fact that those authors did not use specific probes to distinguish PKA regulatory subunits. Since our data depend on the specificity of the antibody against RI, we paid particular attention to confirming or excluding a crossreactivity of anti-RI antibody with the RII subunits. We concluded that the antibody against RI is specific for this subunit and does not crossreact with the RII subunit for the following reasons (results not shown): (1) In sperm cells, the antibody recognizes only a single protein band, although both RI and RII subunits are present. (2) In mouse and human sperm cells, antibody against the RII subunit recognized different proteins than antibody against RI. (3) When reducing agents were omitted from sample preparation, antibody against RI recognized a dimeric form, a specific feature for RI subunits. Unlikely with the RI subunit, the RII subunit has been found in association with insoluble structures in other cell types, and proteins anchoring RII beta subunits (AKAP family) have been identified, cloned, and sequenced (Carr et al., 1992, 1993; Glantz et al., 1992). A protein with sequence homology to the AKAP family also has been found in epididymal mouse sperm and identified as a major component of sperm tail FS (Carrera et al., 1994). It is interesting to note that the human sperm homologue of this protein is one of the major sperm proteins phosphorylated on tyrosine (Carrera et al., 1996; Moos et al., 1995). This observation, together with the finding that the level of tyrosine phosphorylation in mammalian sperm is under control of PKA (Visconti et al., 1995), suggests that a unique crosstalk of kinases and phosphatases may regulate sperm motility.

Si and Okuno (1993) have reported that in vitro sliding of FS on the underlying axoneme is cAMP-dependent. This may explain numerous observations suggesting that detergent-resistant PKA is somehow implicated in regulation of sperm motility (Brokaw, 1987; Lindemann and Kanous, 1989, and references therein), although it is probably not directly implicated in microtubule sliding. The observations reported here, namely, the immunogold localization of detergent-resistant PKA to FS and ODFs, are consistent with the proposed role of detergent-resistant PKA; direct evidence is, however, still needed to prove this hypothesis.

The major protein of FS with a sequence homology to the AKAP family of proteins has been demonstrated recently in mouse sperm (Carrera et al., 1994). This protein is a major structural component of sperm tail FS, and although it binds RII subunit in a gel overlay assay, whether it also anchors PKA to the sperm cytoskeleton in vivo remains to be clarified. We found two reasons suggesting that there must be at least one more mechanism facilitating the association of PKA with the sperm cytoskeleton. First, localization of FS is restricted to the principal piece of the tail, while the detergent-resistant PKA has been localized along the entire tail, connecting piece, and even with the postacrosomal region of the sperm head. Second, the proteins of the AKAP family recognize and bind the RII beta regulatory subunit only, and we clearly demonstrated the presence of detergent-resistant RI subunit in sperm cells of several mammalian species. Since this is the first report showing RI subunit associated with cytoskeleton, it is premature to speculate about the possible nature of this association. To highlight this issue, experiments are in progress in our laboratories aiming to identify the RI binding proteins in mammalian sperm.

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