Studies on [³H]palmitate-binding proteins of rat spermatozoa: a post-translational modification of membrane proteins by fatty acid acylation

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The purpose of the present study was to demonstrate the post-translational modifications of sperm plasma membrane proteins by fatty acid acylation during sperm maturation in the epididymis. Rat epididymal spermatozoa were incubated at 37°C with various concentrations (100 µCi and 1 mCi) of [9–10(*n*)³H]palmitic acid in a medium containing Tyrode's solution supplemented with sodium bicarbonate, sodium pyruvate and sodium lactate. The incorporation of [³H]palmitate *in vitro* was determined in epididymal spermatozoa and an attempt was made to identify the lipid-linked proteins of purified plasma membranes of maturing epididymal spermatozoa by autoradiography. The studies demonstrated that [³H]palmitate was covalently linked to a subset of membrane cytoskeleton proteins of maturing rat spermatozoa. The pattern of incorporation of lipid was a maturation-associated phenomenon as caput spermatozoa incorporated more radioactivity than did caudal spermatozoa. The labelled proteins appeared to be membrane-bound since 82% of radioactivity was associated with membrane fractions. Autoradiograms of SDS-PAGE gels of labelled caput sperm extract showed three prominent palmitate-incorporating protein bands of about 70, 56 and 36 kDa and few minor bands. Most of these proteins were present in the membrane fraction of caput spermatozoa. Labelled gels of both the sperm extracts and of purified membranes showed resistance to hydroxylamine treatment, suggesting that there are amide bonds between lipid and proteins. The higher incorporation of labelled palmitate by immature spermatozoa of the caput epididymis compared with mature spermatozoa from the cauda epididymis and the addition of palmitate to plasma membrane proteins of caput epididymal spermatozoa suggest that fatty acylation is a post-translational modification of sperm membrane proteins.

Introduction

Mammalian spermatozoa cannot fertilize eggs upon leaving the testis, but gradually acquire this ability as they pass through the epididymis. This sperm maturation process includes various morphological, physiological and biochemical changes on the sperm surface (for reviews see Eddy, 1988; Yanagimachi, 1988). Although the structure and function of mammalian spermatozoa have been the subject of intensive study during recent years and there have been numerous reports on the presence and distribution of several cytoskeletal proteins in the mammalian spermatozoa (Primakoff *et al.*, 1987; Lakoski *et al.*, 1989; Camatini *et al.*, 1991; Srivastava and Olson, 1991), how the membrane-associated protein modifications contribute to the maturation of spermatozoa remains largely unresolved. A variety of membrane proteins of cellular and viral origin are modified by covalent attachment of long-chain fatty acids (for reviews see Magee and Schlesinger, 1982; Schultz *et al.*, 1988;

James and Olson, 1990). Several intracellular proteins are acylated by myristate, a process that appears to take place cotranslationally for a variety of soluble and membrane proteins (Carr *et al.*, 1982; Schultz and Oroszlan, 1984; Buss and Sefton, 1985). In contrast, several proteins are acylated by palmitate, a post-translational event that occurs exclusively on membrane proteins, as reported for the transferrin receptor (Omary and Trowbridge, 1981) and the membrane cytoskeleton protein, ankyrin (Staufenbiel and Lazarides, 1986).

The plasma membrane of maturing spermatozoa plays a significant role in the preparation of spermatozoa for fertilization. The phospholipid composition of the plasma membrane as well as the phospholipid distribution in the membrane leaflets are of particular importance for the process of capacitation and fusion (Hinkovska *et al.*, 1986; Hinkovska-Galcheva and Srivastava, 1993). There have been numerous reports on the lipid content of whole spermatozoa from a variety of species (Parks *et al.*, 1987; Lin *et al.*, 1993) and there is a decrease in the amount of sperm lipid during transit through the epididymis in several species (Nikolopoulou *et al.*, 1985; Parks and Hammerstedt, 1985). In addition, alterations in sperm

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membrane lipids have been noted during capacitation and acrosome reaction of spermatozoa (Lee *et al.*, 1986; Wolf *et al.*, 1986). However, very little is known about the fatty acid acylated proteins of the membrane cytoskeleton of mammalian spermatozoa. The purpose of the present study was to identify the post-translationally modified, fatty acylated proteins of sperm membrane during epididymal sperm maturation in rats.

Materials and Methods

Materials

[9-10(n^3 H)]palmitic acid was obtained from Amersham (Searle, Arlington Heights, IL); acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), hydroxylamine hydrochloride and other chemicals for electrophoresis were from Fisher Scientific Company (Norcross, GA). Tyrode's salts, leupeptine, pepstatin and benzamidine hydrochloride were purchased from Sigma Chemical Company (St Louis, MO). For radioactive counting, Ready Solve TM Scintillation Cocktail was purchased from Beckman (Palo Alto, CA). The Kodak X-OMAT C films for autoradiography were obtained from Eastman Kodak Company (Rochester, NY).

Animals

Adult male Sprague-Dawley rats were anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) (30 mg per 0.5 ml saline, i.p.) and were perfused retrograde via the spermatic artery to clear the epididymis of blood with Tyrode's solution containing 12 mmol sodium bicarbonate l^{-1} , 1.0 mmol sodium pyruvate l^{-1} and 9.0 mmol sodium lactate l^{-1} (pH 7.6). The epididymides were removed and the caput and cauda epididymides were dissected out.

Sperm preparation and labelling conditions

Epididymal segments were minced with a razor blade and the spermatozoa released were collected with a Pasteur pipette. An aliquot of the spermatozoa was taken for counting using a haemocytometer under a phase-contrast microscope and for protein estimation by the method of Bradford (1976). The sperm cells were washed three times with Tyrode's solution and were incubated with [9-10(n^3 H)]palmitic acid (specific activity 30 Ci $mmol^{-1}$) at a concentration of 100 μ Ci for 1 h at 37°C in Tyrode's solution containing 1 mg BSA ml^{-1} . To study the effect of time on incorporation of [^{3} H]palmitate, the incubation period was varied from 30 min to 2 h at 100 μ Ci [^{3} H]palmitate. For observation of appreciable amounts of radio-labelled proteins in autoradiography, the spermatozoa were incubated with 1 mCi [^{3} H]palmitate for 2 h at 37°C.

Preparation of sperm extract

At the end of the incubation, the spermatozoa were washed three times in Tyrode's solution and were pelleted by centrifugation at 500 g for 10 min. The resultant supernatant was freeze-dried and reconstituted in Laemmli sample buffer for

electrophoresis. The pellets were boiled in Laemmli sample buffer (0.125 mol Tris-HCl l^{-1} , pH 6.8, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol and 0.1% (w/v) bromophenol blue) and were centrifuged at 8000 g for 5 min in an Eppendorf centrifuge (Model 5415). The supernatant consisting of sperm extract was desalted on a PD10 column (Pharmacia Inc., Piscataway, NJ), freeze-dried and reconstituted in Laemmli sample buffer for determination of amount of radioactivity and SDS-PAGE analysis.

Determination of radioactivity

Radioactivity was determined by incubating 25 μ l labelled sperm extracts with 10% (w/v) trichloroacetic acid (TCA) and BSA (1 mg ml^{-1}) for 15 min at 4°C. The TCA precipitates were collected on Millipore filters (0.45 μ m pore size) and were washed four times each with 10% (w/v) TCA and then by chloroform:methanol (2:1). The filters were dried and dissolved in methyl cellosolve. Radioactivity was determined in a Beckman Counter Model LS 3801 using a Beckman Ready Solve TM scintillation cocktail.

Membrane isolation

The incorporation of radiolabelled palmitate was examined in purified plasma membranes of spermatozoa to demonstrate that lipid-containing proteins were membrane bound. The spermatozoa were labelled with [^{3} H]palmitate at 1.0 mCi for 2 h at 37°C in Tyrode's solution containing BSA as described above. The sperm pellets obtained after washing with Tyrode's solution were resuspended in TNI buffer (25 mmol Tris-HCl l^{-1} pH 7.5, 150 mmol sodium chloride l^{-1} , 2.5 mmol benzamidine l^{-1} , 1 μ g leupeptin ml^{-1} , 1 μ g pepstatin ml^{-1} and 0.05% (w/v) sodium azide) for membrane isolation. The purified plasma membranes were obtained as described by Srivastava and Olson (1991) with minor modifications. Briefly, labelled spermatozoa were disrupted by nitrogen cavitation at 4°C at a pressure of 2760 kPa and an equilibration period of 10 min. The cavitated spermatozoa were then centrifuged for 15 min at 500 g to pellet the spermatozoa (pellet 1). Aliquots of the supernatant fluid were centrifuged at 100 000 g for 1.5 h in a Beckman SW-41 rotor to collect the released membranes. The resultant pellet was reconstituted in TNI buffer and centrifuged at 100 000 g for 1 h in a Beckman 100.3 ultracentrifuge. The membrane pellets obtained were resuspended in TNI buffer and were used for determination of radioactivity and SDS-PAGE analysis. The supernatant (cytosol) was desalted on a PD10 column, freeze-dried and was reconstituted in Laemmli sample buffer for further counting and SDS-PAGE.

Polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography

SDS-PAGE was performed according to the method of Laemmli (1970). The sperm pellet, supernatant and plasma membranes were solubilized in Laemmli sample buffer. The proteins were fractionated on 1.5 mm thick, 10–15% gradient polyacrylamide gels for 3.5 h at 15 W per gel with cooling to

Table 1. Incorporation of [^3H]palmitate (100 μCi for 1 h at 37°C) by maturing spermatozoa from the rat epididymis

Spermatozoa (n)	c.p.m. 10^{-7} spermatozoa (mean $\pm$ SEM)	Percentage incorporation ^a	c.p.m. μg^{-1} protein (mean $\pm$ SEM)	Percentage incorporation ^a
Washed caput (7)	478 327.2 $\pm$ 85 373.6	94.1	1586.8 $\pm$ 283.5	85.6
Washed caudal (7)	29 960.6 $\pm$ 3404.0	5.9	267.3 $\pm$ 30.7	14.4
Unwashed caput (3)	404 380.3 $\pm$ 14 213.2	95.0	1341.1 $\pm$ 471.4	87.6
Unwashed caudal (3)	21 085.8 $\pm$ 4557.0	5.0	189.0 $\pm$ 40.8	12.4

^aThe percentage incorporation of radioactivity by caput and caudal spermatozoa was calculated with the total sum of c.p.m. of two segments considered to be 100%. n: number of observations.

4°C. After electrophoresis the gels were fixed in 50% methanol for 30 min. One set of gels was treated with 1 mol hydroxylamine l^{-1} (pH 8.0) for 2 h to test for ester-linked fatty acids. The dried gels were treated with amplify solution (Amersham) for 30 min and dried in a gel drier at 67°C for 1 h. The dried gels were put for fluorography (Bonner and Laskey, 1974) at -70°C for 50 days using Kodak X-OMAT C film. In a separate set of gels, the proteins were visualized by the silver staining method of Wray *et al.* (1981).

Results

Incorporation of [^3H]palmitate by maturing spermatozoa

Spermatozoa from both caput and cauda epididymides were incubated with [^3H]palmitate at various concentrations and for various periods to investigate whether proteins of the sperm membrane cytoskeleton incorporate long chain fatty acids during epididymal sperm maturation. Preliminary experiments performed with [^3H]palmitate (100 μCi) in incubation medium for 1 h indicated that immature spermatozoa from the caput epididymis incorporated more radioactivity than did mature spermatozoa from the cauda epididymis. The pattern of incorporation did not change when incorporation was expressed as c.p.m. 10^{-7} spermatozoa (caput spermatozoa 94.1%; caudal spermatozoa 5.9%) or as c.p.m. μg^{-1} protein (caput spermatozoa 85.6%; caudal spermatozoa 14.4%). Slightly more incorporation of radioactivity was observed in spermatozoa washed repeatedly with Tyrode's solution before incubation than in unwashed spermatozoa. This finding suggests that some epididymal fluid proteins attached to the sperm surface may compete for the palmitate label (Table 1). The caput spermatozoa continued to show higher incorporation of radioactivity, even when the incubation time was varied from 30 min to 2 h or the concentration of tritiated palmitate was raised to 1.0 mCi in the incubation medium (Fig. 1). This pattern of incorporation was maintained when radioactivity was extracted with TCA, was washed repeatedly with chloroform:methanol and radioactivity was expressed as c.p.m. μg^{-1} protein (Fig. 2), suggesting that radioactivity was specifically and tightly bound to the sperm surface proteins.

Fig. 1. Percentage incorporation of [^3H]palmitate by maturing rat spermatozoa (incorporation of [^3H]palmitate measured in c.p.m. 10^{-7} spermatozoa). Epididymal spermatozoa were incubated with 100 μCi labelled palmitate for 30 min, 1 h and 2 h and with 1.0 mCi for 2 h at 37°C. The percentage incorporation of radioactivity by caput ($\square$) and caudal ($\blacksquare$) spermatozoa was calculated with the total c.p.m. of the two segments considered to be 100%.

Spermatozoa from the caput epididymis seemed to incorporate less palmitate when incubated for a longer period (2 h) at both 100 μCi and 1 mCi of radiolabelled palmitate, as compared with incorporation after 1 h incubation. However, caudal spermatozoa showed slightly higher incorporation at 2 h than at 1 h duration (Figs 1 and 2).

Analysis of acyl proteins by SDS-PAGE

Autoradiograms of SDS-PAGE gel of labelled caput sperm extract showed three prominent palmitate-incorporating bands of about 70, 56 and 36 kDa, and few minor bands of about 92, 48, 31, 29, 22 and 19 kDa (Fig. 3). Detection of these bands required prolonged fluorographic exposure (50 days) of the gel to duplicating film even though 1.0 mCi of tritiated palmitate was used, raising the possibility that only a small fraction of sperm proteins is modified by the addition of lipid and palmitate is incorporated into proteins to a very small degree.

Fig. 2. Percentage incorporation of [^3H]palmitate by maturing rat spermatozoa (incorporation of [^3H]palmitate measured in c.p.m. μg^{-1} protein). Epididymal spermatozoa were incubated with 100 μCi labelled palmitate for 30 min, 1 h and 2 h and with 1.0 mCi for 2 h at 37°C. The percentage incorporation of radioactivity by caput ($\square$) and caudal ($\blacksquare$) spermatozoa was calculated with the total c.p.m. of the two segments considered to be 100%.

Membrane bound acyl proteins

Caput spermatozoa were labelled with 1.0 mCi of palmitate for 2 h and purified plasma membrane fractions were obtained to investigate whether these proteins were associated with plasma membranes. Radioactivity was measured in different subcellular fractions: the sperm pellet (the pellet obtained after releasing membrane supernatant by nitrogen cavitation and centrifugation), the membrane pellet (the pellet obtained after ultracentrifugation of membrane supernatant at 100 000 g) and cytosol (the supernatant obtained after pelleting membrane vesicles). An examination of the pattern of incorporation in different subcellular fractions suggested that the majority of the [^3H]palmitate binding proteins were membrane-bound since 82% of radioactivity (c.p.m. μg^{-1} protein) was recovered in the membrane pellet as compared with 13.0% in the sperm pellet and 5.0% in the cytosol fractions (Fig. 4). Autoradiograms of SDS-PAGE gels of purified plasma membranes from labelled caput spermatozoa revealed two prominent bands of about 70 and 36 kDa, and four minor bands of about 31, 29, 22 and 19 kDa, similar bands to those produced by the caput sperm extracts. However, two proteins of 92 and 56 kDa present on caput sperm extract were not observed in the autoradiogram of membrane fraction (Fig. 5). Figure 6 shows the silver-stained gel of the sperm pellet, supernatant and membrane pellet of spermatozoa from the caput epididymis.

Chemical nature of lipid linkage to sperm cells

The association of sperm membrane proteins with tritiated palmitate appeared to consist of tight, covalent interactions between fatty acid and proteins. The covalent nature of this association was suggested by the fact that the lipid attachment was stable even after repeated extractions of labelled TCA-precipitated proteins with (1) chloroform:methanol (2:1), (2) boiling the extracted proteins with 4% (w/v) SDS and 10% (v/v) β -mercaptoethanol and (3) SDS gel electrophoresis.

Fig. 3. Autoradiograms of SDS-PAGE gel of caput rat spermatozoa labelled with 1.0 mCi of [^3H]palmitate for 2 h at 37°C. The radioactivity was extracted with 10% (w/v) trichloroacetic acid containing BSA (1 mg ml^{-1}) and then repeated washings with chloroform:methanol (2:1). The fluorograms were kept for 50 days at -70°C using Kodak X-OMAT C film. Lane 1, caput sperm extract; lane 2, caput sperm supernatant (gel without hydroxylamine treatment). Lanes 3 and 4, caput sperm extract and supernatant (gel treated with 1.0 mol hydroxylamine l^{-1} , pH 8.0, for 2 h). Arrows show the palmitylated proteins. Molecular mass (MW) markers are indicated on the left.

One set of gels containing [^3H]palmitate-labelled sperm proteins was treated with 1 mol hydroxylamine l^{-1} (pH 8.0), for 2 h to test the chemical stability of the linkage between the label and the protein. This treatment did not release the radioactive label on gels of labelled sperm extracts (Fig. 3, lanes 2 and 4) or on gels of purified sperm plasma membrane (Fig. 5, lane 2). These observations suggest that fatty acids are linked covalently to sperm membranes through tight amide bonds.

Discussion

The data presented here support the hypothesis that fatty acid acylation of membrane proteins is a post-translational modification in rat sperm cells, based on the observation that, in other cell systems, acylation of proteins with palmitate is a post-translational phenomenon. In the present study, palmitic acid labelled a subset of proteins in sperm cells and the label was specifically and tightly bound to sperm surface proteins as evidenced by extraction of radioactivity with TCA and repeated washings with chloroform:methanol (2:1). Most of the

Fig. 4. Distribution of radioactivity of [^3H]palmitate in subcellular fractions of rat caput (□) and caudal (■) spermatozoa. The percentage incorporation was calculated on the basis of total c.p.m. of all the fractions considered to be 100%.

Fig. 5. Autoradiogram of SDS-PAGE gel containing purified plasma membrane of rat caput spermatozoa treated with 1.0 mCi of [^3H]palmitate for 2 h. Lane 1, caput sperm membrane without hydroxylamine treatment; lane 2, caput sperm membrane with hydroxylamine treatment (1.0 mol l^{-1} , pH 8.0) for 2 h.

labelled proteins fractionated with the plasma membrane, as would be expected for proteins modified with a hydrophobic acyl chain. The fatty acid in sperm cells did not appear to be covalently linked by ester bonds, as it was insensitive to hydrolysis with hydroxylamine. However, it was found to be linked via amide bonds, as described for the α -subunit of the insulin receptor (Hedo *et al.*, 1987), *Escherichia coli* lipoprotein (Hantke and Braun, 1973), bovine cardiac muscle cAMP-dependent protein kinase (Carr *et al.*, 1982), murine leukaemia membrane associated proteins P15 and pp60 src (Sefton *et al.*, 1982; Henderson *et al.*, 1983) and the α - and β -subunits of the nicotinic acetylcholine receptor (Olson *et al.*, 1984).

Fig. 6. Silver-stained gel containing rat caput sperm pellet (lane 1), supernatant (lane 2) and purified plasma membrane (lane 3). Molecular mass (MW) markers are indicated on the left.

In initial experiments, the incubation of sperm cells with labelled palmitate (100 μCi) for a period of 1 h showed a high incorporation of radioactivity associated with TCA extracts of caput spermatozoa, but exhibited only faint bands on fluorographs after exposure for 20 days. Hence, both the concentration of palmitate in the incubation medium and the period of incubation were increased (1.0 mCi for 2 h) in subsequent experiments. The incubation of spermatozoa with 1 mCi palmitic acid for 2 h and the prolonged fluorographic exposures of the gel for 50 days resulted in more appreciable protein bands on the fluorograms but raised the possibility that palmitate is incorporated into sperm proteins to a lower degree, with only a small fraction of sperm proteins specifically modified by the addition of lipid.

In the present study, the incorporation of tritiated palmitate in sperm cells appeared to be a maturation-associated phenomenon, as evidenced by the higher incorporation of labelled lipids by immature caput spermatozoa than by mature caudal spermatozoa. The pattern of incorporation of palmitate by maturing sperm cells may be related to decreased amounts of lipid during transport through the epididymis or could be due to barriers to palmitate uptake in caudal spermatozoa. A decrease in the amount of sperm lipid during transit through the epididymis has been ascribed to the utilization of lipids by spermatozoa as energy sources and would explain the greater sensitivity of ejaculated spermatozoa to cold shock and the decreased membrane fluidity of spermatozoa during maturation of epididymal spermatozoa (Vijayasarithi and Balram, 1982; Nikolopoulou *et al.*, 1985). The variation in fluidity may regulate specific functions of sperm membranes associated with

capacitation and membrane fusion at the time of acrosome reaction (Hinkovska-Galcheva and Srivastava, 1993).

In the present study, the possibility of incorporation of radioactivity by plasmalogens of cytoplasmic droplets or caput spermatozoa can be ruled out, as the combination of tritiated palmitate with plasmalogens would produce ester bonds and not amide bonds. These two types of bond can be differentiated by hydroxylamine sensitivity experiments.

The identification of a long chain fatty acid AMP-dependent coenzyme A sulphhydryl ligase (CoASH(AMP)), in human spermatozoa is consistent with the ability of spermatozoa to metabolize palmitic acid. CoASH ligase localized in the plasma membrane, the mitochondria or both, could be involved in energy production, motility or membrane remodelling (Jones *et al.*, 1985). The reduced uptake of tritiated palmitate by caput spermatozoa after incubation for 2 h as compared with 1 h could be due to hydrolysis of labelled palmitate by CoASH ligase in caput spermatozoa.

The highly specific localization of palmitylated proteins in sperm plasma membrane represents a minor fraction of cellular components. This raises intriguing questions about the intracellular pathways followed by acylated proteins and about the role of fatty acylation in sperm cells. Palmitylation of integral membrane proteins in other cell systems may influence protein-protein interaction and protein-lipid interactions within membranes by affecting protein folding or protein orientation (Schlesinger and Malfer, 1982; Olson *et al.*, 1984; Olson and Spizz, 1986). The post-translational addition of lipids may, therefore, be the means by which these proteins acquire affinity for membranes (Olson and Spizz, 1986), or may provide a mechanism for routing nonglycosylated proteins to the cytoplasmic face of the plasma membrane (Wilcox and Olson, 1987). Another role for fatty acids in acylated proteins is to anchor the proteins in the lipid bilayer by providing an additional hydrophobic moiety (Marinetti and Cattieu, 1982; Magee and Schlesinger, 1982; Schmidt, 1983). The fatty acid acylated proteins also play a significant role as components of intracellular signalling pathways (James and Olson, 1990) and may be involved in fusion induction similar to viral membrane glycoproteins (Schmidt, 1983).

It is premature to comment on the role of acylated proteins in membrane-associated changes in spermatozoa during the complex process of maturation. Further studies are needed to establish their role in inducing membrane fusion processes during capacitation and acrosome reaction of spermatozoa and as components of intracellular signalling pathways during fertilization.

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