Cyclic AMP-dependent phosphorylation of epididymal mouse sperm proteins during capacitation in vitro: identification of an Mr 95 000 phosphotyrosine-containing protein

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Cyclic AMP-dependent changes in phosphorylation of epididymal mouse sperm suspensions were examined in media designed to manipulate capacitation and the expression of parameters associated with full fertilizing ability, i.e. hyperactivated motility and the acrosome reaction. After initial assessment of cAMP-dependent protein kinase activity in frozen–thawed and lyophilized sperm suspensions using exogenous substrate, phosphorylation of endogenous sperm phosphoproteins was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography or immunoblotting. Numerous phosphoproteins were detected in both uncapacitated and capacitated suspensions, the majority of which were probably concerned with motility; full expression of fertilizing ability appeared to involve an increase in the amount of endogenous phosphorylation as deduced from the decreased amount of 32P incorporation in these suspensions. The addition of the cAMP-dependent protein kinase inhibitors, H8 and PKI (6–22) amide, demonstrated that most of the phosphoproteins detected were phosphorylated in a cAMP-dependent manner. Of particular interest was a phosphoprotein with an Mr of about 95 000 which was consistently observed in capacitated suspensions. Evidence suggests that this may be phosphorylated on tyrosine residues, since the inclusion of orthovanadate, a phosphoryltyrosine phosphatase inhibitor, altered phosphorylation of this protein. Furthermore, immunodetection using the antiphosphotyrosine antibody, PY-20, identified five proteins with approximate Mr, 116 000, 105 000, 95 000, 86 000, and 76 000, and possibly a sixth at 54 000. The 95 000 protein was consistently diminished in ionophore-treated spermatozoa, indicating that the protein was located in the acrosomal cap region. These results suggest that the protein may be the same phosphotyrosine-containing protein as that described by Leyton and Saling (1989) which has been proposed to play a role in acrosomal exocytosis.

Introduction

When spermatozoa first leave the male reproductive tract they are not immediately fertile. They require a species-dependent period in the female reproductive tract, during which they acquire the ability to fertilize; the changes that occur to promote functional ability collectively constitute capacitation (Austin, 1952). In some species, capacitation can be achieved outside the female reproductive tract under defined culture conditions. Although capacitation has been studied extensively, very little is known of the biochemical events controlling the processes that result in fully fertile sperm cells capable of expressing hyperactivated motility and undergoing the acrosome reaction, obligatory prerequisites for successful fertilization.

There is much evidence that cyclic nucleotides play a role in mammalian sperm capacitation, the acrosome reaction and motility (for reviews see: Garbers and Kopf, 1980; Tash and Means, 1983; Fraser, 1984; Fraser and Ahuja, 1988; Fraser and Monks, 1990). There is also increasing evidence that cyclic adenosine monophosphate (cAMP) may be important in the stimulation of sperm fertilizing ability. Stein and Fraser (1984) reported a capacitation-related increase in adenylate cyclase activity and a decrease in cyclic nucleotide phosphodiesterase activity in mouse spermatozoa. Consistent with this, White and Aitken (1989) reported a progressive increase in intracellular cAMP concentrations preceding the full expression of hyperactivated motility in hamster spermatozoa. Indirect evidence for a role for cAMP in capacitation and fertilization has been found using analogues of cAMP (Fraser, 1981) and inhibitors of the enzymes of cAMP metabolism (Fraser, 1979). In addition, the initiation of flagellar motility in bovine (Lindemann, 1978) and canine (Tash and Means, 1982) spermatozoa depends on the presence of cAMP.

The main function of cAMP in cells is the activation of cAMP-dependent protein kinases (Flockhart and Corbin, 1982),

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and the subsequent phosphorylation events alter the activities of key cellular components or enzymes. Since mature spermatozoa lack any transcriptional or translational abilities, alterations in phosphorylation may play an important role in the regulation of sperm fertilizing ability. Mammalian sperm cells have been shown to possess both isozymes (types I and II) of cAMP-dependent protein kinases (Hoskins et al., 1972; Garbers et al., 1973) and phosphatases that can dephosphorylate proteins (Tang and Hoskins, 1975; Swarup and Garbers, 1982; Tash et al., 1988). Thus phosphorylation/dephosphorylation events could regulate, at least in part, sperm function.

Several phosphoproteins have been detected in spermatozoa of a variety of species (Haucua et al., 1977; Chulavatnatol, 1982; Noland et al., 1984; Haldar and Majumdar, 1986; Wootten et al., 1987; Horowitz et al., 1988). However, few of these have been specifically identified and none has been rigorously associated with specific function. Among those reported is a protein of Mr, of about 55 000 in the cytoplasm of bull spermatozoa, the phosphorylation of which appeared to be related to motility (Brandt and Hoskins, 1980). Many studies on sperm motility have been carried out on detergent demembranated sperm models (reviewed by Tash and Means, 1982). Using such a model, Tash et al. (1984, 1986) identified an Mr 56 000 cAMP-dependent phosphoprotein (axokinin) required for the reactivation of flagellar motility in detergent-lysed canine sperm cells. However, subsequent investigations provided evidence that the major soluble cAMP-dependent phosphoprotein of Mr 56 000 is the regulatory subunit of the type II cAMP-dependent protein kinases (Rn) (Noland et al., 1987; Paupard et al., 1988). Tash (1989) has confirmed the suggestion that axokinin and Rn are the same protein.

Although there is considerable evidence that protein phosphorylation plays a role in the initiation and regulation of flagellar motility (reviewed by Lindemann and Kanous, 1989; Tash, 1989), little is known about the role of phosphorylation during capacitation and the acrosome reaction. Carr and Acott (1990) identified an Mr 255 000 bovine sperm phosphoprotein which may be a sperm microtubule-associated protein 2 and found that the phosphorylation level of this phosphoprotein was sensitive to modulators known to regulate capacitation and the acrosome reaction. Leyton and Saling (1989) detected an Mr 95 000 protein from epididymal mouse spermatozoa that was phosphorylated on tyrosine residues and the level of phosphorylation of which increased following capacitation or exposure to solubilized zona pellucida proteins. They suggested that the 95 000 protein may be a sperm receptor binding ZP3 and that the resulting aggregation of the receptor may stimulate tyrosine kinase activity leading to acrosomal exocytosis.

Using a well-characterized mouse in vitro system (Fraser, 1983, 1987; Fraser and Quinn, 1981), we have looked for capacitation-related changes in the phosphorylation patterns of epididymal mouse spermatozoa. Sperm suspensions incubated for 2 h in the continuous presence of glucose (+G) are fully capacitated, highly motile and highly fertile. In contrast, sperm cells incubated for 2 h in the absence of glucose (−G) are capacitated but non-fertile with sluggish motility. The addition of glucose to −G spermatozoa at 2 h (−G + G) rapidly stimulates motility, and the full expression of hyperactivated motility was observed within a short time; spermatozoa treated in this manner are highly fertile (Fraser and Quinn, 1981). These physiological responses to change in the medium composition have formed the basis for protocols used in our study of phosphorylation events during capacitation.

### Materials and Methods

#### Culture media

The culture medium used to incubate spermatozoa during capacitation in vitro was a modified Tyrode's medium (Fraser, 1983) containing 5.56 mmol glucose l⁻¹ and 4 mg bovine serum albumin ml⁻¹ (BSA, crystalline; Sigma Chemical Co., Poole). A concentrated glucose stock solution was prepared in glucose-free Tyrode's medium such that 10 µl of stock added to 240 µl of glucose-free medium would produce a final concentration of 5.56 mmol glucose l⁻¹.

#### Method of sample preparation

In this study we wished to sample suspensions at precise, multiple time points that correspond to different stages of capacitation. We also wanted to vary the composition of the culture medium in ways known to alter sperm function, e.g. with and without glucose. We considered three possible approaches, namely analysis following permeabilization by either sonication or freeze-thawing/lipophilization or following labelling of intact cells. The last of these was considered to be too limiting since glucose needs to be present to ensure transport of [³²P]Pi into cells and the loading time required is too long to permit precise temporal evaluation, especially since capacitation time in mouse spermatozoa is relatively short. However, we did compare the phosphoproteins detected using this approach with those obtained using lipophilization, the technique chosen for the main part of the study. The array of proteins, based on Mr, was very similar.

Of the remaining two techniques, preliminary gels revealed the same array of phosphoproteins, whether permeabilization was achieved by freeze-thawing/lipophilization or sonication. Protein kinase activity was found to be higher in cells prepared by lipophilization. This, and the fact that we could prepare multiple samples, and then assay all at the same time and so avoid artefacts introduced by assaying at different times, led us to choose freeze-thawing/lipophilization as the primary method of sample preparation.

#### Sperm suspensions

The contents of two cauda epididymides from mature TO male mice (age > 8 weeks) were released into glucose-free medium (−G) in a sterile 30 mm plastic culture dish (Sterilin, Teddington, Middlesex); 1 ml of medium was used for each pair of epididymides to produce a concentration of about 2–3 × 10⁷ cells ml⁻¹. The suspension was left on a warm tray for 5 min to allow dispersal of spermatozoa; an aliquot was removed and glucose stock solution was added to produce a final concentration of 5.56 mmol l⁻¹ (+G). Both suspensions (−G and +G) were incubated at 37°C in 5% CO₂: 5% O₂: 90% N₂ under liquid paraffin. After 120 min incubation, a further aliquot was removed from the −G suspensions; glucose was added to produce a final concentration of 5.56 mmol l⁻¹ (−G + G) and the suspensions were incubated at 37°C for a
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further 10 min. Samples were removed from each suspension at 30 min (−G, +G) and 130 min (−G, −G → +G, +G), frozen–thawed, refrozen and then lyophilized in 100 µl aliquots and stored at −20°C until assay. Thus, a single initial suspension could be divided into the different treatment groups, thereby helping to ensure that sperm concentrations in all were similar. As a further check, sperm counts were carried out on lyophilized samples to verify that approximately the same numbers of cells were used in each assay.

Phosphorylation of exogenous substances

Cyclic AMP-dependent protein kinase activity was assessed using a modification of the method of Corbin and Reimann (1974). Lyophilized sperm suspensions were resuspended to their original volume in a final concentration of 2.5 mmol EGTA l−1; 25 µl was added to 50 µl of incubation medium at 30°C containing 20 mmol potassium phosphate l−1, pH 6.5, 6 mmol magnesium acetate l−1, 50 mmol sodium fluoride l−1, 10 mmol diethiothreitol l−1 (DTT), 100 mmol adenosine triphosphate l−1 (ATP), 1 µCi[γ-32P]ATP (triethylammonium salt, 1 mCi ml−1, Amersham International, Bucks), and 5 mg histone ml−1 (calf thymus type II A, Sigma). Reactions, carried out in the presence or absence of 5 µmol cAMP l−1, were initiated by the addition of sperm suspension and terminated by spotting 50 µl on to filter paper squares (Whatman 3MM: Whatman, Maidstone). These were then washed four times in ice-cold 10% trichloroacetic acid (TCA), followed by 5 min each in 95% ethanol and ethyl ether. After air-drying the papers, radioactivity was determined by liquid scintillation counting. Sperm cell counts were measured using a haemocytometer and specific activity was expressed as pmol phosphate incorporated min−1 10−3 sperm cells.

Phosphorylation of endogenous proteins

Cyclic AMP-dependent protein kinase activity, using only endogenous proteins rather than exogenous histones, was assayed in the presence of 6 µCi[γ-32P]ATP, using the method detailed above. A control sample, with BSA-containing medium added to the incubation medium, was included to take into account any non-specific phosphorylation. The reaction was stopped after 10 min by the addition of sample to a half volume of electrophoresis loading buffer (composition detailed below). The sample was boiled for 5 min and centrifuged at 10 500 g for 5 min, to sediment DNA, before applying equal volumes per lane to a polyacrylamide gel for electrophoresis. In general, 40–50 µl of sample was loaded into each lane. Since each treatment was derived from the same initial suspension, cell counts were not found to vary greatly between the different treatments. Thus, in effect, equal volumes of protein were loaded in each lane within an individual experiment.

Labelling of intact cells

Labelling of intact sperm cells with [32P]Pi, was carried out by an adaptation of the method of Roldan and Harrison (1989). The continuous presence of glucose in the medium was necessary for the incorporation of [32P]Pi into the spermatozoa (Noland et al., 1987). The protocol used for the study of phosphorylation patterns in permeabilized cells prepared after incubation of intact spermatozoa in the presence or absence of added glucose could not therefore be used, and an alternative method of manipulating capacitation was selected. Mouse spermatozoa are fully capacitated after 2 h in complete medium (containing 5.56 mmol glucose l−1 and 1.80 mmol calcium l−1 (+Ca2+)). In the absence of added calcium (−Ca2+), they are not fully capacitated after 2 h incubation and cannot undergo the acrosome reaction. They are, however, highly motile (Fraser, 1982), expressing progressive motility only. Medium containing 90 µmol CaCl2, I−1 (Low Ca2+) allows spermatozoa to complete capacitation, but not to express hyperactivated motility or to undergo the acrosome reaction; however, increasing the extracellular calcium concentration to 1.80 mmol l−1 after 2 h (Low Ca2+ → +Ca2+) enables some of the cells to undergo a spontaneous acrosome reaction immediately and the suspensions are highly fertile (Fraser, 1987). Full labelling of sperm proteins with [32P]Pi, required incubation of at least 45 min and so the early stages of capacitation could not be examined; suspensions were therefore evaluated after 2 h incubation in the various media detailed above.

Sperm suspensions were prepared in −Ca2+ medium. After 5 min to allow dispersal, aliquots were removed and two different concentrations of calcium added to give a Low Ca2+ suspension and a + Ca2+ suspension, the remainder having no added calcium (−Ca2+). [32P]Pi, (carrier free, 10 mCi ml−1, Amersham International) was added to these suspensions to a final concentration of 500 µCi ml−1. Suspensions were incubated in capped tubes in a water bath at 37°C for 2 h. An aliquot was removed from the Low Ca2+ suspension and calcium was added to a final concentration of 1.80 mmol l−1 (Low Ca2+ → +Ca2+); incubation was continued for a further 10 min at 37°C. All suspensions were then diluted tenfold with the appropriate media and centrifuged at 2600 g for 5 min. The supernatant was discarded and 200 µl of sodium dodecyl sulphate (SDS)-extraction buffer (containing 2% SDS and 0.08 mol Tris l−1, pH 6.8) were added to each pellet. The extracted cells were then prepared for electrophoresis as described above.

Electrophoresis and autoradiography

SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out in a Protean IIid tank (Biorad, Hemel Hempstead). Electrophoresis loading buffer contained: 2.7% SDS, 13.5% glycerol, 0.14 mol DTT l−1, 0.1 mol Tris l−1 (pH 6.8), 0.03% bromophenol blue. The resolving acrylamide gel consisted of an 8–15% linear gradient, with a 4% acrylamide stacking gel. Both high and low molecular weight standards (Pharmacia LKB, Milton Keynes) were run on each gel. Routinely, electrophoresis was carried out at 42 mA per gel for approximately 3.5 h. After electrophoresis gels were either stained with Coomassie R250 before autoradiography or silver stained (Biorad Kit 89-0559) as appropriate. Gels were dried and exposed to HRX film (Fuji) for up to 7 days at −70°C with Fuji G8 intensifying screens.

Immunoblotting to detect phosphotyrosine residues

Sperm suspensions were prepared in +G medium containing 100 µmol sodium orthovanadate l−1 and incubated for 105 min. Half the sample was removed and treated with the Ca2+ ionophore A23187 to induce the acrosome reaction (Fraser, 1982). A23187 (Sigma) stock solution in dimethyl sulfoxide...
(DMSO) was added to give a final concentration of 15 µmol l⁻¹. This concentration of ionophore accelerates capacitation as evidenced by the rapid penetration of treated cells into oocytes (Fraser, 1982), indicating that it does not have a deleterious effect on sperm physiology. Both sperm samples were incubated for a further 15 min and were then diluted (each 0.5 ml suspension in 3 ml medium) and washed by gentle centrifugation (750 g max for 15 min) and resuspended to the original volume in fresh medium. This procedure removed numerous proteins of epididymal origin. Samples were frozen–thawed, aliquotted (80 µl per tube) and lyophilized. In one experiment, a sample was removed at 20 min and treated as above.

Electrophoresis was carried out on 6–10% linear gradient gels, to enhance separation of proteins in the higher Mr, range, with a 4% stacking gel as above. Gels and running buffers all contained 100 µmol orthovanadate l⁻¹. Lyophilized samples were reconstituted in 40 µl distilled water (thereby doubling the protein concentration) and a half volume of electrophoresis loading buffer (containing 5.7% SDS because of the increased protein in the sample). Multiple samples were pooled and then 60 µl loaded into each well. This provided more homogeneous patterns in replicate lanes. Molecular weight standards as detailed above were included.

Proteins were then transferred to nitrocellulose (BioRad Transblot, 0.45 µ) using a Pharmacia Novablot system. Blots were probed using anti-phosphotyrosine antibody, either RPN 138 (Amersham International) or PY-20 (ICN Biochemicals, Flow, Irvine, Ayrshire); both are mouse monoclonal antibodies. Two methods were used for immunodetection. In one, a biotinylated anti-mouse antibody followed by streptavidin–alkaline phosphatase was used (blotting detection kit RPN 22, Amersham International). Membranes were blocked in 20% milk (from kit) for 2 h at room temperature, washed in phosphate buffered saline (PBS), incubated overnight at room temperature in a 1:1000 dilution of anti-phosphotyrosine antibody in PBS containing 1% BSA (fraction V). The Amersham kit was used according to instructions provided, with the secondary antibody being used at 1:1000 and the streptavidin–alkaline phosphatase at 1:3000. For some strips, the anti-phosphotyrosine was preincubated with 40 nmol o-phospho-DL-tyrosine l⁻¹ (Sigma) for 1 h prior to use. In the second, an enhanced chemiluminescence (ECL) western blotting detection system (Amersham International RPN 2106) was used: nitrocellulose strips were blocked in milk and incubated in PY-20 overnight as above, washed, incubated for 2 h at room temperature in a 1:1500 dilution of a horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham International NA 931), and finally treated according to the ECL kit instructions.

**Experimental Details and Results**

**Protein kinase activity with exogenous substrate**

A series of experiments was carried out initially to validate the method used to assay protein kinase activity. The kinase activity of spermatozoa incubated in complete medium for 20 min before lyophilization was determined over 60 min in the presence of 5 µmol cAMP l⁻¹ (Fig. 1). Phosphate incorporation increased with time and was linear over the first 10 min; 10 min was therefore the standard incubation time used in all subsequent assays.

Two methods of permeabilizing the cells, to ensure access of protein kinase to the exogenous cAMP and labelled ATP, were compared. After incubating spermatozoa in complete medium for 20 min, one aliquot was frozen in liquid nitrogen, thawed, re-frozen and lyophilized, while the remainder was sonicated (Vibracell Soniprobe) using 3 × 4 s bursts on ice. The results (Fig. 2) indicated that protein kinase activity was greater in the lyophilized samples than in the sonicated samples, both in the presence and absence of added cAMP, indicating adequate permeabilization of membranes in the lyophilized preparations. In addition, this experiment demonstrates the increase in specific activity observed in all samples with the addition of 5 µmol cAMP l⁻¹.

**Phosphorylation of endogenous proteins**

Patterns of proteins phosphorylated in the presence or absence of added glucose. The assay was carried out in the absence of added cAMP. A major phosphoprotein was detected at M₆ of about 70 000 (Fig. 3). Since the same protein was observed in the medium-only control sample (lane k), it probably represents non-specific phosphorylation of the BSA present in the sperm.
incubation medium. Comparison of the phosphorylation patterns observed in $-G$ (lanes c and d) and $-G \rightarrow +G$ (lanes e and f) suspensions at 130 min showed a decreased phosphorylation of endogenous proteins in the latter sample, while comparison of $-G$ incubated suspensions at 30 min (lanes a and b) and 130 min (lanes c and d) revealed an increased intensity of substrate phosphorylation in the latter sample. These results suggest alterations in the activation state of the kinases and hence the available phosphorylation sites on endogenous proteins in capacitated, functional cells, i.e. those incubated for 130 min. Comparison of $-G$ (lanes a and b) and $+G$ (lanes g and h) spermatozoa at 30 min, when both suspensions would be uncapacitated, shows a reduction in intensity of phosphorylation in the latter samples. Since the $+G$ cells would be highly motile compared with $-G$ ones, the majority of phosphoproteins detected may be concerned with motility. The reduced phosphorylation observed in the motile (+G) samples suggests that the endogenous phosphoproteins are already phosphorylated and therefore cannot readily accept $^{32}P$ phosphate groups.

The only apparent change in the pattern of phosphorylation was the appearance of a phosphoprotein at $M_r$ of about 95 000 in all sperm samples at 130 min. The protein was most easily detected in the 130 min $-G$ sperm samples, presumably owing to the generally higher levels of phosphorylation, but was also present in the 130 min $+G$ samples. Since this phosphoprotein was not detected in uncapacitated spermatozoa at 30 min, this may be related to a capacitation event. Silver stained proteins detected in sperm samples after 30 and 130 min incubation in $+G$ medium are shown (Fig. 4). Although the region of $M_r$ 80 000–100 000 has relatively few protein bands, there is one at 95 000 in both the uncapacitated and capacitated suspensions.

**Fig. 3.** Phosphoproteins detected in mouse sperm cells incubated in the absence or presence of glucose for up to 130 min prior to assay. Duplicate samples were run in adjacent lanes. a, b: 30 min and c, d: 130 min incubation in glucose-free medium; e, f: 120 min incubation in glucose-free medium plus 10 min in glucose; g, h: 30 min and i, j: 130 min in glucose-containing medium; k, BSA-containing medium only. The $M_r$ markers ($\times 10^4$) are indicated on the left of this and subsequent figures. * Denotes phosphoprotein of $M_r$ of about 95 000 detected in capacitated samples.

**Fig. 4.** Silver-stained proteins detected in mouse sperm preparations after (a) 30 min and (b) 130 min incubation in glucose-containing medium. * Denotes protein of $M_r$ of about 95 000.
cAMP-dependent changes in phosphorylation. When cAMP was added to the phosphorylation assay the only detectable effect was the appearance of an extra phosphoprotein at M, 42 000 (Fig. 5). The regulatory subunit of the type II cAMP-dependent protein kinase (R) is known to represent a major portion of the sperm protein and has an M of 54 000–56 000. In the presence of cAMP, this protein is susceptible to proteolytic degradation, which results in a major breakdown product of M, 40 000. The possibility that the M, 42 000 phosphoprotein observed was a proteolytic degradation product of the R subunit was investigated by adding the proteolytic inhibitors aprotinin (3.33 µg ml⁻¹), leupeptin (3.33 µg ml⁻¹), soybean trypsin inhibitor (1 mg ml⁻¹), and p-aminobenzamidine (1 mmol l⁻¹) to sperm suspensions just before freezing and at the time of phosphorylation assay. The resulting autoradiograph (Fig. 6) demonstrates that the addition of proteolytic inhibitors either at assay or just before lyophilization of the spermatozoa results in loss of the 42 000 band with no other major change in the phosphorylation pattern, suggesting that this protein is a proteolytic degradation product of the R subunit. The heavy band between 14 000 and 20 000 (Fig. 6b) is due to the inhibitors themselves.

Phosphorylation patterns in intact cells. The array of phosphoproteins detected in whole sperm extracts prepared after incubating intact cells in [³²P]P, was very similar to that obtained with permeabilized cells exposed to labelled ATP, if the M,s are compared. These results thus indicate that treatment undertaken to permeabilize spermatozoa did not significantly alter sites available for phosphorylation. The relative proportions of the various proteins differed markedly, but this is not surprising since the labelling conditions were quite different. Incubation of cells in various concentrations of Ca⁺⁺ to investigate possible capacitation-related changes in the pattern of phosphorylation did not reveal any major alterations. Given the inherent limitations of this approach, e.g. lack of temporal precision due to long [³²P]P, loading time, it would be difficult to detect and quantify specific cAMP-dependent phosphorylation events without further considerable modification of the protocol. A typical pattern is shown (Fig. 7).

Comparison of phosphorylation in particulate and supernatant fractions. Spermatozoa incubated for 130 min in complete medium and then lyophilized were resuspended in 2.5 mmol EGTA l⁻¹ to the original volume; the suspension was centrifuged at 11 600 g for 5 min and the resulting supernatant was
decanted. The pellet (particulate fraction) was resuspended to the original volume with complete medium containing 2.5 mmol EGTA \(1^{-1}\), and both supernatant and particulate fractions were assayed for protein kinase activity in the presence and absence of cAMP as described above. The majority of the phosphoproteins detected were in the particulate fraction of the sperm suspension, and very few phosphoproteins were present in the supernatant fraction. A phosphoprotein with \(M\), 58 000 was present in both particulate and soluble fractions and may represent the R1 subunit of the type II cAMP dependent protein kinase. These results (Fig. 8) suggest that most phosphoproteins detected are associated with major structural elements of the sperm cells.

**Protein kinase inhibitors**

**H8.** The cAMP-dependent protein kinase inhibitor H8 (final concentration 500 µmol \(1^{-1}\)) was added during the assay (in the presence of 5 µmol cAMP \(1^{-1}\)) of spermatozoa incubated in \(-G\) medium for 130 min. The resulting autoradiograph (Fig. 9) demonstrated that the majority of the phosphoproteins detected were phosphorylated in a cAMP-dependent manner. The phosphorylation of BSA present in the medium was not altered by the presence of H8. Although phosphorylation of a major protein of \(M\), 48 000 was unaffected by the presence of H8, most of the minor phosphoproteins could not be detected under these conditions.

**Protein kinase A inhibitor peptide.** The inhibitor peptide, PKI (6–22) amide (Gibco, BRL), is highly selective for cAMP-dependent protein kinases over other kinases. It is one of the most potent inhibitors derived from PKI, the heat stable inhibitor of cAMP-dependent protein kinases. A range of inhibitor concentrations (0, 20 nmol \(1^{-1}\), 200 nmol \(1^{-1}\), 2 µmol \(1^{-1}\)) was added to the incubation mix and the phosphorylation pattern was assessed in spermatozoa incubated in \(-G\) medium for 130 min.

As with H8, there was no effect of the inhibitor on either the non-specific phosphorylation of BSA or the \(M\), 48 000 phosphoprotein (data not shown) in the single experiment carried out. The intensity of phosphorylation of the other phosphoproteins was found to decrease with increasing concentration of inhibitor, although even at the highest concentration used (2 µmol \(1^{-1}\)), the phosphorylation of endogenous substrates was not completely inhibited. At a concentration of 1–2 µmol \(1^{-1}\) complete inhibition of cAMP-dependent protein kinases in somatic cells has been reported (Kemp et al., 1988); it is possible that sperm cell kinases require higher concentrations.

**Protein tyrosine kinase inhibitor.** Since the 95 000 phosphoprotein detected by Leyton and Saling (1989) was found to be
phosphorylated on tyrosine residues, the effect of the addition of the protein tyrosine kinase inhibitor, methyl 2,5-dihydroxy cinnamate (Gibco, BRL) on the appearance of the 95,000 phosphoprotein detected in our capacitated suspensions was examined. The inhibitor was added at a final concentration of 0, 0.1, 1, 10 and 100 µg ml⁻¹ to the incubation mix of the phosphorylation assay and the phosphorylation pattern of -G 130 min spermatozoa was examined. A preliminary experiment indicated that as with the other inhibitors (H8 and PKI cAMP-dependent protein kinase inhibitor peptide) there was no effect on the phosphorylation of BSA or on the M₄ 48,000 protein. There did appear to be a reduction in phosphorylation of the 95,000 phosphoprotein with increasing concentration of inhibitor. However, densitometric scanning of the autoradiograph revealed a reduction in intensity of phosphorylation in all the phosphoproteins detected (data not shown). This was especially apparent in the highest concentration used (100 µg ml⁻¹) which might indicate a non-specific effect of the inhibitor under these conditions.

Orthovanadate effects on phosphorylation

Orthovanadate is reported to be an inhibitor of phosphatases acting on phosphotyrosine (Swarup et al., 1982). Leyton and Saling (1989) routinely included sodium orthovanadate in their capacitation medium and in all manipulations of the sperm cells, e.g. electrophoresis and immunoblotting. The effect of this compound on the detection of the 95,000 phosphoprotein was investigated by preparing sperm suspensions in -G medium. An aliquot was removed after 5 min incubation and sodium orthovanadate (Sigma) was added to a final concentration of 100 µmol l⁻¹ (+ van). The two suspensions were incubated for 120 min, before the addition of glucose to an aliquot of both -van and + van suspensions (-G→ + G ± van). Incubation was continued for a further 10 min before freeze-thawing and lyophilization of all suspensions.

Assay of endogenous phosphorylation was carried out as described above, but with the addition of 100 µmol vanadate l⁻¹ to the incubation mixture where indicated. In the resulting autoradiograph (Fig. 10) the 95,000 phosphoprotein was detected in each of the incubation conditions. However, when vanadate was present continuously during incubation in vitro and during the phosphorylation assay, the intensity of phosphorylation of the protein was reduced compared with that seen when vanadate was absent during incubation or assay. This would suggest that in the presence of orthovanadate, the phosphorylation state of the 95,000 protein is stabilized and therefore more difficult to detect by the addition of [³²P] from ATP during assay. This suggests, indirectly, that the 95,000 phosphoprotein detected was similar to that of Leyton and Saling (1989).
Fig. 9. Phosphoproteins detected in mouse sperm cells incubated in glucose-free medium for 130 min prior to assay in the presence of cAMP and the presence or absence of the cAMP-dependent protein kinase inhibitor H8. Lanes a, b, e, f: medium only; c, d, g, h: spermatozoa.

Fig. 10. Phosphoproteins detected in mouse sperm cells incubated in glucose-free medium for 130 min prior to assay. a: no orthovanadate; b: 100 µmol orthovanadate l⁻¹ added at assay; c: 100 µmol orthovanadate l⁻¹ present during incubation and assay. * Denotes phosphoprotein of M, 95 000.

Fig. 11. Immunoblot of mouse sperm proteins reacting with the anti-phosphotyrosine antibody PY-20, detected using streptavidin–alkaline phosphatase. Control 120 min sperm samples are shown in lanes a, c, e and ionophore-treated samples in lanes b, d, f: a and b, primary antibody used; c and d, primary antibody omitted; e and f, primary antibody preincubated with 40 mmol o-phospho-DL-tyrosine l⁻¹. In this blot, the 116 000 protein is faintly stained. * Denotes protein of M, 95 000.
Immunodetection of phosphotyrosine residues

Of the two anti-phosphotyrosine antibodies used, PY-20 proved to be more sensitive. With the use of both colorimetric and enhanced chemiluminescence methods, a total of eight proteins were detected (Fig. 11), with $M_r$ of about 120 000, 116 000, 105 000, 95 000, 86 000, 76 000, 54 000 and 53 000; one at about 37 000 was seen infrequently. The 116 000 protein was less prominent than the other seven. Of these, the proteins of 120 000 and 53 000 were non-specific, and were observed consistently both when pre-blocked primary antibody was used and when primary antibody was omitted. There appeared to be a non-specific protein of about 76 000 but this was always of considerably lower intensity (Fig. 11, lanes c, d, e, f) than the band observed after incubation in primary antibody (Fig. 11, lanes a and b). This suggests the presence of at least two proteins at this position, one of which is a phosphotyrosine-containing protein. In a minority of samples, the protein of $M_r$ about 54 000 was visible in the absence of primary antibody; this was the case in the sample used for Fig. 11. It is unclear whether this may also indicate the presence of two proteins, one of which contains phosphotyrosine residues. In ionophore-treated samples, the 95 000 protein was the only one consistently reduced (Fig. 11, lane b). In the one uncapacitated (20 min incubation) sample examined, the 86 000, 95 000 and 105 000 proteins were not detectable.

Discussion

Capacitation-related changes in the phosphorylation pattern were investigated by capacitating epididymal mouse spermatozoa in vitro in a controlled environment. Thus, changes in phosphorylation state of the cells could be related to their functional state. Various studies have been carried out to investigate changes in phosphorylation patterns associated with epididymal maturation (Chulavatnatol et al., 1982; Wooten et al., 1987) and initiation of motility (Brandt and Hoskins, 1980; Tash and Means, 1982). However, very few studies investigating the changes in phosphorylation that may occur during capacitation have been attempted.

Incubation of frozen–thawed, lyophilized mouse spermatozoa with $^{32}$P-ATP revealed numerous phosphoproteins (Figs 3 and 5). The majority of these proteins were present in both uncapacitated and capacitated samples, but the incorporation of $^{32}$P differed between highly motile cells incubated in the presence of glucose and poorly motile cells incubated in the absence of glucose. This suggests that most of these proteins are probably concerned with motility, a conclusion consistent with other studies on sperm phosphoproteins (e.g. Brandt and Hoskins, 1980). The apparent inverse relationship between the intensity of the phosphorylation pattern observed and the physiological state of the spermatozoa in vitro suggests that a change in the overall level of phosphorylation of endogenous proteins may be an important component of capacitation. In spermatozoa that were allowed to complete capacitation, undergo the acrosome reaction and express hyperactivated motility (Fig. 3: $-G\rightarrow +G$ 130 min, lanes e and f, and $+G$ 130 min, lanes i and j), the intensity of phosphorylation of substrate was reduced compared with fully capacitated but poorly motile cells that could not undergo the acrosome reaction (Fig. 3: $-G$ 130 min, lanes c and d). Thus completion of the acrosome reaction and expression of hyperactivated motility in vitro would appear to involve an increase in endogenous phosphorylation of protein substrates. This interpretation correlates well with reports of increased intracellular levels of cAMP preceding the onset of hyperactivated motility in hamster spermatozoa (White and Aitken, 1989). Such increased availability of cAMP could promote full activation of cAMP-dependent protein kinases and, consequently, increased phosphorylation of relevant proteins.

The addition of exogenous cAMP to the phosphorylation assay had no major effect on the pattern of phosphorylation. This suggests that the sperm cAMP-dependent protein kinases were already activated to a reasonable extent at the time of assay, and therefore the addition of cAMP could not stimulate enzyme activity in a manner that could be detected using this experimental approach. Tash and Means (1982) calculated that the cAMP concentrations in normal, freshly isolated sperm cells would be sufficiently high to activate cAMP-dependent protein kinases.

Evidence from the studies using the inhibitor H8 suggests that the majority of phosphoproteins detected were phosphorylated in a cAMP-dependent manner, since most of these proteins were missing in H8-treated samples. The preliminary study with the inhibitor PK1 (6–22) amide did not result in complete inhibition of phosphorylation at the concentrations used. However, a gradual decrease in phosphorylation with increasing concentration of inhibitor was evident. Since the highest concentration used was based on studies with somatic cells, it may be that a higher concentration of inhibitor is required to inhibit sperm cAMP-dependent protein kinase. Spermatozoa have been shown to have a relatively high concentration of the enzyme, with 10% of the soluble protein of epididymal bovine spermatozoa represented by cAMP-dependent protein kinase (Hoskins et al., 1972).

The similarity in the array of phosphoproteins detected in permeabilized cell preparations (e.g. Fig. 3) and intact sperm cells (Fig. 7) suggest that the proteins phosphorylated in frozen–thawed, lyophilized cells were not cryptic proteins released during sample preparation. Furthermore, the majority of phosphoproteins were found in the particulate, rather than the supernatant, fraction, suggesting that sample preparations did not result in large-scale release of proteins into the soluble fraction. An example of how preparation might affect the pattern obtained was demonstrated by the addition of proteolytic inhibitors (Fig. 6). In the presence of proteolytic inhibitors, the phosphorylation of a protein of $M_r$ 42 000 was reduced, probably as a result of the inhibition of the proteolytic degradation of the R$_S$ subunit of the type II protein kinase (Noland et al., 1986; Horowitz et al., 1989). However, since no other major changes in the phosphorylation pattern were observed in the presence of proteolytic inhibitors, these were not routinely included in the phosphorylation assay. Although the R$_S$ subunit of the type II cAMP-dependent protein kinase was not specifically identified by immunoblotting in this study, it is thought to be one of the major cAMP-dependent phosphoproteins in mammalian spermatozoa (Noland et al., 1987; Paupard et al., 1988). The R$_S$ subunit is autophosphorylated with a molecular weight of 54 000–56 000 and has been shown to be tightly

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associated with the mammalian sperm flagellum (Horowitz et al., 1984, 1988; Lieberman et al., 1988).

Earlier studies on mouse spermatozoa reported that adenylate cyclase activity was higher in suspensions incubated in calcium-containing medium, which promotes complete capacitation, than in those incubated in calcium-deficient medium, which supports only partial capacitation (Monks et al., 1986); this result suggested a greater availability of cAMP in cells incubated in +Ca\(^{2+}\) medium. Thus, a difference in the phosphorylation pattern of the intact spermatozoon labelled with orthophosphate and incubated in various calcium-containing and calcium-deficient media might have been expected. However, no differences were apparent under these conditions. One explanation may be the difficulty in distinguishing and quantifying cAMP-dependent phosphorylation from cAMP-independent phosphorylation using this technique. Furthermore, the fact that cAMP-dependent protein kinases appeared to be already activated at the time of assay (Fig. 5) suggests that any differences would be subtle and hence difficult to detect with autoradiography. However, perturbing the physiology of the spermatozoon by excluding glucose, which would reduce ATP generation and cAMP production, clearly alters the phosphorylation pattern and therefore supports an important role for these kinases in normal sperm function.

A consistent feature of all the autoradiographs obtained in this study was the non-specific phosphorylation of the BSA in the medium, but the significance, if any, of this is unclear. BSA is an important component of the medium, enabling mouse spermatozoa to undergo the acrosome reaction (Fraser, 1985). However, it is not clear from these experiments whether phosphorylation of the BSA is necessary in vivo or is an artefact of the assay.

The identity of the major phosphoprotein at Mr 48 000 is not known. This protein was not readily detected in Coomassie-stained gels, suggesting that it was highly phosphorylated during assay in vitro and hence easily visible on autoradiographs. Tubulin is a major component of the sperm axoneme and has been shown to be phosphorylated in a cAMP-dependent manner (Tash and Means, 1982). However, immunodetection with various antibodies to tubulin proved negative (data not shown). In addition, the experiments with H8 and the PKI (6-22) amide suggested that the 48 000 phosphoprotein was phosphorylated in a cAMP-independent manner.

The main change to the phosphorylation pattern of epididymal mouse spermatozoa incubated under the various conditions described here was the presence of a phosphoprotein at Mr 95 000 detected in all capacitated suspensions (Figs 3, 5, asterisks). Since Leyton and Saling (1989) had detected a 95 000 phosphoprotein, the level of phosphorylation of which appeared to be related to capacitation events and which was phosphorylated on tyrosine residues, we investigated the possibility that the two might be similar.

Experiments with the tyrosine kinase inhibitor, methyl 2,5-dihydroxy-cinnamate did not show a specific effect on the 95 000 protein detected. Although a reduction in phosphorylation was apparent with increasing concentration of inhibitor, phosphorylation of other proteins was also reduced, suggesting a non-specific effect of the inhibitor. However, experiments with orthovanadate, reported to be a phosphotyrosine phosphatase inhibitor, did show an effect on the 95 000 phosphoprotein. The presence of orthovanadate continuously throughout incubation in vitro and during protein kinase assay resulted in reduced phosphorylation of the 95 000 protein, suggesting that the vanadate acted to stabilize the phosphorylation state of the protein (by inhibiting phosphatase activity), thus making the addition of radiolabelled phosphate groups to the protein more difficult. No major effect of vanadate on other phosphoproteins was detected. The sensitivity of the phosphoprotein to the presence of orthovanadate suggests that it may be phosphorylated on tyrosine residues.

Stronger evidence was provided by immunoblotting experiments using an anti-phosphotyrosine antibody, PY-20. Five proteins reacting specifically with this antibody were identified, with approximate Mr, of 116 000, 105 000, 95 000, 86 000 and 76 000, with possibly a sixth at 54 000. When sperm samples were treated with ionophore A23187 to induce the acrosome reaction, the 95 000 protein was the only one of the six consistently diminished in staining intensity. Leyton and Saling (1989) have localized their 95 000 phosphotyrosine-containing protein to the acrosomal cap region and propose that it is present on the plasma membrane in this region, a position allowing the protein to interact with ZP3 molecules on the zona pellucida of the egg (Saling, 1991). Much of this protein would therefore be lost from the cells as a consequence of the acrosome reaction.

Our observation that the 95 000 phosphotyrosine-containing protein was markedly decreased in acrosome-reacted populations suggests that this protein may be the same as that described by Leyton and Saling (1989). Further support for this suggestion is provided by our observation that the 95 000 phosphotyrosine-containing protein could be detected in capacitated cells incubated either in +G or −G medium, i.e. detection of the protein was not dependent upon motility. Leyton and Saling (1989) came to a similar conclusion.

Saling and her colleagues have formulated two testable hypotheses, namely that either the 95 000 protein is itself a protein tyrosine kinase that can act as a ZP3 receptor or the 95 000 protein is a ZP3 receptor and is phosphorylated by a separate protein tyrosine kinase (Saling, 1991). At present, our data do not allow us to favour one or other of these hypotheses, since our experiments have been directed towards cAMP-dependent protein kinase activity. Preliminary experiments indicated that while assay in the presence of a protein tyrosine kinase inhibitor appeared to inhibit phosphorylation of the 95 000 protein, it also affected phosphorylation of the other detectable proteins. Perhaps assays formulated more specifically to detect protein tyrosine kinase activity could help resolve this question.

In addition to the 95 000 phosphotyrosine-containing protein, Leyton and Saling (1989) identified two others, of Mr 75 000 and 52 000, that were detected only in capacitated spermatozoa. Although we have detected proteins of Mr about 76 000 and 54 000, i.e. possibly the same as those of Leyton and Saling, we have no evidence that their phosphorylation is capacitation-dependent. Both proteins were detected in samples taken at 20 min and 120 min, whereas the 86 000, 95 000 and 105 000 proteins were absent in the uncapacitated 20 min sample. The \(^{32}P\) incorporation studies that did reveal changes in the 95 000 phosphoprotein did not provide evidence for changes in the region of 54 000. The 76 000 protein is more difficult to comment on, since its presence or absence could be obscured by the large area covered by the phosphorylated BSA.
Since ionophore treatment did not result in loss of either of these two phosphotyrosine-containing proteins or the proteins of M1, 116 000, 105 000 and 86 000, it would appear that none is located in the acrosomal cap region.

Little is known about phosphotyrosine-containing proteins in spermatozoa of other species, although Berruti and Martegani (1989) identified three in boar spermatozoa and also demonstrated tyrosine kinase activity. These proteins, of M, 43 000, 40 000 and 36 000, were not lost as a consequence of ionophore-induced acrosome reactions and hence would not appear to be located in the region of the acrosome. None of the phosphotyrosine-containing proteins identified in mouse spermatozoa by the present study and that of Leyton and Saling (1989) correspond to these boar proteins, suggesting species differences. However, we found it difficult to establish optimal conditions for detecting these proteins which may indicate a high degree of lability. Certainly there is some evidence that a 95 000 protein detected in hamster spermatozoa (Moore et al., 1987) may be similar to that reported by Leyton and Saling (H. D. M. Moore, personal communication).

In this study, many phosphoproteins have been detected by the radiolabelling of permeabilized mouse sperm cells with [32P]ATP and we have related overall changes in phosphorylation to the functional state of the spermatozoa. Our evidence suggests that capacitation per se may not necessarily require high levels of protein phosphorylation, since spermatozoa can be essentially fully capacitated yet have a low level of endogenous protein phosphorylation. Although mouse spermatozoa incubated in −G medium are known to be capacitated (Fraser and Quinn, 1981), they could incorporate significantly more 32P into phosphoproteins than their capacitated counterparts incubated in +G medium; this increased incorporation suggests that the proteins were in a relatively unphosphorylated state at the time of assay. However, the introduction of glucose to such −G incubated spermatozoa prior to assay resulted in a marked reduction in 32P incorporation, indicating a high state of phosphorylation at the time of assay. Since the addition of glucose leads to rapid expression of vigorous hyperactivated motility plus the ability to undergo the acrosome reaction, the majority of these phosphorylation events would appear to be associated with the onset of vigorous motility. While most proteins could be detected in both uncapacitated and capacitated cells, one of M, 95 000 was observed routinely only in capacitated cells. Immunodetection experiments indicate that this is a phosphotyrosine-containing protein, present in the acrosomal cap region, since it is lost as a result of the acrosome reaction. The striking similarities between this phosphoprotein and the one described by Leyton and Saling (1989) suggests that it may be involved in interactions with the zona pellucida which play a role in triggering acrosomal exocytosis.

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