Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa

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Summary

Fertilization of the mammalian oocyte depends on the ability of spermatozoa to undergo a process known as capacitation as they ascend the female reproductive tract. A fundamental feature of this process is a marked increase in tyrosine phosphorylation by an unusual protein kinase A (PKA)-mediated pathway. To date, the identity of the intermediate PKA-activated tyrosine kinase driving capacitation is still unresolved. In this study, we have identified SRC as a candidate intermediate kinase centrally involved in the control of sperm capacitation. Consistent with this conclusion, the SRC kinase inhibitor SU6656 was shown to suppress both tyrosine phosphorylation and hyperactivation in murine spermatozoa. Moreover, SRC co-immunoprecipitated with PKA and this interaction was found to lead to an activating phosphorylation of SRC at position Y416. We have also used difference-in-2D-gel-electrophoresis (DIGE) in combination with mass spectrometry to identify a number of SRC substrates that become phosphorylated during capacitation including enolase, HSP90 and tubulin. Our data further suggest that the activation of SRC during capacitation is negatively controlled by C-terminal SRC kinase. The latter was localized to the acrosome and flagellum of murine spermatozoa by immunocytochemistry, whereas capacitation was associated with an inactivating serine phosphosphorylation of this inhibitory kinase.

Key words: Sperm maturation, DIGE, Tyrosine phosphorylation, Capacitation, Hyperactivation, SRC

Introduction

The process of 'capacitation' was first described by Chang (Chang, 1951) who demonstrated that spermatozoa must spend a finite period of time maturing in the female reproductive tract before they become capable of fertilizing the oocyte. The remodelling of ejaculated spermatozoa to produce a functional gamete is routinely recognized in assisted conception therapy where human spermatozoa are incubated in defined media for periods of 3-24 hours to promote capacitation before oocytes are introduced for fertilization. Intriguingly, the acquisition of functional competence during capacitation occurs in the complete (Engel et al., 1973; Hernandez-Perez et al., 1983) or virtual (Gur and Breitbart, 2006) absence of gene transcription and translation. Thus, whereas incubation of human, mouse, rat and bovine spermatozoa with radiolabeled amino acids has recently revealed evidence of limited protein synthesis in these cells, the general consensus is that the acquisition of functionality during capacitation is largely dependent on post-translational modifications to pre-existing proteins (Blaquier et al., 1988a; Blaquier et al., 1988b; Ross et al., 1990).

Analysis of the post-translational modifications that occur during capacitation has revealed a dramatic increase in the tyrosine phosphorylation status of multiple proteins coincident with the attainment of a capacitated state (Visconti et al., 1995a; Baker et al., 2004). Most of these tyrosine-phosphorylated proteins are localized to the sperm tail and are believed to be instrumental in the induction of hyperactivation – a specific form of movement that allows spermatozoa to generate the propulsive forces necessary to penetrate the zona pellucida, a dense glycoprotein shell that surrounds the oocyte. If hyperactivation is prevented, fertilization cannot occur (Amieux and McKnight, 2002).

The control of sperm protein tyrosine phosphorylation involves an unusual signal transduction cascade mediated by protein kinase A (PKA) and driven by increases in intracellular cAMP during capacitation (White and Aitken, 1989; Aitken et al., 1998a). Thus, treatments that increase or decrease the intracellular generation of cAMP have corresponding impacts on the tyrosine phosphorylation status of these cells (Visconti et al., 1995b; Aitken et al., 1995; Rivlin et al., 2003; Baker et al., 2004). Moreover, addition of the cell permeable agent, dibutryl cAMP (dbcAMP), hastens the onset and degree of tyrosine phosphorylation in spermatozoa (Visconti et al., 1995b; Aitken et al., 1995; Thundathil et al., 2002; Baker et al., 2004).

A number of factors are known to impact upon this cAMP-dependent tyrosine phosphorylation cascade. For example, cellular redox status (Aitken et al., 1995), cytoplasmic Ca^{2+} levels (Baker et al., 2004) and extracellular bicarbonate (Visconti et al., 1995b), have all been shown to have dramatic effects on this pathway by a variety of indirect mechanisms, including increased cAMP availability, suppression of tyrosine

phosphatase activity and the buffering of intracellular pH (Aitken et al., 1998b; de Lamirande et al., 1998). While the importance of such modulating factors is clear, the major unresolved problem in this field is the identity of the key intermediate kinase that, once activated by PKA, induces the dramatic increase in tyrosine phosphorylation that characterizes the capacitated state. The clear inhibitory effect of Ca^{2+} on this signal transduction pathway and the fact that two SRC-family tyrosine kinase inhibitors, herbimycin A and erbstatin, downregulate protein tyrosine phosphorylation in human spermatozoa, have lead to the suggestion that the SRC-family tyrosine kinase YES1 is this intermediate kinase (Leclerc and Goupil, 2002). However, recent data, indicating that the negative impact of Ca^{2+} is an indirect consequence of ATP availability rather the inhibition of kinase activity, works against this hypothesis (Baker et al., 2004). Furthermore localization of YES1 to the sperm head, and not the tail where most of the tyrosine phosphorylation events associated with sperm capacitation occur (Sakkas et al., 2003; Urner et al., 2001), is not consistent with a central role for this particular kinase in capacitation, at least as far as the induction of hyperactivated movement is concerned.

Another pathway potentially involved in sperm tyrosine phosphorylation is the extracellular-signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPK) (Luconi et al., 1998a; Luconi et al., 1998b). These enzymes (RAF, MEK and ERK1/2) have all been found in spermatozoa and have been localized to the sperm head. Moreover, addition of PD98059, an inhibitor of ERK1/2, leads to a downregulation of both protein tyrosine phosphorylation and the A23187-induced acrosome reaction, an exocytotoic event that depends on a capacitated state. In addition, five other inhibitors of the ERK1/2 pathway including CGP8793, FTI-277, sulindac sulphide, ZM336372 and U126, all inhibited the lysophosphatidylcholine-induced acrosome reaction (de Lamirande and Gagnon, 2002). Since ERK1/2 are themselves serine/threonine kinases, they cannot be directly responsible for protein tyrosine phosphorylation. Moreover, because this enzyme and other adaptor proteins including RAS (Naz et al., 1992) have been immunolocalized to the sperm head, it can be speculated that the ERK1/2 kinases are important as components of the signal transduction pathways associated with the acrosome reaction, rather than hyperactivated movement, as recently suggested by Liguori et al. (Liguori et al., 2005).

An alternative candidate for the intermediary kinase that regulates sperm capacitation is SRC. Preliminary studies have demonstrated that inhibitors of this kinase suppress the induction of tyrosine phosphorylation in human spermatozoa activated via by a receptor mediated process involving PECAM-1 (Nixon et al., 2005). In this study, we set out to determine whether SRC is the key PKA-regulated intermediary kinase controlling the tyrosine phosphorylation events associated with the expression of hyperactivated movement by capacitating murine spermatozoa. If this were the case, it would have significant implications for our fundamental understanding of the signal transduction mechanisms regulating the hyperactivation-associated with normal sperm capacitation, as well as the pathophysiology of male infertility where this process is commonly impaired (Buffone et al., 2005).

Results

The presence of SRC in mouse sperm and its interaction with PKA

An in silico analysis was performed to determine which kinases of the approximately 300 that are known could potentially be involved in the PKA-mediated increase in tyrosine phosphorylation observed during sperm capacitation. From this bioinformatics search, SRC emerged as a prime candidate because it can be activated by PKA on Ser17 (Patschinsky et al., 1986). Western blot analysis with anti-SRC antibodies generated immediate support for this concept, because extracts of both caput and caudal epididymal spermatozoa possessed a crossreactive band of 60 kDa, exactly the same molecular mass as SRC (Fig. 1A).

Since the anti-SRC antibody could not be used for immunocytochemistry to localize this kinase within spermatozoa, we performed a subcellular fractionation of these cells using isolated highly pure (>95%) preparations of sperm heads (Fig. 1B) and tails (Fig. 1C). Western-blot analysis revealed that SRC was mainly present in the tail preparations (Fig. 1D), the major site of tyrosine phosphorylation in capacitated cells (Sakkas et al., 2003; Asquith et al., 2004). By contrast, very little signal was found in the sperm-head fraction (Fig. 1D). The lack of signal in the sperm head did not appear to be a reflection of a loss of sperm-head plasma membrane during sonication, because very similar results were found when we used antibody against phosphorylated SRC in immunocytochemical studies involving intact cells (see Fig. 3).

The tyrosine kinase of interest in spermatozoa is stimulated by a cAMP-dependent kinase (PKA). Therefore, we sought to determine whether the catalytic domain of PKA (PKA_c) and SRC interacted. To achieve this, anti-SRC antibody was used to immunoprecipitate the kinase and other associated SRC-binding proteins in the head and tail fractions described above. Following elution and separation on an SDS-PAGE, the precipitated proteins were probed with an anti-PKA_c antibody. This analysis clearly revealed a band at 40 kDa in tail preparations of mouse spermatozoa, representing the catalytic subunit of PKA, and which was virtually absent in the head (Fig. 1E). In a follow-up experiment, SRC antibodies were used to immunoprecipitate proteins from populations of capacitated (incubated with 1 mM PTX and 1 mM dbcAMP) and non-capacitated (freshly isolated from the cauda epididymis) spermatozoa; immunoprecipitates were then probed with anti-PKA_c antibodies. Fig. 1F demonstrates that SRC antibodies could immunoprecipitate PKA_c from sperm lysates, but only following capacitation. These results suggested that, within the flagella of capacitating murine spermtozoa, PKA_c and SRC become tightly associated in an ideal position to mediate the tyrosine phosphorylation events associated with hyperactivated movement.

Increase in SRC activity during capacitation

Our initial attempts to measure directly, either by immunoprecipitation or with specific peptides, the level of SRC kinase activity in both capacitated and non-capacitated samples were unsuccessful, probably because of the extremely low amounts of this kinase in spermatozoa. An alternative approach commonly used to overcome this problem is to probe with a antibody that specifically recognizes tyrosine phosphorylated at position 416 (Y416) of SRC (anti-pY416)

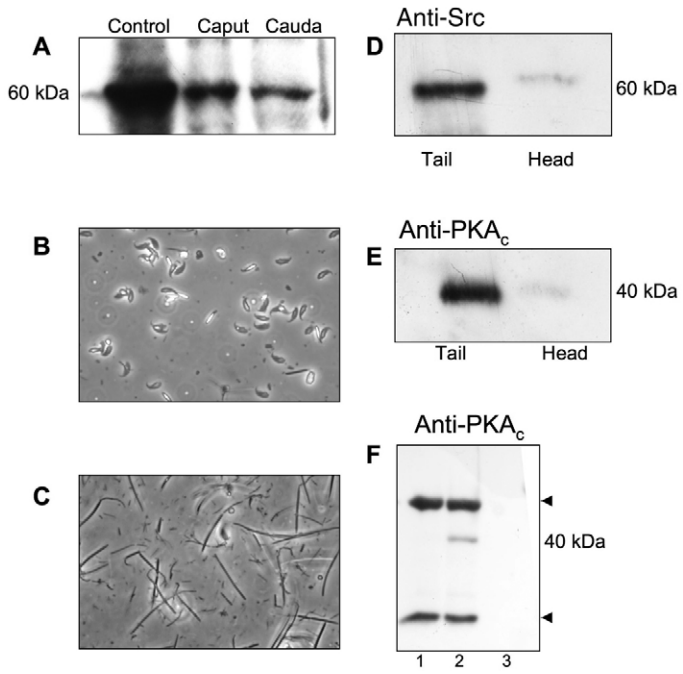

Fig. 1. Identification of SRC and its binding partner PKA_c in murine spermatozoa. (A) Samples were taken from either the caput or caudal regions of the epididymis, lysed and run in a 10% SDS-PAGE. Western blot analysis was performed using the anti-SRC monoclonal antibody. A positive control of SRC (A431 cell lysates) was included to ensure the antibody cross-reacted with a protein of the appropriate size. Visualization was performed with standard ECL chemiluminescence. (B,C) Back-flushed murine spermatozoa were sonicated and Percoll-purified to obtain populations consisting of pure (>95%) (B) sperm heads or (C) sperm tails. (D) Approximately 2 μg of these fractions were then lysed and subjected to 10% SDS PAGE followed by western-blot analysis with anti-SRC antibody. (E) To demonstrate an association between SRC and PKA_c the above fractions were incubated with beads coated with anti-SRC antibodies and the precipitated proteins probed with an antibody against PKA_c. (F) The importance of sperm capacitation in this association between SRC and PKA_c was also confirmed in experiments involving the inmmunoprecitation of SRC-containing complexes from capacitated (incubated with 1 mM PTX and 1 mM dbcAMP for 45 minutes) and non-capacitated cells (freshly isolated from the cauda epididymis without incubation) followed by probing of these immunoprecipitates with anti-PKA_c antibodies. The western blot shows non-capacitated spermatozoa (lane 1) capacitated spermatozoa (lane 2) and a control incubation (lane 3), in which beads were incubated with sperm lysates in the absence of antibody. Arrows indicate the location of the IgG heavy chains (top) and IgG light chains (bottom).

(Bagrodia et al., 1993; Cartwright et al., 1989; Katagiri et al., 1989; Kralisz and Cierniewski, 2000). When mouse SRC becomes activated autophosphorylation of Y416 occurs (Boerner et al., 1996), such that phosphorylation of this residue correlates well with enzyme activity (Amini et al., 1986; Bagrodia et al., 1993; Cartwright et al., 1989; Katagiri et al., 1989; Kralisz and Cierniewski, 2000). To investigate whether SRC activity increased as cells transited from a non-capacitated to a capacitated state, 2D western blot analyses were performed. Spermatozoa undergoing capacitation and exhibiting >95% hyperactivation were compared with non-capacitated control cells exhibiting <5% hyperactivation. Probing the membrane with anti-pY416 resulted in a main spot with the same isoelectric point (8.0) and molecular mass (60 kDa) as SRC in capacitated spermatozoa (Fig. 2A). In non-capacitated cells, staining of this spot was clearly less intense (Fig. 2B). To ensure that equal amounts of the kinase were present in the sample, membranes were stripped and reprobed with anti-SRC antibody (Fig. 2C,D). This demonstrated multiple forms of the enzyme to be present in spermatozoa, visualized as a charge train of spots running along the gel (Fig. 2C,D).

Immunocytochemistry of tyrosine phosphorylated at position 416

To further confirm and validate the western-blot data, non-capacitated (Fig. 3C,D) and capacitated (Fig. 3E,F) spermatozoa were subjected to immunocytochemistry using the specific antibody against tyrosine phosphorylated at position 416 (anti-pY416) (Fig. 3) As shown, the active form of SRC was detected only in the tail of capacitated spermatozoa (Fig. 3E) and not in non-capacitated cells (Fig. 3C). The secondary antibody-only control generated very little background fluorescence (Fig. 3A). These data further support the flagellar localization of this enzyme, as suggested by the western blot analyses of isolated sperm heads and tails (Fig. 1B-E). Moreover, these data are perfectly in keeping with the concept that SRC mediates the generation of phosphotyrosine in the tail region of capacitated murine spermatozoa (Asquith et al., 2004). In the following section, we investigate whether this SRC-mediated increase in tyrosine phosphorylation in the sperm tail is associated with the induction of hyperactivated movement.

Inhibition of tyrosine phosphorylation with SRC inhibitors

Addition of the broad-spectrum SRC-family inhibitor lavendustin A (Fig. 4A) resulted in a reduction of several proteins phosphorylated at their tyrosine residues following stimulation of the spermatozoa with dbcAMP. Although such broad-spectrum inhibitors are known to compromise the function of SRC, they also appear to inhibit a variety of other enzymes (Sepp-Lorenzino et al., 1995). To confirm the involvement of SRC, we used the more specific SRC-family inhibitor SU6656 (Blake et al., 2000). Addition of SU6656 selectively inhibited the tyrosine phosphorylation of several proteins in spermatozoa undergoing cAMP-driven capacitation (Fig. 4B).

Identification of post-translational modifications using difference-in-2D-gel-electrophoresis

To ascertain which proteins become functionally modified during capacitation, we performed a difference-in-2D-gel-electrophoresis (DIGE) analysis on samples of spermatozoa obtained in a capacitated or non-capacitated state. To achieve this, six 2-D gels were created, each containing three populations of proteins (internal standard, capacitated and non-capacitated) labeled with spectrally-resolvable Cy dyes, as set out in Table 1. Each sample comprised pooled spermatozoa from three mice; 36 mice were employed in total. These populations of spermatozoa were either lysed immediately after collection (non-capacitated population) or placed in BWW medium containing dbcAMP and PTX for 90 minutes

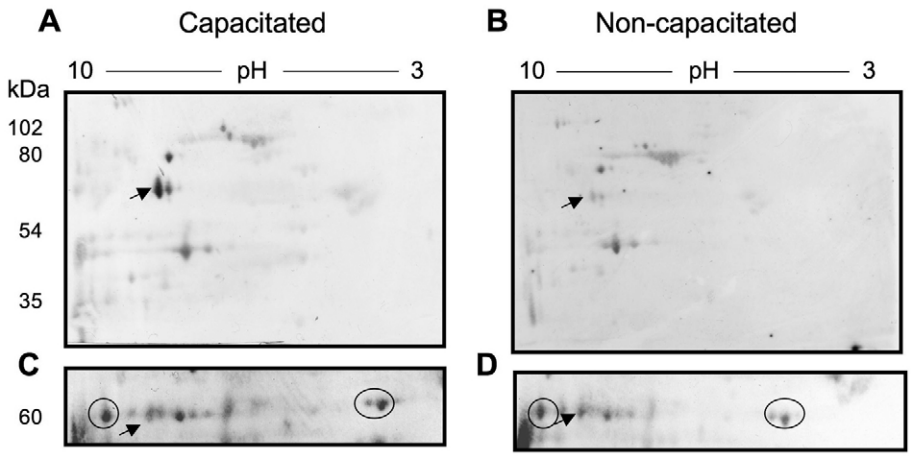

Fig. 2. Identification of the phosphorylated forms of SRC. (A-D) Spermatozoa were pre-incubated for 5 minutes with either 10 μM H89 (B,D, non-capacitated cells) or the vehicle (A,C; capacitated cells) before the addition of pharmacological agents to drive sperm capacitation (1 mM dbcAMP and 1 mM PTX). After a further 40-minute incubation, the cells were assessed for hyperactivated motility. Populations containing at least 95% hyperactivated motility (A,C) or less than 5% hyperactivated motility (B,D) were lysed and subjected to 2D PAGE as described in Materials and Methods. The proteins were then transferred to nitrocellulose membranes and probed with anti-pY416 antibody. Arrows in A and B indicate the position of SRC. The membranes were then stripped and re-probed with anti-SRC as a positive loading control (C,D). Arrows in C and D indicate the position of SRC and its isoform phosphorylated at Y416. The encircled spots were also present when the membrane was probed with secondary antibody alone, indicating that these signals were the result of non-specific interactions.

prior to lysis (capacitated population). Approximately 50 μg of solubilized protein of each pooled sample were labeled with either Cy3 or Cy5; a dye-exchange was performed so that three out of the six non-capacitated samples were labeled with Cy3 while the other three non-capacitated samples were labeled with Cy5 (Table 1). Similarly, three of the capacitated samples were labeled with Cy3 and the remaining three with Cy5. This way, we ensured that no bias was introduced into the analysis due to the nature of the protein label being used. A mixed internal standard was then prepared, consisting of 25 μg of protein from all 12 samples, pooled into one tube and labeled with Cy2 (Table 1).

Following 2D-gel electrophoresis, the six gels were imaged using mutually exclusive excitation and emission wavelengths on the Ettan Typhoon imager system (GE Healthcare, Chalfont St Giles, UK). The images were then analysed with DeCyder

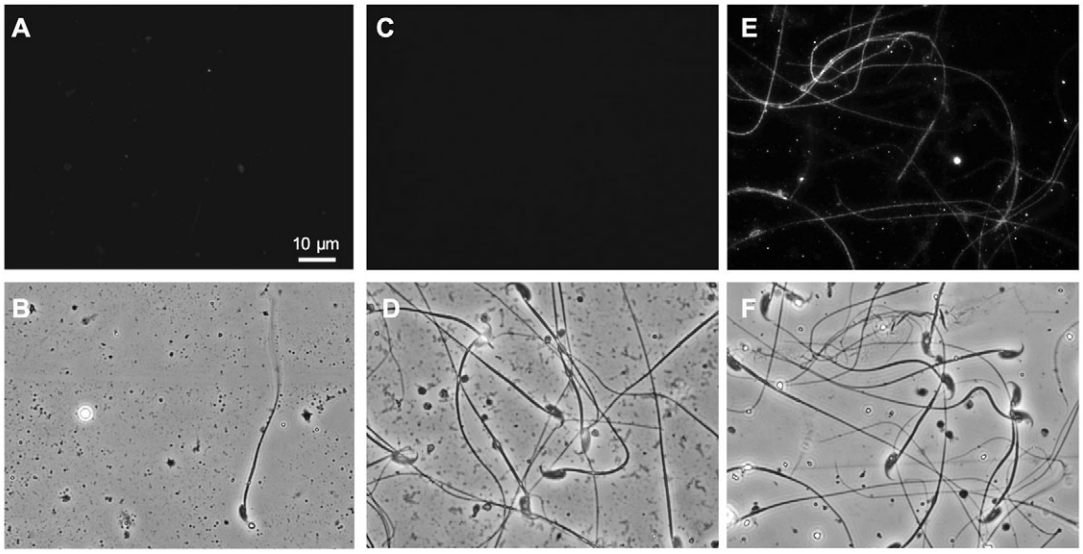

Fig. 3. Localization of pY416 in non-capacitating and capacitating spermatozoon. (A-F) Spermatozoa were pre-incubated for 5 minutes with either 10 μM H89 (C,D) or the vehicle (A,B,E,F) before addition of reagents (1 mM dbcAMP and 1 mM PTX) to drive sperm capacitation. After a further 40-minute incubation, the cells were assessed for hyperactivated motility as described in Materials and Methods. Mouse spermatozoa from the cauda epididymides were fixed, washed and subjected to immunocytochemistry using anti-pY416 antibody in sperm cell populations having less than 5% total hyperactivation (C,D) or at least 95% hyperactivation (E,F). The secondary antibody only controls are shown in panels A and B. The induction of a hyperactivated state is clearly associated with phosphorylated Tyr416 (Y416-*P)* on the sperm tail (E,F). In non-capacitated cells, in which PKA had been blocked with H89, cAMP failed to elicit this response. Corresponding phase-contrast (lower panels) and FITC images (upper panels) are displayed.

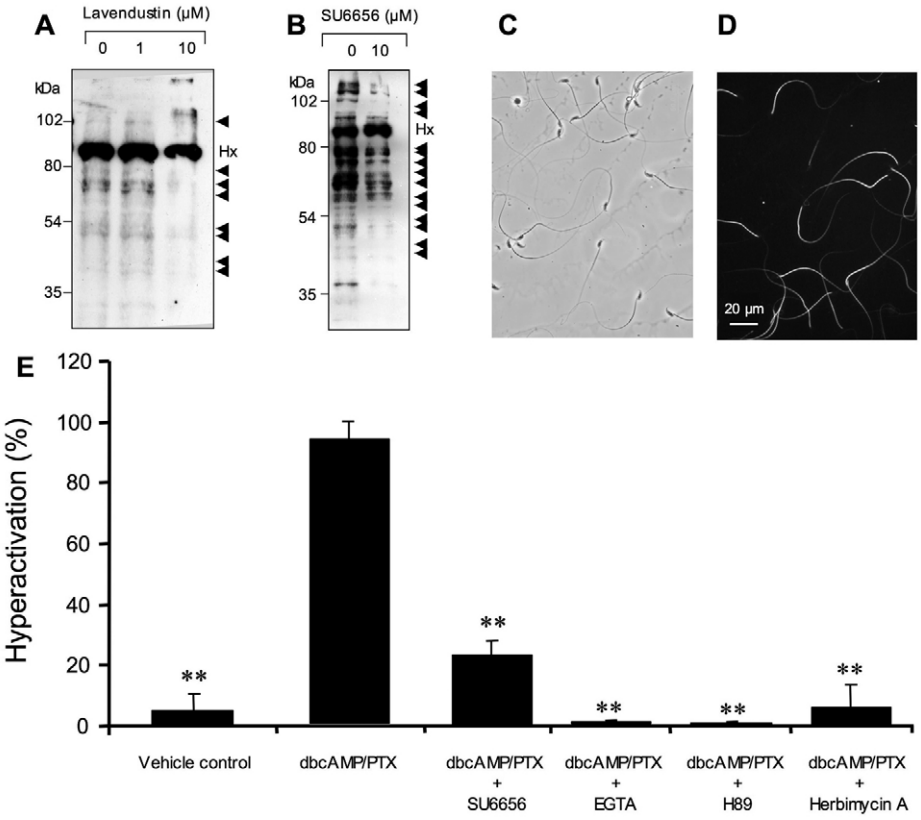

Fig. 4. Tyrosine phosphorylation and sperm hyperactivation. (A,B) Treatment of murine spermatozoa with inhibitors of SRC such as (A) lavendustin A and (B) SU6656 inhibited phosphotyrosine expression. For this analysis, spermatozoa were pre-incubated with the inhibitors for 5 minutes before the addition of 1 mM dbcAMP and 1 mM PTX. After a 40-minute incubation the spermatozoa were analyzed for hyperactivated motility to confirm induction of capacitation in the vehicle-only controls. Approximately 2 μg of lystate was run in a 10% SDS-PAGE and western-blot analysis was performed with anti-phosphotyrosine antibodies as described. Arrowheads indicate the position of protein bands that were reduced in the presence of inhibitor; Hx indicates the location of hexokinase, a constitutively phosphorylated protein that served as a loading control (Nixon et al., 2006). When caudal epididymal murine spermatozoa were treated with PTX (1 mM) and dbcAMP (1 mM) tyrosine phosphorylation was observed along the length of the sperm tail in >95% of cells examined. (C,D) Phase-contrast and phosphotyrosine images of mouse spermatozoa, respectively. (E) Suppression of tyrosine phosphorylation had a dramatic effect on hyperactivated movement. Spermatozoa were pre-incubated for 5 minutes with the inhibitors indicated before the addition of 1 mM dbcAMP and 1 mM PTX to capacitate the cells. Following a 40-minute incubation, a 20 μl aliquot was taken and the spermatozoa were assessed for hyperactivated motility (presented as a percentage of the motile sperm population) as described in Materials and Methods.

5.0 software. Approximately 60 proteins underwent a significant change ($P<0.05$) during the process of capacitation. To clarify the nature of the changes observed, western blot analysis was performed on duplicate gels to identify those sperm proteins that became tyrosine phosphorylated following capacitation. Proteins that could clearly be delineated in both the DIGE analysis as having changed during capacitation, and in the phosphorylated-protein analysis as being tyrosine phosphorylated during capacitation, were identified by MALDI-TOF mass spectrometry and are presented in Table 2. From the perspective of this present study, it is significant to note that several of the proteins identified in Table 2 are known substrates for SRC-mediated phosphorylation, including enolase (Kralisz and Cierniewski, 2000), HSP90 (Hutchison et al., 1992) and tubulin (Matten et al., 1990).

Functional studies

To further understand the functional consequences of SRC activation during capacitation, we again used the inhibitor SU6656 as well as a number of other known capacitation inhibitors, and examined their effect on hyperactivation (Fig. 4). Sperm populations treated with 1 mM dbcAMP and 1 mM PTX together demonstrated high levels (92±6%) of hyperactivated motility, which is in marked contrast with the vehicle controls (Fig. 4E). This high level of hyperactivation reflects the fact that >95% of spermatozoa incubated under these conditions exhibited a strong tyrosine phosphorylation signal along the entire length of the flagellum (Fig. 4C,D). When SU6656 was added to the sperm suspensions and allowed to incubate for 5 minutes before the addition of dbcAMP/PTX, a dramatic reduction in the incidence of hyperactivating cells could be seen (Fig. 4E; 28±6%; $P>0.001$). Since SU6656 is a competitive inhibitor of ATP binding to SRC kinase, it was to be expected that some spermatozoa exposed to this compound are still hyperactivated due to residual kinase activity. We also demonstrated that treatment of spermatozoa with herbimycin A (a less specific SRC-family inhibitor), H89 (a PKA inhibitor) and EGTA (a Ca^{2+} chelator) all suppressed hyperactivation (Fig. 4E). These results are fully consistent with other studies, indicating that the induction of

Table 1. Labelling of murine spermatozoa for the DIGE analysis

Gel number	Pooled internal standard	Capacitated	Non-capacitated
1	Cy2-labelled 1-36	Cy5-labelled 1-3	Cy3-labelled 4-6
2	Cy2-labelled 1-36	Cy5-labelled 7-9	Cy3-labelled 10-123
3	Cy2-labelled 1-36	Cy5-labelled 13-15	Cy3-labelled 16-18
4	Cy2-labelled 1-36	Cy3-labelled 19-21	Cy5-labelled 22-24
5	Cy2-labelled 1-36	Cy3-labelled 25-27	Cy5-labelled 28-30
6	Cy2-labelled 1-36	Cy3-labelled 31-33	Cy5-labelled 34-36

Numbers indicate identities of mice (1-36) contributing to the protein pool

Table 2. Identification of proteins undergoing a functional change using DIGE

Class of protein			
Cytoskeletal	Chaperone	Signal transduction	Unknown
Outer dense fibre from sperm tail 2	HSP-60	Glutathione transferase mu3	Transcript increase in spermatogenesis
α-tubulin	HSP90α	α-enolase	
β-tubulin	HSP70	LDH C3	
AKAP-3	GRP78		
AKAP-4	Calreticulin		
	Endoplasmin		

Proteins seen to undergo a change in intensity during capacitation were matched with replicate gels showing tyrosine phosphorylation status. Proteins that changed in association with tyrosine phosphorylation were analysed by MALDI-TOF.

hyperactivation depends on the concerted action of PKA, Ca^{2+} and the strategic expression of phosphotyrosine residues (Bain et al., 2003; Ho and Suarez, 2003; Nolan et al., 2004; Buffone et al., 2005).

CSK and serine phosphorylation during capacitation

In light of the above, the ability of PKA_c to interact with SRC and stimulate its kinase activity is clearly one of the regulatory processes modulating the tyrosine phosphorylation of mammalian spermatozoa during capacitation. A second enzyme potentially involved in the regulation of SRC is C-terminal SRC kinase (CSK). CSK is known to phosphorylate SRC at Tyr527 and, consequently, inhibit its activity (Bain et al., 2003). To further complicate this entire regulatory process, PKA is also known to interact with and phosphorylate CSK, inhibiting it and thereby promoting activation of SRC (Sun et al., 1997). To determine whether CSK plays a role in capacitation, we probed for the presence of this enzyme using anti-CSK antibodies. Immunocytochemical studies localized CSK predominantly to the sperm tail, in exactly the same position as PKA_c, SRC and tyrosine phosphorylation are located in hyperactivating capacitated murine spermatozoa (Fig. 5). In addition, immunocytochemical analysis revealed a distinct signal in the acrosomal region of the sperm head (Fig. 5), where CSK could be involved in modulating the effects of cAMP on acrosomal exocytosis (Breitbart, 2003; Cohen et al., 2004). Western blot analysis following 2D PAGE confirmed the presence of CSK as a main spot, with an isoelectric point of 7.5 and the molecular mass of approximately 40 kDa, as predicted on the basis of existing data. Probing with the anti-CSK antibody also revealed another less intense charge isomer of the enzyme, presumably reflecting post-translational modifications such as phosphorylation (Fig. 6C,D). Stripping and re-probing the membrane with antibody against phosphorylated serine demonstrated that CSK went from a non-phosphorylated state (Fig. 6A) to a phosphorylated state (Fig. 6B) during capacitation. Since H89 inhibited the serine-phosphorylation status of CSK (data not shown), it appears that this is a consequence of PKA interaction.

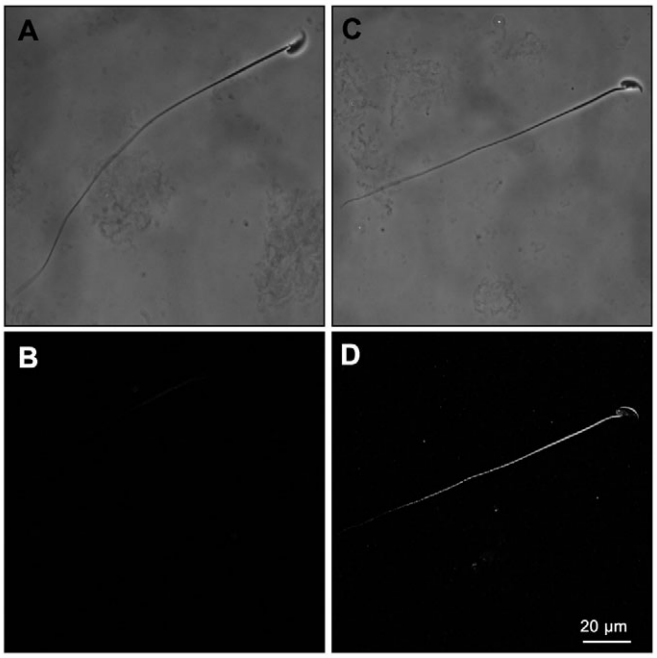

Fig. 5. Localization of CSK in spermatozoa. (A-C) Murine spermatozoa from the cauda epididymis were fixed, washed and subjected to immunocytochemistry with anti-CSK antibody as described in Materials and Methods. The no-primary antibody controls (A,B) and the anti-CSK images (C,D) were visualized by fluorescence (lower panels) or phase-contrast (upper panels) microscopy.

Discussion

Upregulation of tyrosine phosphorylation during capacitation was initially demonstrated several years ago (Visconti et al., 1995b) and later shown to involve a redox-regulated, cAMP-dependent protein tyrosine kinase (Aitken et al., 1995; Aitken

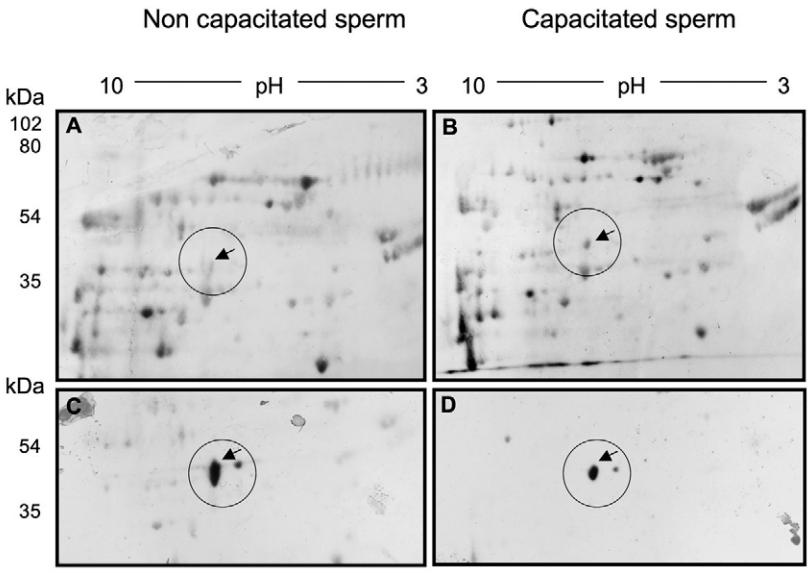

Fig. 6. Phosphorylation of CSK in capacitating murine spermatozoa. (A-D) Spermatozoa were pre-incubated with either 10 μM H89 (A,C) or the vehicle (B,D) for 5 minutes before the addition of 1 mM dbcAMP and 1 mM PTX to drive capacitation. After a further 40-minute incubation, cells were assessed for hyperactivated motility to confirm the attainment of a capacitated state in the vehicle controls. Capacitated and non-capacitated mouse spermatozoa were lysed and subjected to 2D PAGE as described in Materials and Methods. Proteins were then transferred to nitrocellulose membranes and, in the first instance, probed with anti-CSK antibody (C,D). Membranes were stripped and re-probed with the anti-phosphoserine antibody (A,B). Circles and arrows indicate the same position on all four images and indicate the serine phosphorylation of CSK in capacitated spermatozoa (B).

et al., 1998a; Lewis and Aitken, 2001). Importantly, those proteins that become tyrosine phosphorylated during capacitation are clearly present in the flagellum and are involved in the induction of hyperactivated motility (Si and Okuno, 1999). Hence, it is logical to assume that the responsible PKA-activated tyrosine kinase is also localized to the sperm tail during the tyrosine phosphorylation events associated with hyperactivation. Although some researches have focused on YES1 (Leclerc and Goupil, 2002), MAPK (Luconi et al., 1998a) and PI 3-kinase (Luconi et al., 2004) as potential redox-regulated enzymes involved in this highly important maturational event, they seem to be unlikely candidates given that these kinases are located in the acrosomal domain of mammalian spermatozoa. Further, the active YES1 is insensitive to herbimycin A (Fukazawa et al., 1991; Reinehr et al., 2004), a compound that has been found to clearly reduce tyrosine phosphorylation during capacitation of both human and mouse spermatozoa (O'Flaherty et al., 2004) (our unpublished observations).

The hypothesis that SRC is involved in capacitation was initially founded on a bioinformatics search of kinases that are known to be potentially regulated by PKA. The presence of this kinase in murine spermatozoa was subsequently confirmed by western blot analysis (Fig. 1A), whereas the association with PKA_c was demonstrated by experiments confirming the presence of this kinase following immunoprecipitation of sperm lysates with anti-SRC antibodies. Further evidence for the involvement of SRC in mediating tyrosine phosphorylation during sperm capacitation came from the DIGE analysis of proteomic changes associated with this process. In the course of this screen we identified a number of phosphorylated proteins, several of which are known substrates for SRC including enolase, HSP90 and tubulin. In keeping with the global increase in tyrosine phosphorylation observed during

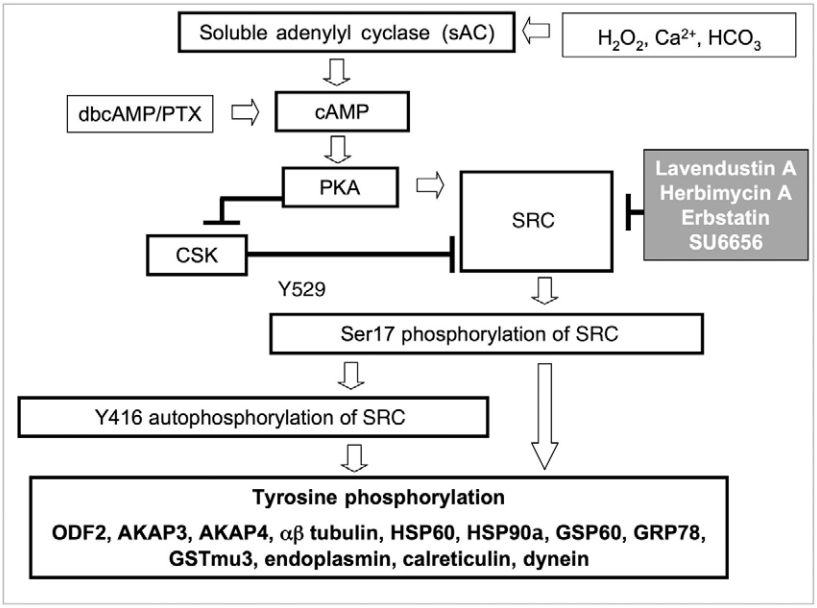

Fig. 7. A biochemical model to explain the tyrosine phosphorylation events leading to sperm hyperactivation. During capacitation, a variety of factors including Ca^{2+}, HCO_3 or H_2O_2 stimulate sAC. This is turn leads to production of cAMP and downstream activation of PKA. PKA is central to the induction of hyperactivation in a two step process: (1) it must phosphorylate SRC and activate this kinase. (2) PKA must also phosphorylate CSK, thereby inhibiting this enzyme and promoting further PKA activity. The activation of SRC by PKA leads to autophosphorylation and consequent tyrosine phosphorylation of a number of sperm targets, including enolase, HSP90 and tubulin.

capacitation, the substrate site specificity of this enzyme is quite promiscuous, even to the point of being able to phosphorylate certain D-amino acids (Lee et al., 1995). Our evidence for the involvement of SRC as a tyrosine kinase during capacitation, is also supported by reports demonstrating that SRC activity (demonstrated by phosphorylation at Y416) becomes upregulated by hydrogen peroxide (Suzaki et al., 2002), a known activator of sperm capacitation (Aitken et al., 1998a; Rivlin et al., 2004).

One vital characteristic of the candidate tyrosine kinase involved in sperm hyperactivation, is the ability to become stimulated upon addition of cAMP. This stimulation does not occur directly, but rather indirectly through PKA, as clearly demonstrated by the fact that the mice whose sperm-specific PKAα is knocked out are infertile, owing to the complete absence of normal sperm movement, including hyperactivated motility (Nolan et al., 2004). Moreover, the same knockout mice demonstrated no increase in tyrosine phosphorylation, consistent with our model. Using a co-immunoprecipitation strategy, we have clearly demonstrated an association between SRC and PKA_c. SRC is known to be phosphorylated on Ser17 by PKA (Patschinsky et al., 1986; Schmitt and Stork, 2002), and on Ser35 by p34 cell-dependent cyclase 2 protein kinase (Shenoy et al., 1992). In our case, because the system is responsive to PKA inhibitors such as H89, it appears likely that Ser17 becomes phosphorylated. Since SRC is expressed at a low level and is apparently difficult to extract from spermatozoa, we were unsuccessful in directly demonstrating an increase in serine phosphorylation together with an increase in enzyme activity. To overcome this problem, we used antibodies that specifically recognize phosphorylated SRC. SRC can be phosphorylated on a number of key residues. Apart from the above-mentioned phosphorylation of Ser17 (and potentially Ser35), phosphorylation also occurs on residues Tyr416 and Tyr529. The total combination of phosphorylation sites would theoretically allow for SRC to exist in many different post-translationally modified states. This may explain why, on 2D PAGE, we see different charge states for SRC (Fig. 2). On the 2D PAGE of spermatozoa treated with the cell-permeable reagents dbcAMP and PTX, we saw a considerable increase in SRC phosphorylated at residue Y416, which corresponded with one of the spots detected with the monoclonal anti-SRC antibody. Additional evidence for the existence of this pathway was the observation that Y416-*P* decreased upon addition of the PKA inhibitor H89 (Fig. 2). In concert with this, H89 inhibited sperm tyrosine phosphorylation and suppressed the concomitant expression of hyperactivated motility (Fig. 4E). In light of these results, we believe that PKA must first phosphorylate SRC on Ser17, which then leads to the phosphorylation of Y416 and the expression of broad-spectrum tyrosine kinase activity.

An additional factor in the activation of SRC tyrosine kinase activity during sperm capacitation is the suppression of the inhibitory kinase CSK, which is present in murine spermatozoa and is a known inhibitor of SRC-family kinases. In the case of SRC, the mechanism by which CSK achieves this suppression is by phosphorylation of Y529. In our hands, spermatozoa treated with dbcAMP/PTX did not display any Y529 phosphorylation (data not shown), suggesting that CSK is somehow inactivated during capacitation. In support of this, the level of serine phosphorylation on CSK appeared to increase during capacitation (Figs 5 and 6). This finding is of interest because it has previously been reported that serine phosphorylation of CSK by PKA suppresses its inhibitory activity (Sun et al., 1997) leading to the consequential activation of SRC.

Based on these findings, we propose a model concerning the regulation of tyrosine phosphorylation during the hyperactivated motility associated with sperm capacitation. Complex relationships exist between soluble adenylyl cyclase (sAC) (Esposito et al., 2004; Hess et al., 2005), PKA (Nolan et al., 2004) and motility. Specifically, the generation of a hyperactivated spermatozoon involves a multitude of intracellular changes, but crucial amongst these is functionally active PKA. The conditional knockout of the PKA $C\alpha_2$ catalytic subunit generated spermatozoa that were able to move but unable to exhibit tyrosine phosphorylation, bicarbonate-evoked Ca^{2+} entry or hyperactivation (Nolan et al., 2004). We therefore propose that, during capacitation a combination of factors – including Ca^{2+}, bicarbonate and a change in redox status – activates sAC, leading to the enhanced production of cAMP (Breitbart, 2003; Hess et al., 2005). This increase in cAMP then activates PKA by inducing dissociation of the catalytic domain from its regulatory subunits. PKA then appears to have a dual role. First, it must phosphorylate SRC at serine residue(s) and hence activate the enzyme, promoting tyrosine phosphorylation and facilitating the onset of hyperactivation. Second, PKA must phosphorylate CSK to inhibit its activity, allowing for optimal SRC activity and the global increase in tyrosine phosphorylation, which characterizes the attainment of a capacitated state. This model is summarized in Fig. 7. This model is not only supported by biochemical events but also by pharmacological studies. Addition of SRC-family inhibitors, such as herbimycin A and SU6656, demonstrated not only a decrease in tyrosine phosphorylation but also a decrease in the number of spermatozoa that undergo hyperactivation. We therefore conclude that, cAMP is a key driver for hyperactivated motility, operating via a tyrosine-phosphorylation signal-transduction cascade that is mediated by the PKA-induced activation of SRC and the concomitant inactivation of CSK.

Materials and Methods

Medium

Biggers-Whitten-Whittingham medium (BWW) consisted of 95 mM NaCl, 44 μM sodium lactate, 25 mM $NaHCO_3$, 20 mM HEPES, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.27 mM sodium pyruvate, 0.3% (w/v) BSA, 5 U/ml penicillin and 5 μg/ml streptomycin, pH 7.4 (Biggers et al., 1971).

Materials

Bovine serum albumin (BSA) was purchased from Research Organics (Cleveland, OH); HEPES, penicillin, and streptomycin were from Gibco (Grand Island, NY); antibody against phosphorylated tyrosine (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY), whereas goat anti-mouse antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SRC antibody was also purchased from Upstate and anti-pY416, which specifically recognizes SRC phoshorylated at Tyr416, was from Calbiochem (Merck, Victoria, Australia); anti-CSK and anti-PKA antibodies were purchased from BD transduction labs (NSW, Australia). All other reagents were obtained from Sigma (St Louis, MO).

Preparation of mouse epididymal spermatozoa

The experiments described here were approved by the University of Newcastle animal ethics committee. Caudal epididymal spermatozoa were obtained from adult, 8-week-old to 14-week-old Swiss mice. The mice were euthanized with carbon dioxide and the reproductive tracts removed. Caudal spermatozoa were collected by back-flushing with water-saturated paraffin oil after which the perfusate was

deposited in 50 μl BWW under oil at 37°C. The sperm suspension was left to disperse in the droplet for 10 minutes at 37°C and then the sperm concentration was assessed with a Neubauer haemocytometer. The cells were aliquoted for various treatments at a final concentration of 10×10^6 sperm/ml and then incubated at 37°C in a 5% CO_2, 95% air atmosphere. Cell viability was assessed after each treatment using the hypo-osmotic swelling (HOS) test (World Health Organization, 1997). To induce sperm capacitation, 1 mM dbcAMP and 1 mM pentoxifylline (PTX) was included in the sperm incubation medium. The attainment of a capacitated state in cells subjected to this treatment regimen was indicated by the presence of high levels of tyrosine phosphorylation and the induction of hyperactivation in more than 95% of the sperm population. Non-capacitated cells were prepared by either taking caudal epididymal spermatozoa and immediately lysing the cells (t=0) or, where indicated, incubating them in the capacitation medium with 10 μM of the PKA inhibitor H89 for 90 minutes. In both cases, the cells typically displayed low levels of tyrosine phosphorylation and less than 5% hyperactivation.

To prepare caput spermatozoa, this region of the epididymis was dissected out and placed in a 500 μl droplet of medium under hydrated liquid paraffin. Multiple incisions were then made in the tissue with a razor blade and spermatozoa gently washed into the medium with mild agitation. The resultant cell suspension was then layered over 30% Percoll and centrifuged (1300 ***g*** for 15 minutes). The pellet consisting of 90% pure caput spermatozoa, was then resuspended in fresh BWW medium and counted as described above.

Protein extraction and concentration

Spermatozoa were pelleted (300 ***g*** for 2 minutes), washed twice (300 ***g*** for 2 minutes) and the supernatant was removed. Approximately 400 μl of lysis buffer, consisting of 50 mM Tris pH 8.5, 4% (w/v) Chaps, 7 M urea and 2 M thiourea was added to 50×10^6 spermatozoa. The sample was then vortexed every 10 minutes and left at 4°C for at least 1 hour. Following this, the sample was centrifuged at 13,000 ***g*** for 10 minutes and the supernatants were removed. Protein concentration was determined in duplicate using the Ettan™ 2D Quant Kit (G.E.Healthcare, Castle Hill, Australia) according to the manufacturer's instructions.

SDS-PAGE and western blotting

SDS-PAGE was conducted with 1-2 μg solubilized sperm proteins using 7.5% or 10% polyacrylamide gels at 25 mA constant current per gel. Proteins were then transferred onto nitrocellulose Hybond super-C membrane (G.E.Healthcare, Castle Hill, Australia) at 350 mA constant current for 1 hour. The membrane was blocked for 1 hour at room temperature with Tris-buffered saline (TBS; 0.02 M Tris pH 7.6, 0.15 M NaCl) containing 3% (w/v) BSA and then incubated for 2 hours at room temperature in a 1:4000 dilution of a monoclonal antibody against phosphorylated tyrosine (clone 4G10), or anti-α-tubulin (Clone B-5-1-2; 1:4000), anti-pY416, anti-CSK, anti-PKA_c(1:1000), anti-SRC (1:500) anti-pY529 (1:570) in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween 20. In addition, certain blots were probed with a 1:1000 dilution of a monoclonal antibody against phosphorylated serine (Sigma; clone PT-66) in TBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20. After incubation, the membrane was washed four times for 5 minutes with TBS containing 0.01% Tween-20, and then incubated for 1 hour at room temperature with either goat anti-mouse (1:3000 in the case of pY416, SRC, phosphoserine, PKA, PT-66 and α-tubulin) or goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (1:2000 in the case of CSK) in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. The membrane was washed again as described above and then HRP was detected with an enhanced chemiluminescence (ECL) kit (G.E.Healthcare, Castle Hill, Australia) according to the manufacturer's instructions. The consistent presence of hexokinase in murine sperm preparations served as an internal loading control (Nixon et al., 2006) for these western blot procedures.

Cy-dye labeling

Aliquots comprising 75 μg of sperm extract were individually precipitated using the Ettan™ 2D clean-up kit (G.E.Healthcare, Castle Hill, Australia). Precipitates were then solubilized in lysis buffer prior to labeling. A pooled internal standard was produced from each of the samples (i.e. 25 μg from each individual sample were pooled). The remaining 50 μg were labeled with 400 pmol of Cy3 or Cy5. To ensure the dye binding showed no bias, replicates of the same sample were labeled with both Cy3 and Cy5 (Table 1). The pooled internal standard containing 300 μg of protein was labeled with 2400 pmol Cy2 (Table 1). Labeling was performed for 30 minutes on ice in the dark, after which the reactions were quenched with 1 μl of 10 mM lysine for 10 minutes on ice in the dark. Fifty μg of the quenched Cy3- and Cy5-labeled samples were then combined and mixed with 50 μg of Cy2-pooled internal standard. Finally, an equal volume of 2× rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% Chaps, 4 mg/ml DTT, 0.5% (v/v) IPG buffer 3-10 was added.

2D-gel electrophoresis

Approximately 125 μl of sample were added to three to ten, 7-cm long, non-linear-immobilized pH-gradient (IPG) strips (G.E.Healthcare, Castle Hill, Australia), following which the strips were covered with mineral oil (Sigma) and left for 10 hours. The samples for each experiment were loaded onto the 24-cm strips from the anodic end. Isoelectric focusing was performed with a IPGphor ceramic manifold (G.E.Healthcare, Castle Hill, Australia) for a total of 55 kVhours (held at 300 V for 0.9 Vhours, ramped to 1000 V for 1 Vhour, ramped to 5000 V for 1 Vhour, held at 5000 V for 5 Vhours). The IPG strips were immediately placed into equilibration buffer [30 % (v/v) glycerol, 2% (w/v) SDS, 6 M urea with trace amounts of Bromophenol Blue] supplemented first with 0.5% (w/v) DTT for 15 minutes at room temperature followed by 2.5% iodoacetamide in fresh equilibration buffer for 15 minutes at room temperature. This had the effect of reducing and carbamido-methylating cysteine sulfhydryls while equilibrating the proteins in the 2D loading buffer. IPG strips were placed on top of 12.5% homogenous polyacrylamide gels that had been set in low fluorescence glass plates using an Ettan™-DALT gel caster (G.E.Healthcare, Castle Hill, Australia). 2D SDS-PAGE was carried out on six gels simultaneously at 1.5 W/gel for 45 minutes followed by 17 W/gel for 4 hours with a Peltier-cooled DALT II electrophoresis unit (G.E.Healthcare, Castle Hill, Australia). The Cy2, Cy3 and Cy5 components of each gel were individually imaged using excitation/emission wavelengths of 480/530 nm, 520/590 nm and 620/680 nm, respectively.

Difference-in-2D-gel-electrophoresis (DIGE) analysis

DeCyder software (5.0, G.E.Healthcare, Castle Hill, Australia) was used to compare abundance changes across the six mature and immature sperm samples (Table 1). The biological variation module was used to match all 18 protein-spot maps from the six gels and calculate average abundance changes; paired Student's t-tests were then employed to secure P-values for the significance of these changes.

In-gel tryptic digest

A number of 2D-pick gels containing between 0.5-2 mg of solubilized protein were produced. Gels was fixed in 50% methanol and 7% acetic acid for 30 minutes and then incubated overnight with Sypro-Ruby (Molecular Probes) in the dark. Protein spots of interest were picked either robotically or manually and equilibrated with 25 mM ammonium bicarbonate for 10 minutes. The spots were then dehydrated three times in 100 μl of 50% methanol in Puregrade Fluka water, 25 mM ammonium bicarbonate for 2 hours and left to dry overnight. Dehydrated plugs were manually digested in-gel with 7.5 μl porcine-modified trypsin protease (Promega) in 25 mM ammonium bicarbonate for 6 hours at 37°C. Tryptic peptides were extracted from the gel by vigorous washing with 25 mM ammonium bicarbonate containing 50% methanol and 0.1% trifluoracetic acid.

Mass spectrometry

Approximately 0.5 μl of the tryptic peptide was mixed with 0.5 μl α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) and 0.5 μl of this mixture was spotted onto the target plate. Matrix-assisted laser desorption ionisation-time-of-flight analysis (MALDI-TOF) mass spectrometry was performed as described (Baker et al., 2005). Peptide-mass fingerprints were acquired in reflectron mode and trypsin autolytic peptides (m/z=842.51 and 2211.10) were used to internally calibrate each spectrum. When trypsin autolytic peptides were not detected, an external standard was applied. Ions from each spectrum were inspected for resolution and isotopic distribution. Those ions specific for each sample were then used to look for a match in the SWISS-PROT and NCBInr databases using the MASCOT and ProFound computer algorithms. Highest confidence identifications were checked for consistency in the gel region from which the protein was excised (MW and pI).

Assessment of hyperactivated movement

A 20 μl droplet of sperm suspension was placed onto a coverslip and viewed under dark-phase magnification. Approximately five fields were video-taped and subsequently assessed for hyperactivated motility as previously described (Yanagimachi, 1994). The results are expressed in terms of the percentage of motile cells exhibiting hyperactivation and, in all cases, the basic level of sperm motility exceeded 90%.

Preparation of mouse sperm-head and -tail fractions

Caudal epididymal mouse spermatozoa (100×10^6/ml) were swum out into 1 ml of BWW supplemented with 0.1% (w/v) polyvinyl alcohol (BWW-PVA) instead of BSA. The sample was transferred to a 1-ml glass tube and sonicated in a Branson sonifier 450 (Branson Ultrasonics. Corp., Danbury, CT) for 15 seconds on ice. Visual inspection of the sample was performed to ensure that >99% of the spermatozoa had been decapitated. The contents was then layered over 75% Percoll (isotonic Percoll, prepared by diluting nine volumes of Percoll with one volume of 10× PBS, was designated 100%) and centrifuged (725 ***g*** for 15 minutes at 37°C). The topmost interface between the BWW-PVA and Percoll, consisting of sperm tails, was taken and washed twice in BWW-PVA by centrifugation (2500 ***g*** for 5 minutes). The pellet, containing sperm heads, was taken and washed in the same manner. Visual inspection and counting was performed to ensure that both samples were >95% pure.

Co-immunoprecipitation studies

Approximately 4 μg of anti-SRC or anti-CSK antibody was added to 60 μl of washed protein G Dyna Bead slurry and gently rocked for 1 hour at 4°C. The beads were isolated using a magnet to allow the removal of the supernatant and subsequent washing of the beads (2×). The spermatozoa were then lysed [0.1% (v/v) Triton X-100, 300 mM NaCl, 20 mM sodium orthovanadate, 20 mM Tris, protease inhibitor tablet, pH 7.4] and 50-100 μg of soluble lysate were added to the pre-absorbed beads. The sample was left to incubate overnight at 4°C on a rotator (60 rpm) following which the slurry was washed twice (300 mM NaCl, 20 mM Tris, pH 7.5) using the magnet as described above. Following complete removal of the supernatant, the beads were resuspended in 2× SDS lysis buffer. The sample was boiled (5 minutes at 100°C) prior to SDS PAGE. In control incubations, beads were incubated with sperm lysate in the absence of antibody and then processed as described above.

Immunocytochemistry

Extracted spermatozoa were incubated with 1 mM dbcAMP and 1 mM PTX in BWW-BSA buffer at 37°C for 45 minutes in an incubator with an atmosphere of 5% CO_2, 95% air and 100% humidity. Following incubation, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. After three washes in 0.05 M glycine-PBS, cells were air-dried onto 0.1% (w/v) poly-L-lysine coated slides and then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Following a PBS rinse, cells were blocked with 3% BSA in PBS at 37°C for 60 minutes and then incubated with 1:100 anti-phosphorylated tyrosine antibody in PBS overnight at 4°C. After three washes in PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (1:64) in 1% BSA-PBS for 60 minutes at 37°C. After three PBS washes, cover slips were mounted on 5 μl of anti-fade reagent [13% Mowiol 4-88, 33% glycerol, 66 mM Tris (pH 8.5), 2.5% 1,4 diazobcyclo-[2.2.2]octane] and cells examined and photographed with a Carl Zeiss MC 200 Chip microscope camera on an Axiovert S100 inverted-phase-contrast microscope (Zeiss, Jena, Germany). Fluorescent images were captured through a Zeiss No. 9 (FITC) filter system with blue excitation at 450-490 nm.

Confocal microscopy

Confocal images were obtained with a Zeiss LSM 510 scanning microscope incorporating a Zeiss axiovert stand (exitation wavelength, 488 nm; emission wavelength, 500-530 nm).

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