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Cyclic-AMP initiates protein tyrosine phosphorylation independent of cholesterol efflux during ram sperm capacitation

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Abstract. Unlike most other species, ram spermatozoa are difficult to capacitate *in vitro*. Bicarbonate and Ca^{2+} are necessary, whereas bovine serum albumin does not appear to be obligatory. In the present investigation we have assessed (*1*) the ability of the cholesterol-sequestering agent, methyl-β-cyclodextrin (M-β-CD), to initiate protein tyrosine phosphorylation, and (*2*) the importance of phosphodiesterases (PDEs) in controlling the levels of cAMP. Results show that despite removing significant amounts of membrane cholesterol, as assessed by filipin staining, M-β-CD treatment did not stimulate major increases in protein tyrosine phosphorylation. Addition of a cocktail of PDE inhibitors (theophylline and caffeine), a phosphatase inhibitor (okadaic acid) and dibutyryl-cAMP (db-cAMP), however, stimulated specific tyrosine phosphorylation of several proteins between 30 and 120 kDa. On their own, none of the above reagents were effective but a combination of db-cAMP + PDE inhibitors was sufficient to achieve a maximal response. H-89, a protein kinase-A inhibitor, suppressed tyrosine phosphorylation significantly. Immunofluorescence revealed that the newly-phosphorylated proteins localised mainly in the sperm tail. These findings suggest that in ram spermatozoa cAMP levels are too low to initiate tyrosine phosphorylation of flagellar proteins that are indicative of the capacitation state and that this is caused by unusually high levels of intracellular PDEs.

Additional keywords: cAMP agonists, immunocytochemistry, methyl-β-cyclodextrin.

Introduction

After ejaculation, mammalian spermatozoa have to undergo a maturational process called capacitation either *in vivo* during transit through the female genital tract (Austin 1951; Chang 1951) or *in vitro* in defined artificial media (Yanagimachi 1994). The composition of media that support *in vitro* capacitation typically approximates that of oviduct fluid (Yanagimachi 1994), but the exact composition is variable for different species. The role of the different medium constituents is not well defined. Several reports indicate that bicarbonate is an essential capacitating agent (Harrison *et al*. 1996; Visconti *et al*. 1999*c*; Holt and Harrison 2002; Harrison 2004; Harrison and Gadella 2005) and that bovine serum albumin (BSA) is necessary for the removal of cholesterol from the sperm plasma membrane (Davis *et al*. 1979; Davis 1981; Langlais *et al*. 1981; Go and Wolf 1985; Suzuki and Yanagimachi 1989; Visconti *et al*. 1999*b*). Although capacitation is considered to be mediated by various second messengers, such as cAMP, (Visconti *et al*. 1998), the molecular mechanisms have yet to be fully elucidated. It has been suggested that the sperm cell can intrinsically control capacitation and that aspects of this control lie within the sperm plasma membrane (Visconti *et al*. 1999*b*).

Capacitation has also been correlated with an increase in protein tyrosine phosphorylation of a restricted subset of proteins (Visconti *et al*. 1995*a*; Carrera *et al*. 1996; Leclerc *et al*. 1996; Galantino-Homer *et al*. 1997; Tardif *et al*. 2001). It has generally been accepted that the increase in tyrosine-phosphorylated proteins is associated with the activation of protein tyrosine kinase (PTK), the inactivation of protein tyrosine phosphatases (PYP), or a combination of these that are controlled by the action of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) (Visconti *et al*. 1998; Urner and Sakkas 2003). The first indications that cAMP might be involved in capacitation were provided by Toyoda and Chang (1974) and Rosado *et al*. (1974), who showed that inclusion of dibutyryl-cAMP (db-cAMP) increased capacitation in rat and rabbit sperm, respectively. Further evidence accrued from numerous studies that showed that cAMP plays a major secondary messenger role in the initiation of capacitation (Fraser and Monks 1990; Visconti *et al*. 1995*a*; Leclerc *et al*. 1996; Aitken *et al*.

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1998; Wennemuth *et al*. 2003; Harrison 2004; Tardif *et al*. 2004).

Currently, there are conflicting results that may be due to differences between species. We have demonstrated in ram spermatozoa that tyrosine phosphorylation of membrane proteins is related to the sperm capacitation state (Perez-Pe *et al*. 2002), both in the absence and presence of BSA, and that the addition of increasing concentrations of cholesterol sulfate to samples containing BSA has little or no effect (Grasa *et al*. 2006). Furthermore, we have shown that ram spermatozoa incubated in the absence of both bicarbonate and calcium are capacitated to a lesser extent than those in the presence of these compounds. Our findings suggest that Visconti's model (Visconti *et al*. 1995*b*) might function in ram sperm capacitation with an interdependent role for calcium and bicarbonate that might exert their effects through adenylyl cyclase (AC), thereby increasing sperm cAMP levels.

Cyclodextrins are water-soluble cyclic heptasaccharides consisting of β (1–4)-glucopyranose units (Pitha *et al*. 1988) that are able to promote cholesterol efflux from a variety of somatic cells (Kilsdonk *et al*. 1995; Yancey *et al*. 1996), including spermatozoa (Choi and Toyoda 1998; Cross 1999; Osheroff *et al*. 1999; Visconti *et al*. 1999*b*; Iborra *et al*. 2000). Their cholesterol-binding efficiency correlates directly with their ability to increase protein tyrosine phosphorylation in sperm (Visconti *et al*. 1999*a*; Pommer *et al*. 2003; Shadan *et al*. 2004; Galantino-Homer *et al*. 2006). Methyl-β-cyclodextrin (M-β-CD) is the most potent of the cyclodextrins with respect to its affinity for cholesterol binding (Yancey *et al*. 1996).

In previous studies on capacitation of ram spermatozoa we demonstrated a requirement for extracellular bicarbonate and Ca^{2+} (Perez-Pe *et al*. 2002; Grasa *et al*. 2006). Unlike other species, however, capacitation (as assessed by CTC staining patterns) appeared to be relatively independent of BSA and the addition of cholesterol sulfate had no effect. Some stimulation of protein tyrosine phosphorylation was observed but the magnitude of the response was modest compared with that in the mouse, bull and human (Osheroff *et al*. 1999; Visconti *et al*. 1999*b*, 1999*c*). In the present study we have investigated (*1*) the requirement for cholesterol efflux during capacitation using the specific reagent M-β-CD, and (*2*) the hypothesis that in ram spermatozoa cAMP levels are limiting and that it is the availability of this second messenger that is crucial for initiating protein tyrosine phosphorylation.

Materials and methods

Sperm preparation and media

Ejaculated semen was collected from Suffolk rams maintained at the Babraham Institute, UK, using an artificial vagina. Maintenance of animals and all animal procedures were carried out with approval of local ethical committees in accordance with Home Office (UK) regulations. Seminal plasma-free spermatozoa were obtained by a dextran/swim-up procedure (Garcia-Lopez *et al*. 1996) and used as the control sample. This procedure was performed with a swim-up medium (SM) devoid of $CaCl_2$ and $NaHCO_3$ (Grasa *et al*. 2004), which consisted of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 1.5 UI mL^{-1} Penicillin, and 1.5 μg mL^{-1} Streptomycin, pH 7.2 (adjusted using NaOH). The osmotic pressure was 320 mOsm kg^{-1}.

Swim-up-selected spermatozoa were washed once in TALP medium (for composition see In vitro *capacitation*) by gentle centrifugation (300*g* for 10 min at room temperature) and the pellet resuspended in 1 mL of TALP medium. Sperm concentration was determined using a hemocytometer.

Cholesterol labelling and release determination

Cholesterol distribution in the sperm plasma membrane was investigated by staining with filipin (Polysciences Inc., Warrington, PA, USA). Sperm aliquots (100 μL, 10^7 cells) were mixed with 100 μL of filipin (200 μg mL^{-1} in TALP containing 4% (v/v) ethanol), incubated for 15 min at room temperature in the dark and washed twice by centrifugation (200*g* for 3 min and pellet resuspended in 500 μL mHtf) in TALP. Spermatozoa were viewed either by fluorescence microscopy (excitation 365 nm, emission 420 nm) or analysed by a fluorescence-activated cell sorter (FACS) with a Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA) using a FL4 530/30 nm band-pass filter. The system collects fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50–500 events per second. Data were analysed using the Cell Quest package.

To investigate the effectiveness of methyl-β-cyclodextrin (M-β-CD, Sigma, Poole, UK) for removing cholesterol, spermatozoa were incubated with different concentrations (0.1–20 mM) of M-β-CD in capacitating conditions. Cholesterol remaining in the plasma membrane was quantified by FACS analysis after staining with filipin as described above.

In vitro *capacitation*

For the induction of *in vitro* capacitation, aliquots of 4×10^7 cells mL^{-1} were incubated at 39°C in a humidified incubator with 5% CO_2 in air (capacitated sample). Incubations were performed in complete TALP medium (Parrish *et al*. 1988) containing 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM Hepes, 1 mM Na pyruvate, and 5 mM glucose, pH adjusted to 7.3 by gassing with 5% CO_2 in air, and sterilised using Stericup filtration (0.22 mm). The osmotic pressure was measured at 295–305 mOsm L^{-1}.

To evaluate the presence and role of the cAMP-PKA pathway, we tested the effects of dibutyryl-cAMP (db-cAMP, Sigma Chemical Co., Madrid, Spain; 1 mM), caffeine and theophylline (both inhibitors of phosphodiesterases, Sigma; 1 mM each), and okadaic acid (OA, a broad spectrum phosphatase inhibitor, Sigma; 0.2 μM). In some experiments a cocktail of all of these compounds was added to the sperm samples.

Extraction of ram sperm proteins

Aliquots of 2×10^6 cells of control or capacitated samples diluted in PBS containing 0.2 mM Na_3VO_4 1 : 10 were centrifuged at 14 000*g* in a microfuge for 10 min at room temperature and the supernatant discarded. The resulting sperm pellet

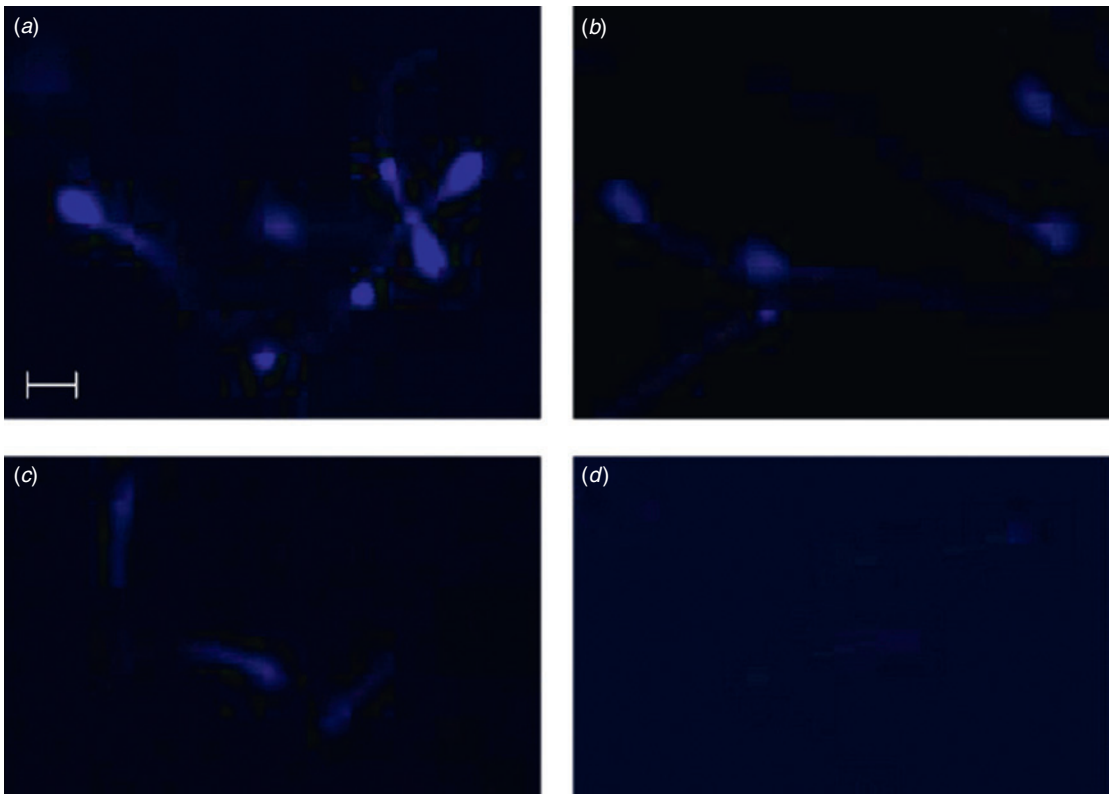

Fig. 1. Effect of M-β-CD on cholesterol levels in ram sperm plasma membranes as detected with filipin. Fluorescence pictures of filipin-stained spermatozoa before (*a*) and after 4 h of incubation in capacitating conditions with (*b*) 2.5 mM, (*c*) 5 mM, and (*d*) 20 mM M-β-CD. Scale bar = 10 μm.

was frozen by snap-freezing in dry ice until extraction. Proteins were obtained by resuspending sperm pellet in 100 μL of extraction medium (2% SDS (w/v), 0.0626 M TRIS-HCl (pH 6.8), 0.002% bromophenol blue in 10% glycerol (final glycerol concentration 1%)) and immediately incubated for 5 min at 100°C. After centrifugation at 14 000*g* for 5 min at room temperature, the supernatant was recovered and 2-mercaptoethanol and glycerol were added to a final concentration of 5% and 1%, respectively.

SDS–PAGE and immunoblotting

Proteins were separated in one dimension on 10% SDS–PAGE gels and either transferred to nylon membranes (Immobilon; Millipore, Bedford, MA, USA) by western-blotting techniques or visualised by staining with 0.1% Coomassie Blue R-250.

For detection of phosphorylated proteins, non-specific sites on the blotting membranes were blocked for 1 h with 5% BSA in blocking buffer (25 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.1% Tween). The blots were incubated with the monoclonal antibody horseradish-peroxidase-conjugated anti-phosphotyrosine (Monoclonal Ab; clone 4G10, Upstate, Santa Cruz, CA, USA, cat. no. 16–105), diluted 1 : 5000, for 1 h at 25°C. After extensive washing, the proteins that bound the antibody were visualised by chemiluminescence procedures (Perkin-Elmer, Boston, MA, USA).

Western-blot images were quantified using Quantity One software (Bio Rad, Hércules, CA, USA) to determine the relative intensity of the tyrosine-phosphorylated protein bands. The phosphorylation signal of a single band (p100), of some molecular regions, and of all bands was evaluated as peak intensity. Changes in intensity were evaluated relative to comparable regions in control blots and presented as a percentage.

Immunolocalisation of tyrosine-phosphorylated proteins

Aliquots of 10^7 spermatozoa were fixed with paraformaldehyde at a final concentration of 3% for 30 min at room temperature and further permeabilised with 0.1% TX-100 for 30 min at room temperature. After washing in TALP, non-specific antibody-binding sites were blocked by incubation with 5% BSA for 1 h at room temperature followed by 4G10 monoclonal antibody diluted 1 : 100 in PBS with 1% BSA for 1 h at room temperature. The second layer antibody was rabbit anti-mouse conjugated with Alexa^{488} (Molecular Probes, Leiden, The Netherlands), diluted 1 : 500 in PBS with 1% BSA, and incubated for 1 h at room temperature protected from light. To eliminate excess reagents after every incubation step, cells were washed by gentle centrifugation (200*g* for 3 min at room temperature and pellet was resuspended in 300 μL PBS) in PBS. As negative controls, either the primary or the secondary antibody was omitted, to prove the specificity of each.

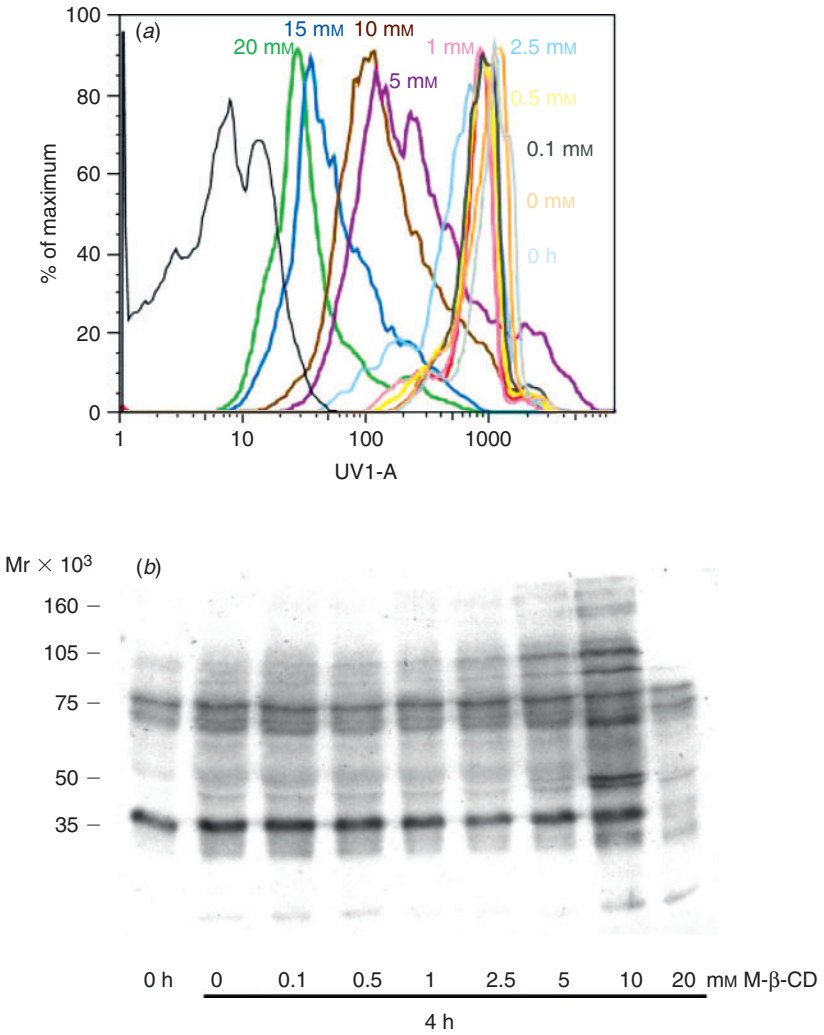

Fig. 2. (*a*) FACS-sorted spermatozoa after incubation with M-β-CD followed by staining with filipin. Each colour represents different concentrations of M-β-CD. (*b*) Western-blot analysis of protein tyrosine phosphorylation in sperm incubated 4 h at 39°C in the presence of different concentrations of M-β-CD. Proteins were extracted and analysed by SDS–PAGE/western blotting with 4G10 monoclonal Ab. The experiment was performed five times, and a representative membrane is shown.

Cells were examined by epifluorescence microscopy (Zeiss Axiophot using a fluorescein filter (465–495 nm)). At least 100–200 cells were scored for each sample depending on the density of cells in the preparation. Images were captured on a digital camera (Sony 3CCd Colour Video Camera and Sony Digital Still Recorder) and were analysed with the Visilog 5.1 (Noesis SA, Orsay, France) program.

Fluorescence from 4G10-labelled spermatozoa was quantified by FACS Vantage flow cytometer (Becton Dickinson) using a 530 ± 15 nm band-pass filter.

Statistical analysis

Results are shown as mean ± s.e.m. of the number of samples indicated in each case. ANOVA test was performed to determine whether there were significant differences between samples, and *post hoc* comparisons were made using the Tukey's test. Software used was GraphPad (InStat, San Diego, CA, USA).

Results

Fluorescence microscopy of filipin–cholesterol complex in ram sperm plasma membranes

Fresh control spermatozoa showed strong fluorescence over the whole cell surface (Fig. 1*a*) that did not change appreciably after 4 h of incubation in capacitating conditions (data not shown). The addition of a low concentration (2.5 mM) of M-β-CD to the incubation medium caused barely-detectable changes in fluorescence intensity (Fig. 1*b*), but at 5 and 20 mM an important decrease became apparent (Fig. 1*c*, *d*).

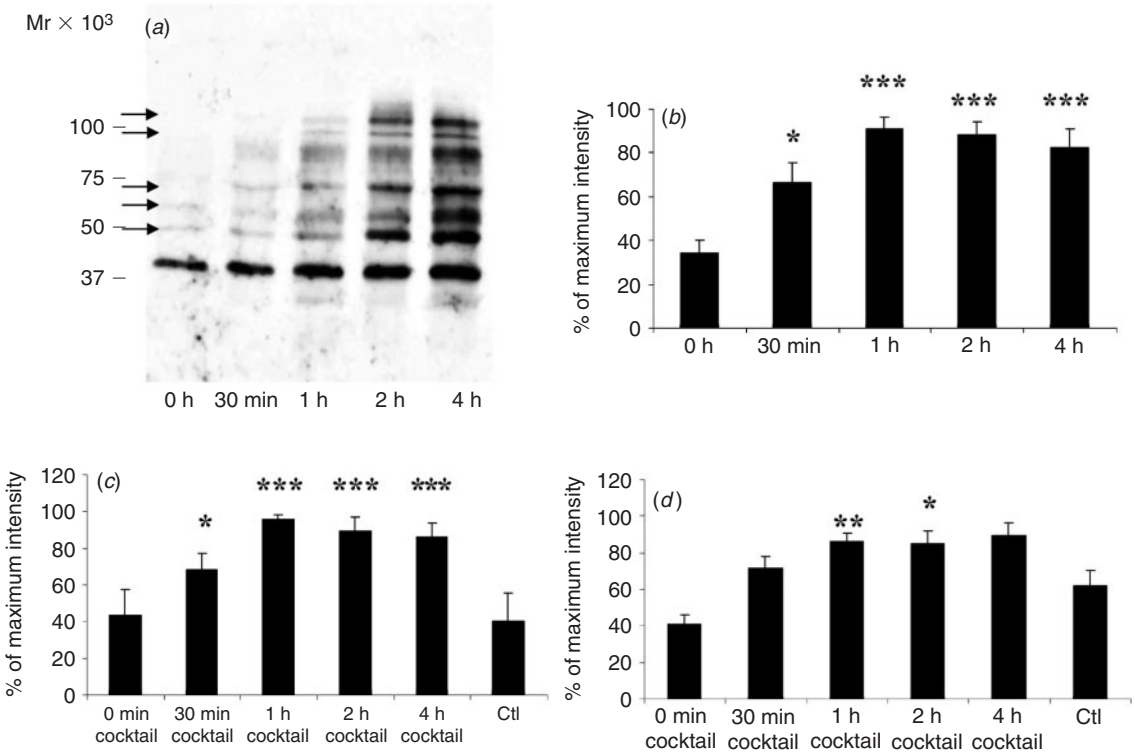

Fig. 3. Time course of protein tyrosine phosphorylation in the presence of a cocktail containing 1 mM db-cAMP, 1 mM theophylline and 1 mM caffeine (both inhibitors of phosphodiesterases) and 0.2 μM okadaic acid (OA, a broad spectrum protein phosphatase inhibitor). (*a*) Western blot analysis of protein tyrosine phosphorylation. (*b–d*) Densitometric quantification of (*b*) total phosphotyrosine proteins; (*c*) 30–60 kDa molecular region; (*d*) 60–120 kDa molecular region. Spermatozoa were incubated 0–4 h at 39°C, proteins extracted and analysed by SDS–PAGE/western blotting with 4G10 monoclonal Ab. The experiment was performed seven times, and a representative membrane is shown. Data presented represent mean ± s.e.m. Significant differences relate to control (time 0) samples: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Release of cholesterol from ram sperm membranes by methyl-β-cyclodextrin

FACS analysis of filipin-stained spermatozoa revealed no appreciable differences between controls and samples containing up to 1.0 mM M-β-CD (Fig. 2*a*). With increasing concentrations of M-β-CD (2.5–20 mM), however, there was a progressive displacement of the fluorescence peak to the left as a result of a reduction in cholesterol associated with the sperm membrane. Conversely, sperm incubated in a medium containing 5 mg mL^{-1} BSA showed no significant release of cholesterol (results not shown).

Effect of methyl-β-cyclodextrin on protein tyrosine phosphorylation in ram spermatozoa

In a previous study, we showed that the induction of *in vitro* ram sperm capacitation in a medium containing calcium and bicarbonate without BSA promoted a small but measurable increase in protein tyrosine phosphorylation (Perez-Pe *et al*. 2002). Likewise, in a more recent study, in which we validated the chlortetracycline-fluorescence (CTC) assay for the evaluation of capacitation and acrosome reaction-like changes in ram sperm, we found a time-dependent increase in the proportion of sperm displaying the capacitation pattern of CTC staining and protein tyrosine phosphorylation in the absence of BSA (Grasa *et al*. 2006). Therefore, we examined whether cholesterol removal by M-β-CD in a medium devoid of BSA was sufficient to activate the signal transduction pathway that lead to an increase in protein tyrosine phosphorylation. As shown in Fig. 2*b*, M-β-CD induced a slight increase in protein tyrosine phosphorylation at concentrations up to 5 mM. At higher concentrations (10 mM) there was an overall increase but this declined at 20 mM.

Effect of cAMP-elevating agents and a PKA inhibitor on protein tyrosine phosphorylation in ram spermatozoa

Although we showed in a previous study (Grasa *et al*. 2006) that protein tyrosine phosphorylation in ram spermatozoa increased slightly in samples containing dibutyryl-cAMP (db-cAMP) and isobutyl-methylxanthine, tyrosine phosphorylation of specific protein bands appeared to be independent of induced changes in cAMP levels. In the present study, we evaluated the effect of several compounds expected to upregulate cAMP on protein tyrosine phosphorylation in ram spermatozoa.

A time-dependent increase in protein tyrosine phosphorylation occurred after incubation in the presence of a cocktail

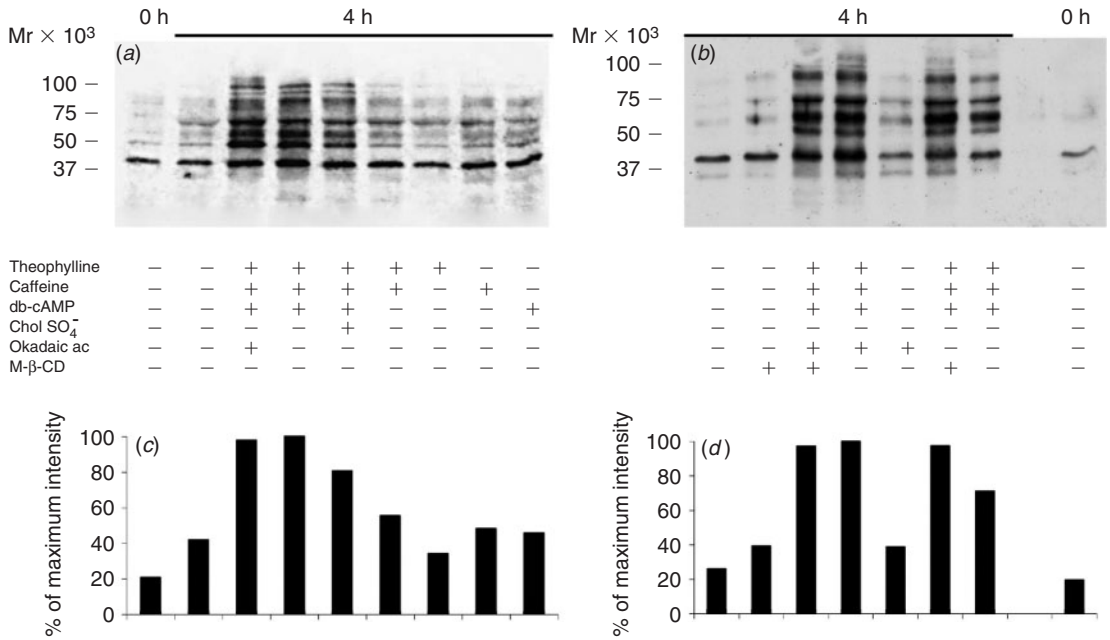

Fig. 4. Effects of theophylline, caffeine, db-cAMP, okadaic acid, cholesterol sulfate and M-β-CD on protein tyrosine phosphorylation after incubation for 4 h. (*a*, *b*) Western blot analysis and (*c*, *d*) densitometric quantification of total phosphotyrosine proteins. Each assay was performed at least twice and a representative membrane is shown.

containing db-cAMP, theophylline + caffeine (both inhibitors of phosphodiesterases) and okadaic acid (OA, a broad spectrum phosphatase inhibitor) (Fig. 3*a*). Densitometric quantification of the immunoblots revealed that the total protein tyrosine phosphorylation signal increased during the course of incubation in the cocktail-containing samples (Fig. 3*b*). Likewise, densitometric quantification of the signal at the 30–60 kDa (Fig. 3*c*) and 60–120 kDa (Fig. 3*d*) molecular regions showed that the maximum phosphorylation value was achieved after 1 h of incubation, and that it was maintained for up to 4 h.

To investigate if all constituents of the cocktail were necessary to stimulate tyrosine phosphorylation, individual components were added alone or in different combinations. On their own theophylline, caffeine, db-cAMP, okadaic acid and M-β-CD had little or no significant effect (Fig. 4*a*, *b*, *c*, *d*). Similarly, removal of okadaic acid from the cocktail had no apparent effect but omission of db-cAMP reduced tyrosine phosphorylation considerably (~45%, Fig. 4*a*). To stimulate phosphorylation maximally it was necessary to have a combination of db-cAMP, caffeine and theophylline. Consistent with earlier results in Fig. 3, the presence of 2.5 mM M-β-CD in the cocktail had no observable effect (Fig. 4*c*) nor did the addition of 30 μM cholesterol sulfate (Fig. 4*a*).

The addition of a PKA inhibitor (H-89, 100 μM) caused a slight decrease in tyrosine-phosphorylated proteins as deduced by densitometric analysis of the western-blots (Fig. 5). The assessed percentages of inhibition were 19.4 ± 2.0 and 17.0 ± 2.0 in control and cocktail-containing samples, respectively.

In one experiment we investigated whether immature spermatozoa collected from the rete testis could respond to the

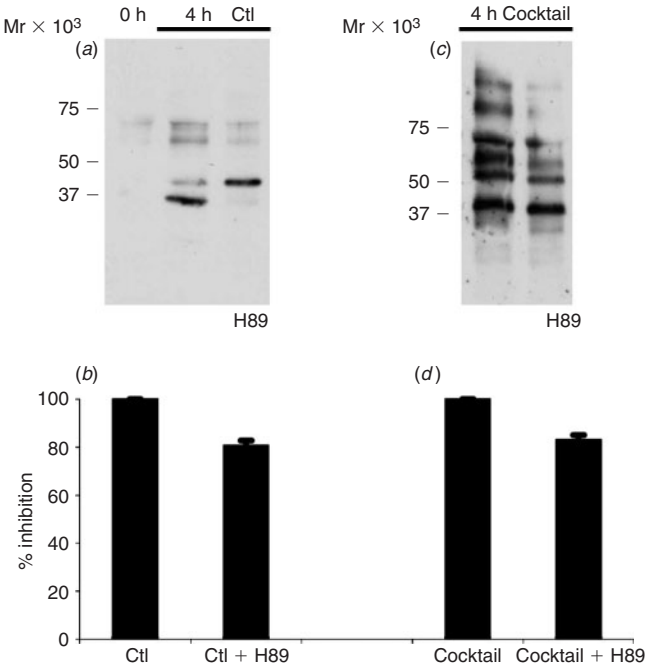

Fig. 5. Effect of H89 (a PKA inhibitor) on protein tyrosine phosphorylation. (*a*, *c*) Western blot analysis and (*b*, *d*) densitometric quantification of total phosphotyrosine proteins in control samples at (*a*) 0 h and (*b*) after 4 h incubation, and in cocktail-containing samples (*c*, *d*) after incubating for 4 h. Each assay was performed four times and a representative membrane is shown.

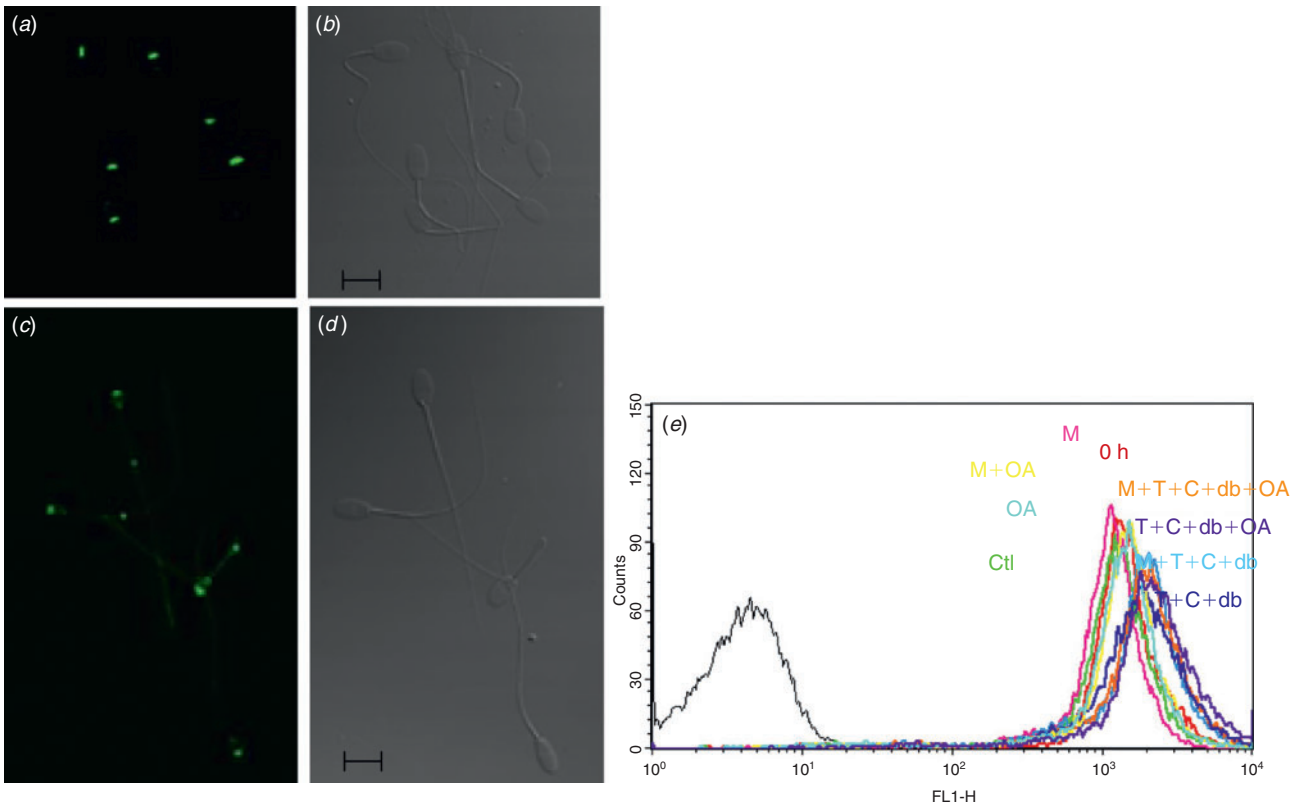

Fig. 6. Immunofluorescence detection of phosphotyrosine proteins in ram spermatozoa before and after incubation for 4 h in capacitating conditions. Paired fluorescent and brightfield photomicrographs of (*a*, *b*) a control spermatozoa and (*c*, *d*) spermatozoa incubated in the cocktail-containing medium. (*e*) FACS-sorting of spermatozoa of both samples. Each colour represents incubation under capacitating conditions with different components of the cocktail. M: Methyl-β-cyclodextrin; T: theophylline; C: caffeine; OA: okadaic acid; db: db-cAMP. Scale bar = 10 μm.

cocktail-containing medium. No stimulation in tyrosine phosphorylation was observed (results not shown) although mature cauda epididymidal spermatozoa from the same animal initiated tyrosine phosphorylation of the same proteins to a similar extent as ejaculated spermatozoa. This suggests that cAMP-dependent signalling pathways in spermatozoa become functional during maturation in the epididymis.

Visual inspection of 4G10-stained spermatozoa from the experiments in Fig. 4 by epifluorescence microscopy showed strong staining in the equatorial subsegment of control spermatozoa (Fig. 6*a*, *b*). After incubation in the cocktail-containing medium, however, there was a noticeable increase in fluorescence on the flagellum, with a marked signal appearing at the level of Jensen's ring (Fig. 6*c*, *d*). Quantitation by FACS corroborated these findings with an increase in fluorescence signal in the cocktail-containing samples as shown by a shift in the curve to the right relative to controls (Fig. 6*e*).

Discussion

This work has shown that increasing cAMP levels in ram spermatozoa with phosphodiesterase inhibitors and exogenous db-cAMP initiates capacitation-associated tyrosine phosphorylation of flagellar proteins. Unlike the situation in most other species, removal of cholesterol from the plasma membrane with M-β-CD has only weak stimulatory effects on phosphorylation.

Previous studies on mouse (Visconti *et al*. 1995*a*, 1995*b*), rat (Lewis and Aitken 2001*a*, 2001*b*), boar (Flesch *et al*. 1999; Harrison and Miller 2000; Shadan *et al*. 2004; Bravo *et al*. 2005), bull (Visconti *et al*. 1999*a*; Marquez and Suarez 2004) and human (Aitken *et al*. 1995, 1996; Carrera *et al*. 1996; Emiliozzi and Fenichel 1997) spermatozoa have demonstrated a close correlation between an increase in protein tyrosine phosphorylation and capacitation. The obligatory requirement for an external macromolecule such as BSA (or HSA) has frequently been explained on the grounds that, amongst other things, it causes efflux of cholesterol from the plasma membrane. This reasoning is supported by strong experimental evidence. Addition of cholesterol sulfate to the medium inhibits capacitation and protein tyrosine phosphorylation (Osheroff *et al*. 1999) while M-β-CD, a carrier compound that specifically removes sterols such as cholesterol from membranes, is very effective in initiating protein tyrosine phosphorylation and other capacitation-associated responses, such as changes in CTC staining (Choi and Toyoda 1998; Shadan *et al*. 2004). It has long been a puzzle, therefore, why ram spermatozoa do not respond, or at best respond very poorly, to cholesterol depletion irrespective of whether it is mediated with BSA (Grasa *et al*. 2006) or M-β-CB (present study).

One explanation that has been proposed is that in ram sperm the cholesterol : phospholipid ratio is already very low (White and Darin-Bennett 1976; Darin-Bennett and White 1977) and that any further reduction would severely compromise membrane integrity and subsequent survival. More likely, it is related to the macromolecular organisation of the membrane (Jones *et al*. 2007). Cholesterol is an important constituent of membrane lipid rafts, which are enriched in signalling molecules such as src protein kinases (Simons and Ikonen 1997; Harder *et al*. 1998). In boar spermatozoa, removal of small amounts of cholesterol has been shown to enhance raft formation, implying recruitment of new molecules into the raft that are required for activation of signalling pathways (Shadan *et al*. 2004). It has also been shown that sphingomyelinase will initiate protein tyrosine phosphorylation without loss of membrane cholesterol (Shadan *et al*. 2004). One explanation is that the ceramide generated by sphingomyelinase displaces cholesterol from the raft (Megha and London 2004) thereby permitting recruitment of non-raft molecules required for downstream signalling. Interestingly, substantial sphingomyelinase activity has been reported in ram sperm membranes (Hinkovska *et al*. 1987). In our experience ram sperm membranes contain a lower proportion of lipid rafts than spermatozoa from most other species, which may be related to their relative insensitivity to cholesterol-depleting reagents for initiating capacitation.

The addition of exogenous db-cAMP enhanced capacitation in rat (Toyoda and Chang 1974) and rabbit (Rosado *et al*. 1974) spermatozoa. Further evidence for the involvement of cAMP in fertilisation has accrued in subsequent years (Visconti *et al*. 1995*a*, 1995*b*, 1998, 1999*c*; Harrison *et al*. 1996; Harrison 2003; Tardif *et al*. 2004; Harrison and Gadella 2005; Fraser *et al*. 2006). However, despite a great deal of research, our understanding of the cell biology of cAMP in spermatozoa, particularly in ram, remains incomplete. The levels of intracellular cAMP are regulated by the activity of two enzymes that are implicated in its generation (adenylate cyclase, AC) and degradation (phosphodiesterase, PDE). Bicarbonate stimulates AC activity and transiently increases cAMP levels, which favours protein tyrosine phosphorylation during capacitation (Okamura *et al*. 1985; Visconti *et al*. 1998; Harrison and Miller 2000). Our results show that addition of db-cAMP on its own to ram spermatozoa has little or no effect on protein tyrosine phosphorylation. Only in the presence of PDE inhibitors + db-cAMP (the cocktail) was there significant stimulation of tyrosine phosphorylation of several specific proteins of approximately 50, 60, 65, 80 and 110 kDa. In agreement with other species (Ficarro *et al*. 2003; Harayama *et al*. 2004; Nagdas *et al*. 2005), immunofluorescence data indicate that these phosphoproteins are found mostly in the sperm tail where they have an implied role in stimulating and modulating motility. PDEs are a diverse enzyme group containing 11 different families of which six have been identified in mouse spermatozoa in different subcellular localisations (Baxendale and Fraser 2005). The sperm tail seems especially rich in PDEs. In ram spermatozoa in particular, cAMP breakdown by PDE activity is ~100 times greater than its synthesis by adenylyl synthetase (Tash 1976). This explains why added db-cAMP is only effective in the presence of PDE inhibitors like caffeine and theophylline.

The implication of the cAMP-dependent PKA pathway in protein tyrosine phosphorylation in ram spermatozoa was confirmed by assessing the effect of PKA inhibition. Our results showed that H-89, a relatively specific PKA inhibitor (Chijiwa *et al*. 1990), caused a decrease in the level of protein tyrosine phosphorylation in accordance with our previous reports in ram (Grasa *et al*. 2006), and others in mouse (Visconti *et al*. 1995*b*) and boar (Tardif *et al*. 2004) spermatozoa. It is fair to say, however, that the decrease was modest, suggesting that other pathways may be involved. Recently, Branham *et al*. (2006) reported that acrosomal exocytosis in human spermatozoa is mediated via cAMP activation of Epac, a guanine nucleotide exchange factor for the GTPase Rap, rather than protein kinases. It is not known whether this pathway is involved in the capacitation events that precede the acrosome reaction or not, but if so, it would help to explain the relative insensitivity of tyrosine phosphorylation to PKA inhibition and the critical importance of cAMP levels in ram spermatozoa.

Our current efforts are directed towards identifying the specific molecules that undergo differential phosphorylation at tyrosine residues during ram sperm capacitation.

Acknowledgements

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