Calmodulin antagonists differentially affect capacitation-associated protein tyrosine phosphorylation of mouse sperm components

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Accepted 28 January 2003
doi:10.1242/jcs.00396

Summary
Sperm capacitation in vitro is thought to be correlated with the increased protein tyrosine phosphorylation of a subset of sperm components. Our group recently used a pharmacological approach to demonstrate that calmodulin (CaM), a 17 kDa calcium sensor protein, has a role in sperm capacitation. In the present study, we have used several CaM antagonists in an attempt to characterize further the role of CaM in capacitation-associated protein tyrosine phosphorylation of sperm components. Our data demonstrate, first, that mouse spermatozoa incubated in a medium that favors capacitation undergo increased protein tyrosine phosphorylation in a time-dependent manner. Second, inclusion of six CaM antagonists individually in an in vitro incubation medium prevented sperm capacitation, as demonstrated by their diminished ability to undergo agonist-induced acrosome reaction. Third, half of the CaM antagonists (compound 48/80, W13 and CaM-binding domain) had no effect on protein tyrosine phosphorylation or sperm motility. Fourth, by contrast, three CaM antagonists (W7, ophiobolin A and calmidazolium) significantly inhibited protein tyrosine phosphorylation of sperm components (42, 56, 66, 82 and 95 kDa) and adversely affected their motility without altering viability as assessed by propidium iodine staining. Finally, inclusion of purified CaM in the capacitation medium significantly increased tyrosine phosphorylation of 82 kDa and 95 kDa components. Combined, these data suggest that CaM antagonists prevent capacitation by interfering with multiple regulatory pathways, and do so either with or without adverse effects on sperm motility and protein tyrosine phosphorylation.

Key words: Sperm capacitation, Protein tyrosine phosphorylation, Mammalian spermatozoa, Calmodulin, Calmodulin antagonists, Sperm mobility

Introduction
Testicular spermatozoa are morphologically differentiated cells; however, they are neither motile nor are they able to bind to zona-intact eggs. They acquire progressive motility during transit from the proximal to the distal region of the epididymis. In addition to the maturation in the epididymis, spermatozoa must undergo functional changes between the events of mating and fertilization. During residence in the female genital tract, they undergo a series of biochemical and functional modifications collectively referred to as capacitation (Yanagimachi, 1994; Tulsiani et al., 1997). The known changes associated with sperm capacitation include: (1) increased adenylyl cyclase activity and increased levels of cyclic adenosine monophosphate (cAMP) (Parish et al., 1994); (2) protein tyrosine phosphorylation of a subset of sperm components (Visconti et al., 1995a); (3) elevated intrasperm pH (Tulsiani et al., 1998; Abou-Haila and Tulsiani, 2000); (4) Ca2+ influx (Singh et al., 1978); (5) loss of surface components (Fraser, 1984); (6) modification/alteration of the sperm plasma membrane (PM) (O’Rand, 1982); and (7) changes in the lectin-binding pattern of spermatozoa (Lee and Ahuja, 1987). However, the sequence of these modifications and their significance in sperm capacitation remains unclear.

All mammalian spermatozoa studied thus far undergo capacitation after residing in the female genital tract for a certain period of time (Yanagimachi and Chang, 1963; Yanagimachi, 1994). Sperm cells can also be capacitated in vitro by incubating in a chemically defined medium supplemented with serum albumin, usually BSA (Dow and Bavister, 1989), or methyl-β-cyclodextrin (Visconti et al., 1999) and energy substrates, such as glucose and pyruvate as well as reagents used in the Krebs-Ringer bicarbonate medium. It should be noted that albumin is a major protein both in the female genital tract and in the in vitro capacitation medium. The protein is believed to facilitate capacitation by efflux of sterols (mainly cholesterol) from the sperm PM (Cross, 1998; Visconti et al., 1999). The in vivo/in vitro removal of sterols is believed to increase fluidity and permeability of the sperm PM, initiating capacitation.

It is important to emphasize that capacitation is correlated with protein tyrosine phosphorylation of a subset of sperm molecules (Visconti and Kopf, 1998; Visconti et al., 1999) as well as with hyperactivated motility (Fraser, 1995). Although, in some instances, changes in sperm motility pattern (hyperactivity) and capacitation can be separated, the two events are generally considered dependent since one of the
features of sperm capacitation is hyperactivated mobility (Yanagimachi, 1994). Capacitation, therefore, is the net result of changes that occur (1) on the sperm head, which enables it to bind to the extracellular coat of the egg, the zona pellucida, and undergo the acrosome reaction (AR) and (2) in the flagellum, which facilitates hyperactivated sperm motility.

In a previous report (Bendahmane et al., 2001), we demonstrated the functional significance of calmodulin (CaM), a 17 kDa Ca2+-binding protein, in capacitation and in the neoglycoprotein-/zona pellucida-induced AR. To characterize further the involvement of CaM in capacitation-associated protein tyrosine phosphorylation, we have examined the effect of CaM antagonists on the biochemical changes in capacitating spermatozoa. Data included in this report demonstrate that CaM inhibitors differentially affect capacitation-associated protein tyrosine phosphorylation of a subset of sperm components, and hyperactivated motility.

Materials and Methods

Animals and reagents

Mature Swiss (ND4) male mice (9–10-weeks old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed under 16L:8D conditions with free access to food and water for at least 3 days before experiments were begun. The animals were sacrificed by CO2 asphyxiation. All procedures using animals were approved by the Institutional Animal Care and User Committee. Methyl-β-cyclodextrin, neoglycoproteins [ngps; including p-aminophenyl-N-acetyl-β-D-glucosaminidase-BSA (GlcNAc-BSA), p-aminophenyl-N-acetyl β-D-galactosaminidase-BSA (GalNAc-BSA) and p-aminophenyl-α-D-mannopyranosidase-BSA (Man-BSA)] and CaM purified from bovine brain were purchased from Sigma (St Louis, MO). CaM antagonists, namely calmodulin-binding domain CaM inhibitors differentially affect capacitation in the absence or presence of CaM antagonists or purified CaM by incubation at 37°C under 5% CO2 in air as described (Bendahmane et al., 2001).

Sperm motility

Spermatozoa were examined by phase-contrast microscopy, and the percentage of motile spermatozoa (a combined measure of flagellum beat and forward motility) was determined by scoring 100 spermatozoa.

Sperm viability

Sperm samples (300 µl) incubated in the absence (control) or presence (experimental) of CaM antagonist were stained at 36°C with 12 µM propidium iodide for 15 minutes as described (Garner and Johnson, 1995). Spermatozoa (200) were observed and counted under phase-contrast/epifluorescence illumination with a Zeiss Axiophot photomicroscope, and the percentage of dead (red stain on the nucleus) and live (nonstained) spermatozoa were calculated.

Assessment of acrosomal status

The status of the sperm acrosome was assessed using the Coomassie brilliant blue G-250 dye method of Larson and Miller (Larson and Miller, 1999) as described (Bendahmane et al., 2001). Spermatozoa (200) were scored in duplicate, and the percentage of spermatozoa that had undergone the AR was calculated.

Preparation and use of CaM antagonists

A 100-fold concentrated stock solution was prepared either in distilled water (compound 48/80, CBD, W7, and W13), methanol (OA), or 10% DMSO (CZ). Aliquots of the stock solution were mixed with the sperm suspension to achieve the desired concentration. In experiments where antagonists were solubilized in DMSO or methanol, the sperm cells were incubated concurrently with the solvent as a control. The final concentration of DMSO and methanol was 0.1% and 1%, respectively. At these concentrations, the organic solvents had no effect on capacitation, the AR, sperm motility, cell viability, or protein tyrosine phosphorylation.

Preparation and capacitation of the cauda spermatozoa

Caudal epididymidis was excised and freed of the fat-pad, blood vessels and connective tissue under a dissecting microscope. The tissue was transferred to a 3 ml Petri dish containing 1 ml EKRB medium pre-warmed to room temperature (24°C), and cut in several places with iridectomy scissors to release the spermatozoa into the medium. After 5 minutes, the sperm suspension was transferred to a 15 ml centrifuge tube. The concentration of the spermatozoa dispersed in the medium was assessed using a Neubauer hemocytometric chamber. The sperm concentration was adjusted to 1×10⁶/ml by adding EKRB medium containing 6 mg BSA/ml (the final concentration of BSA was 3 mg/ml) or 2 mM methyl-β-cyclodextrin (the final concentration of cyclodexrin was 1 mM) and subjected to capacitation in the absence or presence of CaM antagonists or purified CaM by incubation at 37°C under 5% CO2 in air as described (Bendahmane et al., 2001).

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the method of Towbin et al. (Towbin et al., 1979), as described (Tulsiani et al., 1995). Following the transfer, the membrane was blocked for 1 hour in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 and 30 mg BSA/ml at 25°C, and incubated with monoclonal anti-phosphotyrosine antibody PY20 at 1:5000 dilution for 1 hour at room temperature. After this incubation, the membrane was washed for 30 minutes in TBS/0.1% Tween 20 (once for 15 minutes and three times for 5 minutes each), followed by incubation with anti-mouse IgG-HRP conjugate at 1:3000 dilution for 1 hour. The membrane was washed for 30 minutes as above and the protein-tyrosine-phosphorylated components were revealed using the ECL kit according to the manufacturer’s instructions. Tyrosine-phosphorylated bands were scanned and their intensities quantified using IPLab Gel H Program.

Statistical analysis
The results are presented as mean±s.d.; means of control and experimental groups were compared using the Newman-Keuls test after one-way ANOVA to determine statistically significant differences (P<0.05).

Results
Tyrosine phosphorylation of mouse sperm components
It has been demonstrated that the cauda epididymal spermatozoa incubated in a defined medium supplemented with BSA or methyl-β-cyclodextrin undergo a capacitation-associated increase in protein tyrosine phosphorylation of a subset of molecules (Visconti et al., 1995a; Visconti and Kopf, 1998). Our first approach was to confirm these studies using the EKRB solution as a capacitation medium. Mouse cauda epididymal spermatozoa were capacitated in vitro by incubating in the above medium containing 3 mg BSA/ml or 1 mM methyl-β-cyclodextrin at 37°C under 5% CO2 in air. At indicated time intervals, aliquots were withdrawn and sperm cells were washed and extracted in Laemmli’s buffer. The sperm extract was electrophoresed, and the protein-tyrosine-phosphorylated molecules were revealed using anti-phosphotyrosine antibody. Data presented in Fig. 1 show a time-dependent increase in tyrosine phosphorylation of several sperm molecules in the medium containing BSA (Fig. 1A) or methyl-β-cyclodextrin (Fig. 1B). The increase in phosphorylation was significant after 60 minutes and 90 minutes of incubation, a result that is in agreement with the published reports in several species including the mouse (Visconti et al., 1995a; Visconti et al., 1999).

CaM antagonists block capacitation of the mouse spermatozoa
In a previous report, we demonstrated that inclusion of CaM antagonists during in vitro capacitation of mouse spermatozoa inhibited/prevented the physiological priming of the cells. Since the CaM inhibitors used in this report were from new lots, it was important to demonstrate that all of the inhibitors were effective in preventing sperm capacitation. Mouse spermatozoa in EKRB supplemented with 3 mg BSA/ml were incubated in the absence or presence of a known concentration of the CaM antagonist for 30 minutes at 37°C under 5% CO2 in air (Bendahmane et al., 2001). The concentration of each of the six antagonists used here was demonstrated by us to have a maximum effect on the capacitation of spermatozoa from mice (Bendahmane et al., 2001) and rats (Bendahmane et al., 2002). Following this incubation, the spermatozoa were pelleted, gently suspended in an equal volume of EKRB medium supplemented with 3 mg BSA/ml, and incubated in the absence or presence of agonists (ngps) for 30 minutes at 37°C under 5% CO2 in air to determine whether CaM antagonists have effectively blocked the ability of cells to become acrosomally responsive, an accepted criteria for capacitation (Cross and Razy-Faulkner, 1997; Cross, 1998). The ngps used in this report were demonstrated by our group to mimic mouse zona pellucida (ZP) and induce the AR (Loeser and Tulsiani, 1999; Loeser et al., 1999; Bendahmane et al., 2001). Following this incubation, the cells were fixed, stained and the acrosomal integrity examined by criteria described previously (Larson and Miller, 1999; Bendahmane et al., 2001). Data presented in Table 1 demonstrate the effectiveness of all inhibitors in preventing or significantly inhibiting the agonist (ngp)-induced ability of spermatozoa to undergo the AR.

Effects of CaM antagonists on capacitation-associated sperm protein tyrosine phosphorylation
In preliminary studies, we incubated cauda epididymal spermatozoa in EKRB medium supplemented with 3 mg BSA/ml or 1 mM methyl-β-cyclodextrin in the absence or presence of six CaM antagonists for 60 minutes and 90 minutes at 37°C under 5% CO2 in air. Interestingly, three of the six

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**Fig. 1.** Time-dependent protein tyrosine phosphorylation of mouse spermatozoa. Cauda epididymal spermatozoa were suspended in EKRB medium supplemented with 3 mg BSA/ml (A) or 1 mM methyl-β-cyclodextrin (B) and incubated at 37°C for 60 minutes or 90 minutes as described under Materials and Methods. At the indicated time intervals, spermatozoa were washed, extracted and subjected to SDS-PAGE. The resolved components were transferred to a nitrocellulose sheet, and the tyrosine-phosphorylated bands were revealed. A representative experiment, out of a total of at least three experiments, is shown. The sharp band at the position of 116 kDa appears in all lanes and is nonspecific. This band is also present in all lanes in Figs 2 and 3. Spermatozoa incubated in EKRB medium (without BSA or methyl-β-cyclodextrin) for 60 minutes or 90 minutes revealed little or no phosphorylation.
antagonists used (W7, OA and CZ) showed a significant inhibitory effect on the tyrosine phosphorylation of all sperm components (Fig. 2A). By contrast, the other three CaM antagonists (compound 48/80, W13 and CBD) had no effect on tyrosine phosphorylation of sperm (Fig. 3A). These incubations were carried out for 90 minutes, the time needed for optimal levels of protein tyrosine phosphorylation. Data presented in Figs 2A and 3A demonstrate differential affects of the two sets of CaM antagonists on protein tyrosine phosphorylation of the mouse sperm molecules.

Since the presence of BSA or methyl-β-cyclodextrin in the capacitation medium had similar effects on sperm protein tyrosine phosphorylation, it was of interest to determine the effect of CaM antagonists on tyrosine phosphorylation of the sperm molecules. Spermatozoa were capacitated in EKRB medium supplemented with methyl-β-cyclodextrin (instead of BSA) in the absence or presence of CaM antagonist. After 90 minutes of incubation, sperm cells were extracted, electrophoresed, transferred to a nitrocellulose membrane and the phosphorylated bands were revealed as above. Data demonstrate that tyrosine phosphorylation of sperm proteins, capacitated either in medium supplemented with BSA (Fig. 2A; Fig. 3A) or methyl-β-cyclodextrin (Fig. 2B; Fig. 3B) responded to the CaM antagonists in a similar manner.

Do CaM antagonists have an adverse effect on sperm motility and sperm viability?

Our next approach was to examine whether CaM inhibitors have any effect on sperm viability and sperm motility. Spermatozoa were capacitated in EKRB medium supplemented with 3 mg BSA/ml with or without the known concentration of CaM antagonists. After 90 minutes incubation at 37°C in 5% CO2 in air, aliquots were checked for sperm motility and sperm viability. The remaining cells were pelleted and used to examine protein tyrosine phosphorylation by the procedures described above. Data presented in Table 2 demonstrate that, whereas OA, W7 and CZ inhibited/prevented sperm motility and protein tyrosine phosphorylation, the other three antagonists (compound 48/80, W13 and CBD) had no effect on these parameters (Table 2). Furthermore, OA, W7 and CZ inhibited/prevented motility without affecting sperm

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**Table 1. Effect of CaM antagonists on capacitation of mouse spermatozoa**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antagonist concentration (μM)</th>
<th>Ngp concentration (μg/ml)</th>
<th>Man-BSA</th>
<th>GlcNAc-BSA</th>
<th>GalNAc-BSA</th>
<th>Acrosome reacted spermatozoa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>–</td>
<td>–</td>
<td>25.8±2.0</td>
<td>26.2±1.9</td>
<td>25.5±2.5</td>
<td></td>
</tr>
<tr>
<td>Agonist alone</td>
<td>–</td>
<td>10</td>
<td>41.8±3.3</td>
<td>45.7±4.0</td>
<td>44.5±5.4</td>
<td></td>
</tr>
<tr>
<td>Ophiobolin A</td>
<td>20</td>
<td>10</td>
<td>28.0±1.3</td>
<td>28.2±2.5</td>
<td>26.2±2.4</td>
<td></td>
</tr>
<tr>
<td>W7</td>
<td>25</td>
<td>10</td>
<td>34.2±1.9</td>
<td>34.7±2.5</td>
<td>32.3±2.1</td>
<td></td>
</tr>
<tr>
<td>CZ</td>
<td>2.5</td>
<td>10</td>
<td>25.5±3.3</td>
<td>25.3±2.0</td>
<td>25.7±2.5</td>
<td></td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>2.0</td>
<td>10</td>
<td>26.2±2.6</td>
<td>26.7±3.1</td>
<td>27.2±2.3</td>
<td></td>
</tr>
<tr>
<td>W13</td>
<td>1.58</td>
<td>10</td>
<td>28.7±1.8</td>
<td>27.5±2.2</td>
<td>28.8±2.8</td>
<td></td>
</tr>
<tr>
<td>CBD</td>
<td>0.16</td>
<td>10</td>
<td>27.7±2.3</td>
<td>27.8±2.0</td>
<td>30.5±2.2</td>
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</tbody>
</table>

*Mouse cauda epididymal spermatozoa were incubated in EKRB medium supplemented with 3 mg BSA/ml in the absence or presence of the indicated concentration of antagonists at 37°C for 30 minutes under 5% CO2 in air. Following this incubation, the cells were pelleted by centrifugation and the antagonists were removed by aspirating the supernatant. The pelleted cells were suspended in an equal volume of EKRB with 3 mg BSA/ml and incubated in the presence of ngps (10 μg/ml) at 37°C for 30 minutes as above. The three ngps were previously shown to mimic mouse ZP and induce the AR (Loeser and Tulasi, 1999; Bendahmane et al., 2001). The cells were fixed, stained and the acrosomal integrity assessed. Data reported are an average of three independent experiments carried out in triplicate with ± indicating s.d.

†These values are significantly different (P<0.05) from the control spermatozoa or spermatozoa capacitated in the presence of CaM antagonists.
viability, as monitored by propidium iodide staining; approximately 50% of the sperm cells remained viable after 90 minutes of incubation in the presence or absence of these antagonists.

Purified CaM increases tyrosine phosphorylation of two sperm proteins

In a previous report (Bendahmane, 2001), we demonstrated that the inclusion of purified CaM in the incubation medium largely reversed the AR-blocking effects of antagonist during in vitro sperm capacitation. Furthermore, our group has recently reported that the inclusion of purified CaM in in vitro incubation medium did not alter the overall rate of capacitation; however, its presence accelerated initial stages of capacitation-associated membrane primings (Abou-Haila and Tulsiani, 2002), a result consistent with the suggestion that CaM has a role in sperm capacitation. In this study, we have attempted to assess the role of CaM in protein tyrosine phosphorylation of sperm molecules. Mouse spermatozoa were incubated in the capacitation medium in the absence or presence of 10 μM purified CaM. Following this incubation, the sperm cells were pelleted, extracted, electrophoresed and the tyrosine-phosphorylated bands were revealed. The bands present in the control and experimental groups were scanned and quantitated by densitometric analyses. Each band in the control group was considered 100% and the relative intensity of the same molecular weight band in the experimental group was calculated. Data presented in Fig. 4 demonstrate that CaM increased capacitation-associated tyrosine phosphorylation of

<table>
<thead>
<tr>
<th>Table 2. Effect of CaM antagonists on viability, motility and protein tyrosine phosphorylation of mouse spermatozoa*</th>
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</thead>
<tbody>
<tr>
<td><strong>Addition</strong></td>
</tr>
<tr>
<td>None (control)</td>
</tr>
<tr>
<td>Ophiobolin A</td>
</tr>
<tr>
<td>W7</td>
</tr>
<tr>
<td>CZ</td>
</tr>
<tr>
<td>Compound 48/80</td>
</tr>
<tr>
<td>W13</td>
</tr>
<tr>
<td>CBD</td>
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</table>

*Mouse spermatozoa were capacitated by incubating with or without the indicated concentration of CaM antagonists at 37°C for 90 minutes under 5% CO2 in air. Following this incubation, aliquots were checked for sperm viability, sperm motility and sperm protein tyrosine phosphorylation.
†Values are the mean of three independent assays.
‡Most of the motile spermatozoa (>95%) displayed hyperactivated motility.
§Normal levels of protein tyrosine phosphorylation (see Fig. 3).
¶Significantly different from the control group.
**Traces.

Fig. 3. Effects of CaM antagonists on tyrosine phosphorylation of the mouse sperm components. Spermatozoa were incubated at 37°C for 90 minutes in EKRB medium supplemented with 3 mg BSA/ml (A) or 1 mM methyl-β-cyclodextrin (B) in the absence (control) or presence of different concentrations (μM) of CaM antagonists (compound 48/80, 2; W13, 1.58; CBD, 0.16). Tyrosine phosphorylation was revealed as above. The figure shows data of a representative experiment. Similar results were obtained in two other experiments. A slight variation in phosphorylated proteins around 80 kDa in this figure (compare A versus B) is probably due to different running conditions.

Fig. 4. Effect of CaM on protein tyrosine phosphorylation of mouse sperm components. Cauda epididymal spermatozoa were incubated in EKRB medium supplemented with 3 mg/ml BSA in the absence or presence of 10 μM CaM. After 90 minutes at 37°C under 5% CO2 in air, sperm cells were pelleted, washed, extracted, resolved by SDS-PAGE, and protein-tyrosine-phosphorylated components were revealed as above. Bands from the exposed X-ray film were scanned and the intensities of control and CaM-treated groups quantified. The asterisk (*) indicates a significant difference (P<0.05) between the control and experimental groups.
Discussion

Although the significance of capacitation has been known for over 50 years (Chang, 1951; Austin, 1951), the intracellular and extracellular changes that render sperm physiologically primed and fertilization competent (i.e. capacitated) are poorly understood. Most experts agree that capacitation is the end result of multiple molecular changes in the sperm flagellum and head. These changes lead to a hyperactivated motility and enable sperm surface receptor(s) to bind to its complementary ligand(s) on the ZP and undergo a Ca2+-dependent signal transduction cascade. The net result is fusion of the outer acrosomal membrane (OAM) and overlying PM at multiple sites and release of acrosomal contents (glycohydrolases, proteinases, etc.) by regulated exocytosis (Tulsiani et al., 1998; Abou-Haila and Tulsiani, 2000). The hydrolytic action of glycohydrolases and proteinases, released at the site of sperm-zona binding, along with the enhanced thrust generated by the hyperactivated beat pattern of the bound spermatozoa, are important factors that regulate the penetration of ZP (Suarez, 1996; Yanagimachi, 1970; Katz and Drobnis, 1990). Although hyperactivated motility is associated with capacitation, the two phenomena are thought to be related but independent events (Olds-Clarke, 1990).

Several recent studies have demonstrated that in vitro capacitation of spermatozoa from several species is also associated with increased protein tyrosine phosphorylation of a subset of macromolecules (Visconti et al., 1995a; Visconti et al., 1999). In this study, we have used a pharmacological approach in an attempt to characterize further the inter-relationship between capacitation and protein tyrosine phosphorylation. First, we confirmed published reports by demonstrating that there was indeed a time-dependent increase in tyrosine phosphorylation of a subset of molecules when the mouse spermatozoa were incubated in a medium that favors capacitation (Fig. 1).

The capacitation-associated tyrosine phosphorylation of the sperm components has been reported to be dependent on Ca2+ and NaHCO3. The concentration of these components needed in the incubation medium for the protein tyrosine phosphorylation to occur was reported to be correlated with that needed for sperm capacitation, a result consistent with the suggestion that tyrosine phosphorylation of sperm proteins and sperm capacitation are highly correlated (Visconti et al., 1995a; Visconti et al., 1999). Thus, our next goal was to determine the inter-relationship between capacitation and protein tyrosine phosphorylation. This was accomplished by incubating the cauda epididymal spermatozoa in EKR-2, supplemented with BSA or methyl-β-cyclodextrin, in the absence and presence of several CaM antagonists, and examining sperm capacitation and protein tyrosine phosphorylation. The six CaM inhibitors used in two previous reports (Bendahmane et al., 2001; Bendahmane et al., 2002) and in the present study (Table 1) were effective in inhibiting/preventing sperm capacitation as evident by their poor response to the agonist-induced AR. Three of the six antagonists used (W7, OA and CZ) inhibited/prevented capacitation (Table 1), sperm motility (Table 2), and tyrosine phosphorylation of all sperm components (Fig. 2). However, the effect of these inhibitors varies, with OA and W7 having a greater effect on motility and tyrosine phosphorylation than CZ (Table 2). The adverse effect of these antagonists on sperm motility was not due to altered cell viability, as assessed by the propidium iodine protocol, since the sperm cells in the absence or presence of CaM antagonists displayed similar viability (Table 2). By contrast, the other three CaM antagonists (compound 48/80, W13 and CBD) inhibited capacitation (Table 1) without adversely affecting sperm motility or cell viability (Table 2) or protein tyrosine phosphorylation (Fig. 3). Taken together, data from the two sets of CaM antagonists suggest that they inhibit/prevent capacitation by at least two regulatory mechanisms (Fig. 5). The first event might adversely affect sperm motility before inhibiting protein tyrosine phosphorylation (Fig. 5A) or vice versa (Fig. 5B). The second event could be inhibition/prevention of capacitation without affecting sperm motility or protein tyrosine phosphorylation (Fig. 5). These data imply that all CaM antagonists used in this study act on the sperm head (inhibiting/preventing capacitation as evident by the failure of the sperm to respond to agonist), but only W7, OA and CZ have an adverse effect on the sperm tail (Table 2). These results suggest a close inter-relationship between sperm protein tyrosine phosphorylation and motility. The proposed relationship is consistent with the experimental evidence from many investigators suggesting that protein tyrosine phosphorylation plays an important role in the control of the hyperactivated motility (Vijayaraghavan et al., 1997; Si and Okuno, 1999; Holt and Harrison, 2002). Our data,

![Fig. 5](image_url)

**Fig. 5.** Proposed regulatory pathways to explain the effect of CaM antagonists on sperm capacitation, sperm motility and protein tyrosine phosphorylation. All antagonists used inhibited/prevented capacitation, as evident by the poor response of spermatozoa to agonist-induced AR (Table 1). Three antagonists (W7, OA and CZ) inhibited sperm motility before protein tyrosine phosphorylation (A) or vice versa (B), and capacitation. The second set of antagonists (i.e. compound 48/80, W13 and CBD) adversely affected capacitation without altering sperm motility or protein tyrosine phosphorylation. Inclusion of purified CaM in the capacitation medium significantly enhanced tyrosine phosphorylation of the 95 kDa and 82 kDa proteins (A).
However, do not allow us to conclude whether protein tyrosine phosphorylation precedes motility or vice versa.

The fact that tyrosine phosphorylation and sperm capacitation are stimulated by cAMP analogues, but inhibited by CaM antagonists or protein kinase A (PKA) inhibitors, strongly suggests that the CaM/PKA signaling pathways are involved in both processes (Galantino-Homer et al., 1997; Visconti et al., 1999; Leclerc et al., 1996). Further support for the role of CaM-dependent protein tyrosine phosphorylation during sperm capacitation comes from the observation that calyculin A, an inhibitor of protein phosphatases 1 and 2A (Ishihara et al., 1989), enhances protein phosphorylation and sperm capacitation (Leclerc et al., 1996; Furuya, 1993). The present pharmacological approach demonstrates that a set of CaM antagonists (compound 48/80, W13 and CBD) can inhibit/prevent capacitation without adversely affecting sperm motility or protein tyrosine phosphorylation. These data raise questions about the proposed tight correlation between the tyrosine phosphorylation and capacitation of mouse spermatozoa. Future studies should be directed to determine whether the antagonists that affected both processes have additional molecular targets beyond CaM. New data will provide insights into the mechanisms underlying protein tyrosine phosphorylation and sperm capacitation.

Mammalian spermatozoa contain two main parts: (1) the head with the acrosomal (anterior head) and post-acrosomal (posterior head) regions, and (2) the flagellum (Yanagimachi, 1994; Tulsi and Abou-Haila, 2001). Whereas the receptor(s) responsible for binding to the ZP and initiating a signal transduction cascade prior to induction of the AR are present on the anterior head region of the capacitated spermatozoa (Tulsi and Abou-Haila, 2001), the hyperactivated motility is a net result of molecular changes on the flagellum (Yanagimachi, 1970; Yanagimachi, 1994; Suarez, 1996). Since all antagonists used in this study inhibited/prevented capacitation, it is reasonable to suggest that their blocking effects are due to inhibition of CaM in the sperm head. However, why only W7, OA and CZ adversely affected sperm motility and protein tyrosine phosphorylation or why OA and W7 (but not CZ) nearly blocked motility is not known at the present time. One possibility is that these three reagents might not elicit their effects through CaM alone, but have other molecular targets such as FSP95 (Mandal et al., 1999), a testis-specific fibrous-sheath protein. Alternatively, perhaps only some of the reagents are able to enter the tail and block CaM. Additional studies will be needed to resolve these issues.

It should be noted that in vitro capacitation requires BSA (Dow and Bavister, 1989) or methyl-β-cyclodextrin (Visconti et al., 1999) in the incubation medium. The two reagents are thought to promote capacitation through their ability to efflux cholesterol from the sperm PM (Visconti et al., 1999). The loss of cholesterol increases fluidity and permeability of the sperm PM, initiating the signaling events leading to capacitation. Since the two sets of CaM antagonists have similar effects on BSA- or methyl-β-cyclodextrin-supplemented medium during in vitro capacitation, it is reasonable to conclude that these antagonists inhibit/prevent capacitation in these media by blocking the same signaling pathway. It would be interesting to determine if CaM antagonists inhibit/prevent capacitation by blocking the loss of cholesterol from the sperm membrane. Data from this approach will provide new insights into the inter-relationship between the loss of cholesterol, protein tyrosine phosphorylation and sperm capacitation.

How does CaM influence protein tyrosine phosphorylation? The acidic protein has been reported to stimulate adenyl cyclase (Gross et al., 1987; Kopf and Vacquier, 1984), a PM enzyme responsible for the synthesis of cAMP. Thus, it seems likely that the three antagonists that inhibit/prevent the protein tyrosine phosphorylation do so by their inhibitory effect on CaM-dependent adenyl cyclase activity. The inhibition of this enzyme activity is expected to start a chain reaction by reducing levels of cAMP and inhibiting the proposed cross-talk between cAMP and tyrosine kinase second messenger systems (Visconti and Kopf, 1998), thereby inhibiting protein phosphorylation and sperm capacitation. As a corollary, it seems likely that the inclusion of purified CaM in the capacitation medium will stimulate adenyl cyclase, which in turn will increase the levels of cAMP. The increased cAMP levels, suggested to act upstream on the protein tyrosine phosphorylation (Visconti et al., 1995b; Visconti and Kopf, 1998), might be important for the observed increases in protein tyrosine phosphorylation of the 95 kDa and 82 kDa sperm molecules. Whether the 95 kDa component is a unique hexokinase (Kalab et al., 1994), FSP95 (Mandal et al., 1999) or has both ZP3-binding and a tyrosine kinase activity (Leyton and Salting, 1989) is not yet known.

Does CaM enter the capacitating spermatozoa and trigger intracellular signaling that promotes the AR? A published report provided evidence suggesting that inclusion of pure CaM in the culture medium promotes DNA synthesis and cell proliferation in human leukemia lymphocytes (Crocker et al., 1988). These data strongly suggest that CaM can influence mitosis through an extracellular mechanism. Furthermore, extracellular CaM was reported to inhibit monocyte tumor necrosis factor release and augment neutrophil elastase release (Houston et al., 1997). These data, in conjunction with the reported occurrence of receptor(s) on monocytic cell lines, strongly suggest that CaM possesses an extracellular signaling role in addition to its intracellular regulatory functions (Houston et al., 1997). Whether CaM functions via sperm surface receptors is not yet known.

The differential effects of the two sets of CaM antagonists on sperm capacitation and protein tyrosine phosphorylation raise many interesting questions on the inter-relationship between sperm cholesterol, protein tyrosine phosphorylation and capacitation. Cross has reported that, when human spermatozoa are incubated in capacitating medium in vitro, there is a gradual loss of cholesterol; however, the acrosomal responsiveness does not develop for some time (Cross, 1998). This result is consistent with the author’s suggestion that cholesterol loss precedes capacitation. It would be interesting to see if CaM acts upstream or downstream to the cholesterol efflux or if there are regional modifications of the sperm membranes.

In summary, this work uses a pharmacological approach to examine the inter-relationship between motility, tyrosine phosphorylation of sperm components and sperm capacitation. Our data provide evidence suggesting the occurrence of multiple pathways that regulate sperm components and sperm capacitation. It would be interesting to use CaM inhibitors to determine whether the loss of cholesterol and sperm capacitation is tightly correlated.
from these studies will allow new insights for blocking capacitation and altering sperm function.

The secretarial assistance of Loreita Little is gratefully acknowledged. We are indebted to Malika Bendahmane for her technical support and to Aida Abou-Haila, Malika Bendahmane and Benjamin J. Danzo for critically reading the manuscript. This work was supported in part by Grants HD25869 and HD34041 from the National Institute of Child Health and Human Development.

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