Different Signaling Pathways in Bovine Sperm Regulate Capacitation and Hyperactivation

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ABSTRACT

Hyperactivated sperm motility is characterized by high-amplitude and asymmetrical flagellar beating that assists sperm in penetrating the oocyte zona pellucida. Other functional changes in sperm, such as activation of motility and capacitation, involve cross talk between the cAMP/PKA and tyrosine kinase/phosphatase signaling pathways. Our objective was to determine the role of the cAMP/protein kinase A (PKA) signaling pathway in hyperactivation. Western blot analyses of detergent extracts of whole sperm and flagella were performed using antiphosphotyrosine antibody. Bull sperm capacitated by 10 μg/ml heparin and/or 1 mM dibutyryl-cAMP plus 100 μM 3-isobutyl-1-methylxanthine exhibited increased protein tyrosine phosphorylation without becoming hyperactivated. Procaine (5 mM) or caffeine (10 mM) immediately induced hyperactivation in nearly 100% of motile sperm but did not increase protein tyrosine phosphorylation. After 4 h of incubation with caffeine, sperm expressed capacitation-associated protein tyrosine phosphorylation but hyperactivation was significantly reduced. Sperm initially hyperactivated by procaine or caffeine remained hyperactivated for at least 4 h in the presence of Rp-cAMPS (cAMP antagonist) or PKA inhibitors H-89 or H-8. Pretreatment with inhibitors also failed to block induction of hyperactivation; however, the inhibitors did not block protein tyrosine phosphorylation when sperm were incubated with capacitating agents, thereby verifying inhibition of the cAMP/PKA pathway. While induction of hyperactivation did not depend on cAMP/PKA, it did require extracellular Ca++. These findings indicate that hyperactivation is mediated by a Ca++ signaling pathway that is separate or divergent from the pathway associated with acquisition of acrosomal responsiveness and does not involve protein tyrosine phosphorylation downstream of the actions of procaine or caffeine.

calcium, cyclic adenosine monophosphate, signal transduction, sperm, sperm capacitation

INTRODUCTION

In the mammalian oviduct, as the time of ovulation approaches, sperm begin to hyperactivate, initiating a vigorous swimming pattern characterized by high flagellar bend amplitude and beat asymmetry [1, 2]. In the mouse, hyperactivated sperm can be observed through the wall of the oviduct, pulling off of attachments to the mucosal epithelium and rapidly escaping into the central lumen from peripheral storage sites [3, 4]. In vitro, hyperactivated hamster sperm penetrate the zona pellucida far more effectively than sperm prevented from undergoing hyperactivation [5].

Although physiological functions of hyperactivated motility have been established, little is known about the mechanism by which it is initiated. Hyperactivation has been considered part of the capacitation process because sperm have been observed to hyperactivate while undergoing capacitation in vitro. Capacitation is generally defined as the changes a sperm must complete to become competent to fertilize an oocyte; however, for the sake of clarity, we will use the more restrictive definition of acquisition of acrosomal responsiveness [2]. During capacitation, several sperm proteins become phosphorylated on tyrosine residues and this phosphorylation has been demonstrated to be regulated by a cAMP pathway through activation of protein kinase A (PKA) [6–8]. Some of the proteins that become tyrosine phosphorylated during capacitation have been localized to the flagellum, and therefore it has been proposed that they are involved in hyperactivation [9–11]. Despite the apparent relationship between hyperactivation and capacitation, it has been demonstrated that one can occur without the other under certain conditions [12–15]. Furthermore, the expression of protein tyrosine phosphorylation is observed during capacitation of hamster sperm hours before the onset of hyperactivated motility [10, 16, 17]. The role of cAMP-regulated protein tyrosine phosphorylation in mediating hyperactivation therefore remains unclear.

Bull sperm can be incubated in media that induce capacitation without producing hyperactivated motility [15]. Hyperactivation can be induced in uncapacitated bull sperm by procaine [18, 19] or caffeine [15]. Because these agents can hyperactivate sperm without capacitation as a prerequisite, they provide a model system for elucidating the signaling pathway downstream of their points of action and for distinguishing the regulatory pathway of hyperactivation from that of capacitation. We used this model system to examine the role of cAMP-dependent protein tyrosine phosphorylation in the regulation of hyperactivation.

MATERIALS AND METHODS

Chemicals and Media

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) with the following exceptions. BSA and HEPES were purchased from Calbiochem Corporation (La Jolla, CA). Molecular Probes (Eugene, OR) provided 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA). Antiphosphotyrosine antibody (clone 4G10) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody were obtained from Upstate Biotechnology (UBI, Lake Placid, NY).

A modified Tyrode balanced salt solution (TALP) [20] was used for washing and incubating sperm. The medium consisted of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.4 mM NaH2PO4, 1.1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 1 mM pyruvate, 25.4 mM lactate, 50 μg/ml gentamycin, and 6 mg/ml fraction V BSA (pH 7.45, 290–300 mOsm/kg).
Sperm Preparation and Capacitation

Bovine semen was collected from bulls of proven fertility and generously donated by the bovine artificial insemination service Genex Cooperative, Inc. (Ithaca, NY). Each bull was used only once in a set of replicate experiments. Semen was diluted 1:5 in TALP immediately after collection and transported to the laboratory within 60 min in a 39°C (bovine body temperature) water jacket. Sperm were washed free of seminal plasma by centrifugation (170 × g, 10 min) followed by two 1:10 dilutions and centrifugations in TALP. Washed sperm were incubated at 39°C under 5% CO₂.

Sperm were capacitated by 4 h of incubation at 50 × 10⁶/ml in TALP containing 10 μg/ml heparin [20] and/or 1 mM dibutyryl-c-AMP (db-cAMP) plus 100 μM 3-isobutyl-1-methylxanthine (IBMX) [8]. To assess capacitation, samples of sperm were treated with 100 μg/ml lysophosphatidylcholine (LPC) [20] or TALP alone (solvent control) for 30 min to induce acrosome reactions and then fixed in 1% paraformaldehyde in PBS. Aliquots of sperm suspensions were placed on slides and covered with coverslips. Acrosome status was visually assessed for at least 200 sperm per treatment using 640X differential interference contrast (DIC) microscopy.

Induction of Hyperactivation

Sperm numbers were adjusted to 100 × 10⁶/ml and added to an equal volume of 10 mM procaine [19], 20 mM caffeine [15], or TALP alone (solvent control). Samples of sperm were videotaped within 15 sec (designated 0 h) and then every 30 min for 4 h. To test the effects of extracellular Ca²⁺ on procaine- or caffeine-induced hyperactivation, treatments were prepared in TALP that lacked 2 mM Ca²⁺ and contained 10 mM BAPTA (Ca²⁺-deficient) to buffer extracellular Ca²⁺ below the intracellular Ca²⁺ level of nonhyperactivated sperm (~30 mM) [21, 22] (calculated using Max Chelator: www.stanford.edu/~cpaton/maxc.html).

Analysis of Sperm Motility

Treated sperm were placed on slides on a 39°C stage of a Zeiss Axi-135 microscope and videotaped using 400X differential interference microscopy (Carl Zeiss, Inc., Thornbrook, NY) and stroboscopic illumination at 30 Hz provided by a xenon flash tube (Chadwick-Helmuth Co., El Monte, CA). Videotaping was conducted using a black-and-white Dage CCD 72 video camera (Dage-MTI, Inc., Michigan City, IN) connected to a Panasonic AG-7300 Super VHS videocassette recorder (Panasonic Industrial Co., Secaucus, NY). Videotapes were used to determine percent motility and hyperactivation of at least 200 sperm per treatment.

In bull sperm, hyperactivated motility can be easily distinguished from activated motility. Activated motility is characterized by symmetrical beating of the flagellum that results in a linear swimming trajectory (Fig. 1A). Hyperactivated motility is characterized by asymmetrical flagellar beating that produces circular or figure-eight swimming patterns (Fig. 1, B and C). In a figure-eight swimming pattern, sperm complete a circle in two flagellar beats. In our experiments, less than 2% of motile sperm were sluggish or showed an abnormal pattern.

To provide images of flagellar movement patterns for Figure 1, segments of videocassette recorded using stroboscopic illumination at 60 Hz were digitized using a CG-7 frame grabber (Scion Corp., Frederick, MD) in a Macintosh G4 computer (Apple Computers, Cupertino, CA). A video frame was selected from the sequence of digitized images in which the principal and reverse flagellar bends could be clearly distinguished.

Sperm Incubated Under Capacitating Conditions

Sperm capacitated by incubation for 4 h with heparin and/or db-cAMP plus IBMX (Fig. 2) did not hyperactivate (Table 1). Motility was checked every half hour, and at no time point were more than 2% of motile sperm hyperactivated. Including the protein tyrosine phosphatase inhibitor sodium orthovanadate (0.2 mM) with the capacitating treatments did not induce hyperactivated motility nor did increasing concentrations of db-cAMP and IBMX to 2 mM
and 200 μM, respectively (data not shown). In sharp contrast, procaine stimulated hyperactivated motility in 100% of motile sperm within 15 sec of addition (Table 1). Hyperactivated sperm swam in a vigorous figure-eight pattern (Fig. 1C). After 1 h of incubation in procaine, the movement pattern changed from figure eight to circular, apparently resulting from a decrease in maximal flagellar bend amplitude; however, flagellar beating remained asymmetrical, as is characteristic of hyperactivation. Hyperactivated motility was maintained for at least 4 h without negatively affecting overall sperm motility.

When uncapacitated sperm were treated with 10 mM caffeine, 98% displayed hyperactivated motility within 15 sec (Table 2). Sperm treated with caffeine also remained hyperactivated for at least 4 h, but hyperactivation was significantly reduced when compared with 0 h. Lower doses of caffeine produced lower incidence of hyperactivation; therefore, 10 mM was used to produce the maximal response and reveal any protein tyrosine phosphorylation.

Capacitation But Not Procaine- or Caffeine-Induced Hyperactivation Is Associated with Increased Protein Tyrosine Phosphorylation

Three different protein extraction protocols yielded three different sets of proteins (Fig. 3).

Examination of SDS extracts revealed that sperm capacitated by heparin in TALP medium exhibited a pattern of higher protein tyrosine phosphorylation than those incubated in TALP alone (Fig. 4A), as previously reported [8, 27]. Addition of db-cAMP and IBMX further increased tyrosine phosphorylation; however, it did not result in increased capacitation, as assessed by the incidence of LPC-induced acrosome reactions (Fig. 2). Extracts of Triton X-100-soluble proteins or extracts of flagellar proteins only revealed increases in tyrosine phosphorylation when sperm were incubated with db-cAMP plus IBMX or heparin and db-cAMP plus IBMX (Fig. 4, D and G).

Sperm hyperactivated by procaine did not exhibit any change in phosphotyrosine content in either whole sperm or flagellar protein extracts (Fig. 4, B, E, and H) even though 100% of motile sperm were hyperactivated. No change in protein tyrosine phosphorylation was detected in sperm that remained hyperactivated for 4 h in the presence of procaine. Sperm incubated in procaine for 4 h did not capacitate (LPC-induced acrosome reactions: procaine, 29.6 ± 5.0; heparin, 48.6 ± 0.7; control, 25.6 ± 3.2). When sperm incubated for 4 h with heparin were treated with procaine to induce hyperactivation, there was no further elevation of protein tyrosine phosphorylation (data not shown).

Noncapacitated sperm were also induced to hyperactivate using 10 mM caffeine (Fig. 1B), and although 98% of motile sperm hyperactivated within 15 sec of caffeine addition, extracts of these sperm showed no increase in protein tyrosine phosphorylation (Fig. 4, C, F, and I). Sperm treated with caffeine for 4 h did capacitate, according to the LPC assay (Fig. 2), as well as display elevated protein tyrosine phosphorylation similar to heparin or db-cAMP plus IBMX treatments (Fig. 4, C and F). Similar results were obtained whether gel lanes were loaded with identical amounts of protein or with extracts from identical numbers of sperm. Additionally, capacitated and hyperactivated sperm were also extracted with SDS sample buffer in the presence of 40 mM dithiothreitol, which resulted in the extraction of several more tyrosine phosphoproteins; however, the same protein tyrosine phosphorylation patterns were produced (data not shown).

Inhibition of the cAMP/PKA Pathway Eliminates Increased Protein Tyrosine Phosphorylation Associated with Capacitation But Has No Effect on Hyperactivated Motility

Pharmacological agents were used to suppress components of the cAMP-dependent PKA pathway. Sperm initially hyperactivated by procaine or caffeine (5 min) remained hyperactivated for at least 4 h after Rp-cAMPS (a

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**TABLE 1. Effects of procaine and capacitating agents on bull sperm hyperactivated motility.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Control</td>
<td>79.7 ± 0.3</td>
<td>72.7 ± 2.3</td>
</tr>
<tr>
<td>Procaine</td>
<td>76.7 ± 1.8</td>
<td>71.0 ± 1.5</td>
</tr>
<tr>
<td>Heparin</td>
<td>81.3 ± 1.3</td>
<td>72.7 ± 1.2</td>
</tr>
<tr>
<td>db-cAMP, IBMX</td>
<td>82.3 ± 1.2</td>
<td>71.2 ± 0.9</td>
</tr>
<tr>
<td>Heparin, db-cAMP, IBMX</td>
<td>81.7 ± 2.0</td>
<td>72.7 ± 1.3</td>
</tr>
</tbody>
</table>

* a Sperm were treated with 5 mM procaine or 10 μg/ml heparin with or without 1 mM db-cAMP plus 100 μM IBMX (mean ± SEM, n = 3 bulls).

* b Procaine treatment different from all other treatments (P < 0.01).

**TABLE 2. Effects of caffeine on bull sperm hyperactivated motility.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Control</td>
<td>92.3 ± 1.4</td>
<td>85.3 ± 2.9</td>
</tr>
<tr>
<td>Caffeine</td>
<td>90.0 ± 2.3</td>
<td>83.7 ± 2.9</td>
</tr>
</tbody>
</table>

* a Sperm were treated with or without 10 mM caffeine (mean ± SEM, n = 3 bulls).

* b Caffeine treatment different from control; caffeine at 4 h different from 0 h (P < 0.01).
Sperm treated with caffeine or heparin and/or db-cAMP plus IBMX failed to exhibit increased protein tyrosine phosphorylation when incubated for 4 h with the inhibitors (Fig. 5), thereby verifying inhibition of the cAMP/PKA pathway.

**Extracellular Ca²⁺ Is Required for Procaine or Caffeine Induction of Hyperactivation**

Sperm treated with procaine or caffeine did not hyperactivate while in Ca²⁺-deficient TALP (Tables 5 and 6). When the same sperm were resuspended in medium containing procaine or caffeine and 2 mM Ca²⁺, they immediately expressed hyperactivated motility. Upon transferring sperm back to Ca²⁺-deficient medium containing procaine, hyperactivated motility almost immediately reverted to activated motility. In a separate set of experiments, sperm treated with procaine or caffeine in Ca²⁺-deficient medium containing 1 mM db-cAMP and 100 μM IBMX did not hyperactivate during 4 h of incubation (data not shown).

**DISCUSSION**

Our results indicate that hyperactivation induced by procaine or caffeine is mediated by a pathway requiring Ca²⁺ signaling but is not dependent on the cAMP/PKA-regulated protein tyrosine phosphorylation associated with capacitation (that is, acquisition of acrosomal responsiveness). These findings suggest that the pathways regulating acrosomal responsiveness and hyperactivation diverge downstream of the actions of procaine or caffeine or that they are entirely separate.

Increased intracellular cAMP has been correlated in sperm with development of both capacitation [28] and hyperactivated motility [17, 29, 30]. Nevertheless, in those studies, hyperactivation and capacitation occurred concurrently, making it difficult to determine whether cAMP is involved in signaling capacitation, hyperactivation, or both. Unlike rodent [12, 17] and human [31] sperm, bull sperm capacitated in vitro do not express hyperactivated motility. Heparin-induced capacitation of bull sperm is associated with increased intracellular cAMP [28], and agents designed to elevate cAMP such as db-cAMP and IBMX, have also been shown to capacitate bull sperm [8]. These capacitating agents, however, did not promote hyperactivated motility in our experiments. In sharp contrast, procaine immediately stimulated noncapacitated sperm to hyperactivate in a vigorous figure-eight pattern. Evidence suggests that the downstream action of procaine is not mediated by cAMP because procaine treatment of noncapacitated guinea pig sperm did not elevate intracellular cAMP [19] nor was increased cAMP-dependent protein kinase activity detected in noncapacitated bull sperm hyperactivated by local anesthetics [32]. Thus, hyperactivation can be stimulated to occur independently of cAMP-regulated capacitation.

In the present study, capacitation of bull sperm was associated with increased protein tyrosine phosphorylation, but hyperactivated motility was not. It has been proposed that tyrosine phosphorylation of flagellar proteins observed during capacitation is related to the development of hyperactivated motility [9–11]. Increased tyrosine phosphorylation of flagellar proteins after treatment with cAMP-elevating drugs has further fueled support for involvement of tyrosine phosphorylation in regulating hyperactivation [29, 33, 34]; however, these same cAMP-elevating drugs simultaneously supported capacitation. Our use of procaine to induce hyperactivation eliminated the confounding factor.

**FIG. 4.** Phosphotyrosine content of protein extracted from capacitated or hyperactivated sperm. Sperm were capacitated for 4 h in TALP containing 10 μg/ml heparin and/or 1 mM db-cAMP plus 100 μM IBMX (A, D, G), hyperactivated by 5 mM procaine (B, E, H), or 10 mM caffeine (C, F, I). SDS extracts of whole sperm (5 × 10⁶ sperm/lane) (A–C), 1% Triton X-100 extracts of whole sperm (25 × 10⁶ sperm/lane) (D–F), and flagellar extracts (25 × 10⁶ sperm/lane) (G–I) were separated by SDS-PAGE and analyzed by immunoblotting with antiphosphotyrosine antibody. Numbers to the left of the blots represent molecular mass standards (× 10⁻³). 

CAMP antagonist) or PKA inhibitors H-89 or H-8 were added (Tables 3 and 4). Control treatments consisting of TALP plus inhibitors (without procaine or caffeine) had no effect on motility. Induction of hyperactivation was not inhibited when sperm were pretreated for 30 min (data not shown) or 4 h with inhibitors before exposure to procaine or caffeine (Tables 3 and 4). A range of concentrations of 37.5–75 μM for H-89 and 0.5–1 mM for Rp-cAMPS and H-8 were tested, and data for 1 mM Rp-cAMPS, 75 μM H-89, and 1 mM H-8 are presented in Tables 3 and 4.
of capacitation. Western blot analyses revealed no change in protein phosphotyrosine levels in bull sperm hyperactivated by procaine. This is significant, considering that 100% of the motile sperm hyperactivated in response to procaine. Based on these results, we employed two additional protein extraction protocols in hopes of identifying tyrosine-phosphorylated substrates associated with hyperactivation; however, neither Triton X-100-soluble proteins nor flagellar extracts revealed increased protein tyrosine phosphorylation in sperm hyperactivated by procaine. These results are consistent with recent reports that sperm from CatSper1- and CatSper2-null mutant mice fail to hyperactivate but exhibit normal protein tyrosine phosphorylation [35, 36].

In our study, procaine could have bypassed a physiological signal transduction pathway involving cAMP-dependent protein tyrosine phosphorylation; therefore, we also examined caffeine-induced hyperactivation of noncapacitated sperm and obtained the same results. Caffeine did elevate protein tyrosine phosphorylation after 4 h of incubation with sperm, but hyperactivation was significantly reduced while acrosomal responsiveness was augmented, suggesting that increased protein tyrosine phosphorylation was most likely associated with capacitation and not hyperactivation.

Our data confirmed that inhibiting the cAMP/PKA pathway eliminates the increased protein tyrosine phosphorylation associated with capacitation, as previously reported [8]; however, inhibiting this pathway did not prevent hyperactivation from occurring in response to procaine or caffeine treatment. It is well established that capacitation and protein tyrosine phosphorylation are regulated by cAMP at the level of PKA. PKA is a serine/threonine protein kinase, but substrates associated with its activity are actually phosphorylated on tyrosine residues of sperm proteins. Thus, there appears to be cross talk between the cAMP/PKA and tyrosine kinase and/or phosphatase signaling pathways in sperm. Capacitation [37] and increased protein tyrosine phosphorylation are prevented when sperm are treated with Rp-cAMPs or H-89 [6–8]. Because agents that block capacitation did not affect hyperactivated motility, these results further suggest that separate or diverging regulatory pathways mediate hyperactivation and capacitation.

Induction of hyperactivation by procaine or caffeine was dependent on the availability of extracellular Ca2+. Sperm hyperactivated by procaine almost immediately reverted to activated motility upon transfer to medium in which extracellular Ca2+ was buffered below 50 nM. These results confirm the key role of Ca2+ in regulating hyperactivation established in earlier studies. For example, hamster sperm hyperactivated motility was not maintained in extracellular medium deficient in Ca2+ [38]. Elevated intracellular Ca2+ levels were detected in the flagella of hyperactivated (~200 nM) compared with activated (~50 nM) hamster sperm [21]. Bull sperm were stimulated to hyperactivate when treated with thimerosal or thapsigargin, which release Ca2+ from intracellular stores [15]. While treatment of guinea pig sperm with procaine did not increase intracellular cAMP, it did increase intracellular Ca2+ when extracellular Ca2+ was available [19]. It has already been demonstrated that extracellular Ca2+ is required for caffeine-induced hyperactivation and that caffeine promotes a rise in intracellular Ca2+ in the flagellum [15]. Thus, procaine and caffeine may induce hyperactivation by raising intracellular Ca2+. Procaine is known to stimulate Ca2+ influx by acting on membranes to increase permeability to Ca2+ [39–42]. Evidence indicates that caffeine is neither working as a phosphodiesterase inhibitor nor via a ryanodine receptor when it rapidly stimulates hyperactivation [15]. Caffeine, can, however, activate Ca2+-permeable cation channels in the plasma membrane [43, 44]. On the other hand, long-term incubation with caffeine did produce capacitation and protein tyrosine phosphorylation, indicating that caffeine was working slowly as a phosphodiesterase inhibitor. Additional experiments are required to determine exactly how procaine and caffeine affect flagellar Ca2+ to stimulate hyperactivation in sperm.

### Table 3. Effects of inhibitors on procaine-induced hyperactivated motility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87.3 ± 1.9</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>Proxaine</td>
<td>71.3 ± 2.3</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>+ Rp-CAMPS</td>
<td>77.0 ± 0.6</td>
<td>99.3 ± 0.7</td>
</tr>
<tr>
<td>+ H-8</td>
<td>81.0 ± 5.6</td>
<td>99.7 ± 0.3</td>
</tr>
<tr>
<td>+ H-8</td>
<td>73.3 ± 4.9</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

Inhibitors, 1 mM Rp-cAMPs, 75 μM H-89, or 1 mM H-8, were added to sperm hyperactivated by 5 mM procaine or sperm were preincubated with inhibitors for 4 h before exposure to procaine (mean ± SEM, n = 3 bulls).

### Table 4. Effects of inhibitors on caffeine-induced hyperactivated motility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.3 ± 2.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Caffeine</td>
<td>87.7 ± 4.7</td>
<td>98.3 ± 1.2</td>
</tr>
<tr>
<td>+ Rp-CAMPS</td>
<td>86.3 ± 6.7</td>
<td>99.7 ± 0.3</td>
</tr>
<tr>
<td>+ H-8</td>
<td>89.7 ± 3.0</td>
<td>97.0 ± 0.6</td>
</tr>
<tr>
<td>+ H-8</td>
<td>84.7 ± 6.9</td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

Inhibitors, 1 mM Rp-cAMPs, 75 μM H-89, or 1 mM H-8, were added to sperm hyperactivated by 10 mM caffeine or sperm were preincubated with inhibitors for 4 h before exposure to caffeine (mean ± SEM, n = 3 bulls).

No difference among caffeine treatments (P > 0.05); all caffeine treatments different from control (P < 0.01).
sperm. Recently, CatSper1, a voltage-gated Ca\textsuperscript{2+} channel located on the mouse sperm flagellum, was found to be critical for hyperactivated motility, providing further insight into the mechanism of Ca\textsuperscript{2+} entry and signaling of hyperactivation [35].

Like hyperactivation, capacitation is dependent on extracellular Ca\textsuperscript{2+} and is associated with elevated intracellular Ca\textsuperscript{2+} [45, 46]. Alterations in sperm plasma membranes, such as loss of cholesterol, initiate the signal transduction pathway that promotes capacitation [47, 48]. These membrane alterations are believed to increase permeability to ions such as Ca\textsuperscript{2+} and HCO\textsubscript{3}\textsuperscript{-}, which enter the cytoplasm and stimulate adenyl cyclase [49–51] to promote cAMP production, leading to the stimulation of PKA and, ultimately, protein tyrosine phosphorylation. Nevertheless, the rise in intracellular Ca\textsuperscript{2+} associated with capacitation must not be sufficient or it must not take place in the proper cytoplasmic compartment to initiate hyperactivation.

There is evidence that increases in intracellular cAMP depend on availability of extracellular Ca\textsuperscript{2+} [17], but db-cAMP plus IBMX can substitute for Ca\textsuperscript{2+} in supporting protein tyrosine phosphorylation and capacitation in rodent sperm [6]. For this reason, we tested whether db-cAMP plus IBMX could enable bull sperm to hyperactivate in the absence of available extracellular Ca\textsuperscript{2+}. Inhibition of hyperactivation in medium devoid of Ca\textsuperscript{2+} could not be overcome by db-cAMP plus IBMX. This indicates that inhibition of hyperactivated motility was not attributed to a decline in cAMP production due to a lack of available extracellular Ca\textsuperscript{2+}.

If procaine or caffeine bypass the portion of a signaling pathway involving cAMP-dependent protein tyrosine phosphorylation, this would suggest that Ca\textsuperscript{2+} operates downstream of tyrosine phosphorylation to promote hyperactivation. A possible example of tyrosine phosphorylation occurring upstream of Ca\textsuperscript{2+} signaling is the phosphorylation of CABYR, a protein that has been localized to the principal piece of the flagellum [11]. CABYR binds Ca\textsuperscript{2+} after becoming tyrosine phosphorylated during capacitation and could therefore serve to sequester Ca\textsuperscript{2+} in the flagellum, raising it to the levels that induce asymmetrical bending. It is important to point out, however, that because CABYR is restricted to the principal piece of the flagellum, it cannot account for changes in the flagellar midpiece, the site of initiation of the flagellar bend. Additional phosphorylated flagellar proteins could act to increase available Ca\textsuperscript{2+} in the axonemal compartment of the midpiece. In any case, our results indicate that, while cAMP/PKA-mediated tyrosine phosphorylation could occur upstream, it does not occur downstream of Ca\textsuperscript{2+} signaling during induction of hyperactivation.

In summary, our results indicate that, in bull sperm, 1) hyperactivation can occur independently of capacitation (acquisition of acrosomal responsiveness), 2) activating the

### Table 5. Effects of extracellular Ca\textsuperscript{2+} on procaine-induced hyperactivation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 2 mM Ca\textsuperscript{2+}</td>
<td>79.0 ± 2.6</td>
<td>2.3 ± 1.4</td>
</tr>
<tr>
<td>Control, 10 mM BAFTA</td>
<td>83.3 ± 2.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Procaine, 2 mM Ca\textsuperscript{2+}</td>
<td>81.0 ± 1.5</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>Procaine, 10 mM BAFTA</td>
<td>81.7 ± 2.7</td>
<td>0.7 ± 0.7</td>
</tr>
</tbody>
</table>

* Sperm were treated with 5 mM procaine while in TALP medium containing 2 mM Ca\textsuperscript{2+} or 10 mM BAFTA (mean ± SEM, n = 3 bulls).

### Table 6. Effects of extracellular Ca\textsuperscript{2+} on caffeine-induced hyperactivation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility (%)</th>
<th>Hyperactivation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 2 mM Ca\textsuperscript{2+}</td>
<td>86.3 ± 0.3</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Control, 10 mM BAFTA</td>
<td>84.7 ± 2.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Caffeine, 2 mM Ca\textsuperscript{2+}</td>
<td>86.7 ± 1.4</td>
<td>98.0 ± 1.0</td>
</tr>
<tr>
<td>Caffeine, 10 mM BAFTA</td>
<td>85.7 ± 1.7</td>
<td>1.0 ± 0.6</td>
</tr>
</tbody>
</table>

* Sperm were treated with 10 mM caffeine while in TALP medium containing 2 mM Ca\textsuperscript{2+} or 10 mM BAFTA (mean ± SEM, n = 3 bulls).

* Procaine (2 mM Ca\textsuperscript{2+}) treatment different from all other treatments (P < 0.01).
signal transduction pathway conducive to capacitation is not sufficient to stimulate hyperactivation, 3) hyperactivated motility is not associated with increased protein tyrosine phosphorylation downstream of the actions of procaine or caffeine, 4) inhibiting the cAMP/PKA signaling pathway has no effect on hyperactivation stimulated by procaine or caffeine, and 5) Ca^{2+} plays an essential role in procaine or caffeine induction of hyperactivation. Hence, we conclude that hyperactivation is mediated by a Ca^{2+} signaling pathway that is separate or divergent from the pathway associated with acquisition of acrosomal responsiveness.

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