BIOLOGY OF REPRODUCTION **65,** 1606–1615 (2001)

An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca^{2+} Store Is Involved in Regulating Sperm Hyperactivated Motility[1]

Han-Chen Ho and Susan S. Suarez[2]

Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

ABSTRACT

Hyperactivated motility, a swimming pattern displayed by mammalian sperm in the oviduct around the time of ovulation, is essential to fertilization. Ca^{2+} has been shown to be crucial for the initiation and maintenance of hyperactivated motility. Nevertheless, how Ca^{2+} reaches the axoneme in the core of the flagellum to switch on hyperactivation is unknown. Ca^{2+}-releasing agents were used to determine whether an intracellular store provides Ca^{2+} to the axoneme. Hyperactivation was induced immediately in bull sperm by thapsigargin, caffeine, and thimerosal. The responses were dose-dependent and were induced in both capacitated and uncapacitated sperm. When external Ca^{2+} was buffered below 50 nM with 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, the response to caffeine was significantly reduced; however, the responses to thapsigargin and thimerosal were not affected. This indicates caffeine-induced hyperactivation depends on external Ca^{2+} influx, whereas hyperactivation by thapsigargin and thimerosal do not. Acrosome reactions were not induced by these treatments; therefore, an acrosomal store was probably not involved. Indirect immunofluorescence labeling showed type I inositol 1,4,5-trisphosphate receptors (IP_3R) in the acrosome and neck region, but no ryanodine receptors (RyR) were found using anti-RyR antibodies or BODIPY FL-X ryanodine. These data indicate that there is an IP_3R-gated Ca^{2+} store in the neck region of sperm that regulates hyperactivated motility.

calcium, gamete biology, signal transduction, sperm, sperm motility and transport

INTRODUCTION

In mammals, fertilization takes place deep inside the female reproductive tract, in the oviduct. Sperm observed at the time of fertilization within the oviduct [1, 2] or after retrieval from the oviduct [3–5] display hyperactivated motility. This motility is characterized by high amplitude, asymmetrical flagellar bends, and a circular or erratic swimming trajectory [6]. There is a close correlation between the ability of sperm to display hyperactivated motility and the ability of sperm to fertilize zona-intact eggs [7, 8]. There is strong evidence that hyperactivated motility is highly advantageous in vivo for getting sperm to the site of fertilization and penetrating the cumulus matrix and zona pellucida [9–12]. Apparently, sperm from *t* haplotype mice fail to fertilize because they hyperactivate prematurely within the female reproductive tract [13, 14]. Therefore, hyperactivated motility is necessary and must be regulated precisely to achieve fertilization.

Despite the importance of hyperactivation to fertilization, very little is known of the biochemical pathways regulating its expression. Although hyperactivation has been described as a subset of capacitation, which is a sequence of events that enable sperm to undergo the acrosome reaction [6], the two activities have been demonstrated to occur independently [8]. It is known that extracellular Ca^{2+} is required to maintain hyperactivation in hamster sperm [15], and that cytoplasmic Ca^{2+} concentration within the flagella of hyperactivated sperm is increased [16, 17]. When sperm are treated with Triton X-100 to disrupt plasma and mitochondrial membranes, essentially leaving only the axoneme and cytoskeletal support, they will show the hyperactivated pattern of flagellar beating when ATP and micromolar amounts of Ca^{2+} are added [18, 19]. The crucial site for the action of Ca^{2+} is therefore the axoneme in the core of the flagellum [19]. Thus, Ca^{2+} is a key regulator of hyperactivation, acting directly on the axoneme. Nevertheless, the means by which Ca^{2+} reaches the axoneme to switch on hyperactivation in intact sperm are still unknown.

Elevation of cytoplasmic Ca^{2+} concentration can occur by entry of Ca^{2+} into cells through the plasma membrane or release of Ca^{2+} from membrane-bounded internal stores. Entry of external Ca^{2+} can occur through several types of Ca^{2+} channels: voltage-gated [20], receptor-linked [21], store-operated [22], or cyclic nucleotide-gated [23]. Ca^{2+} can be released from internal stores via receptor-operated channels. Inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) represent the two principal intracellular Ca^{2+} channels responsible for releasing stored Ca^{2+}. Both IP_3R- and RyR-gated stores are reloaded by thapsigargin-sensitive Ca^{2+}-ATPases [24].

Sperm flagellar beating initiates at the neck region, the base of the flagellum, and propagates through the midpiece and principal piece of the tail to its end. The axoneme at its origin in the flagellar base is completely surrounded by a redundant nuclear envelope (RNE) and closely associated mitochondria of the proximal midpiece. Structurally, these organelles appear to create an internal cytoplasmic compartment with no immediate, direct access to extracellular Ca^{2+}. Therefore, we undertook this study to determine whether Ca^{2+} reaches the axoneme via an internal store in order to switch on hyperactivation.

MATERIALS AND METHODS

Media and Chemicals

A modified Tyrode balanced salt solution (TALP, consisting of 99 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 1 mM pyruvate, 25.4 mM lactate, 50 ng/ml gentamycin, and 6 mg/ml fraction V BSA, pH 7.45, 290–300 mOsm/kg), sterilized through a 0.22-μm Nalgene cellulose acetate filter (Nalge Co., Rochester, NY), was used for washing and incubating sperm. TALP was

[1]Supported by National Science Foundation grant MCB-9818512 to S.S.S.
[2]Correspondence: Susan S. Suarez, Department of Biomedical Sciences, T5 006 Vet Research Tower, Cornell University, Ithaca, NY 14853. FAX: 607 253 3541; e-mail: sss7@cornell.edu

Received: 10 May 2001.
First decision: 12 June 2001.
Accepted: 10 July 2001.

ISSN: 0006-3363. http://www.biolreprod.org

equilibrated in a humidified atmosphere at 39°C containing 5% CO_2 before use.

Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) with the exception of those noted here. BSA, Hepes, and thapsigargin were from Calbiochem Corporation (La Jolla, CA). Glutaraldehyde, osmium tetroxide (OsO_4), sodium cacodylate, uranyl acetate, lead citrate, and Spurr embedding medium were from Electron Microscopy Sciences (Fort Washington, PA). Fluo-3FF/AM was from Teflabs (Austin, TX). Pluronic F-127 and BODIPY FL-X ryanodine were from Molecular Probes (Eugene, OR).

Sperm Preparation

Semen was collected and generously donated by Genex Cooperative, Inc. (Ithaca, NY). Fresh ejaculated bull semen was diluted 1:5 with TALP shortly after collection and transported to the laboratory in a 39°C water bath. Sperm were washed free of seminal plasma by centrifuging twice in fresh TALP at 170 × *g* for 10 min each. Washed sperm were adjusted to 40 × 10^6/ml in TALP and kept at 39°C under 5% CO_2 for the duration of the experiment.

Treatment of Sperm with Pharmacological Agents

Sperm were treated with Ca^{2+}-releasing agents thapsigargin, caffeine, and thimerosal to determine their effectiveness at inducing hyperactivation. Dose-response curves were first constructed for each pharmacological agent tested: thapsigargin (0–20 μM), caffeine (0–10 mM), and thimerosal (0–100 μM). To test the effect of removing extracellular Ca^{2+}, 2 mM extracellular Ca^{2+} originally present in TALP was buffered below 50 nM by 1:1 addition of 20 mM 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) in Ca^{2+}-free TALP (calculated by MaxChelator) [25], then sperm were treated with 10 μM thapsigargin, 2 mM caffeine, 25 μM thimerosal, or vehicles (0.04% dimethyl sulfoxide [DMSO] or Ca^{2+}-free TALP). Thapsigargin was dissolved in DMSO and aliquots were stored frozen at −20°C. The working thapsigargin solution was prepared immediately before addition to the sperm suspension by diluting the stock solution in Ca^{2+}-free TALP. Caffeine and thimerosal were prepared in Ca^{2+}-free TALP just before use.

Effects of capacitation on hyperactivation were tested to determine whether induction of hyperactivation might be dependent on the capacitation state. Following the methods used by Galantino-Homer et al. [26], with some modifications, bull sperm were capacitated in TALP with 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 1 mM dibutyryl-cAMP (db-cAMP) for 2.5 h, while control sperm were incubated in TALP alone. Capacitation was tested in preliminary experiments by using lysophosphatidylcholine (LPC) to induce acrosome reactions [27]. After a 2.5-h incubation in capacitating medium, more than 70% of sperm were acrosome-reacted after LPC treatment. Sperm samples were treated with pharmacological agents right at the beginning of the 2.5-h incubation (0 h) or at its conclusion (2.5 h). An aliquot of sperm suspension and the tested agent or its solvent were mixed 1:1 on a warm slide and rapidly covered with a coverslip. The slide was videotaped immediately as described below.

Analysis of Motility and Acrosomal State of Sperm

Treated sperm were examined on a 39°C stage on a Zeiss Axiovert microscope (Carl Zeiss Inc., Thornbrook, NY). They were videotaped using differential interference contrast (DIC) at 320× magnification. Superimposed on the recorded image was time-date information provided by a videotimer (Model VTG33; For-A Co., Ltd., Newton, MA). A black-and-white Dage CCD 72 video camera (Dage-MTI, Inc., Michigan City, IN) was used with a Panasonic AG-7300 Super VHS videocassette recorder (Panasonic Industrial Co., Secaucus, NJ). Videotaping of each slide was completed in approximately 2 min. The percentage of hyperactivation was determined from the videotapes by categorizing 200 motile sperm for each treatment. Figure 1, B and C, illustrates the movement patterns classified as hyperactivated. Figure 1 was obtained by videotaping sperm using 400× DIC combined with stroboscopic illumination (Chadwick-Helmuth Co., El Monte, CA) at 30 Hz. Tracings of sperm were made on clear acetates from the videotapes by following several successive images along with the positions of sperm head/midpiece junctions from individual frames.

The incidence of acrosome reactions was assessed using 640× DIC for videotaping, and 100 motile sperm were categorized as acrosome-intact or acrosome-reacted in each sample, according to the criteria of Saacke and Marshall [28].

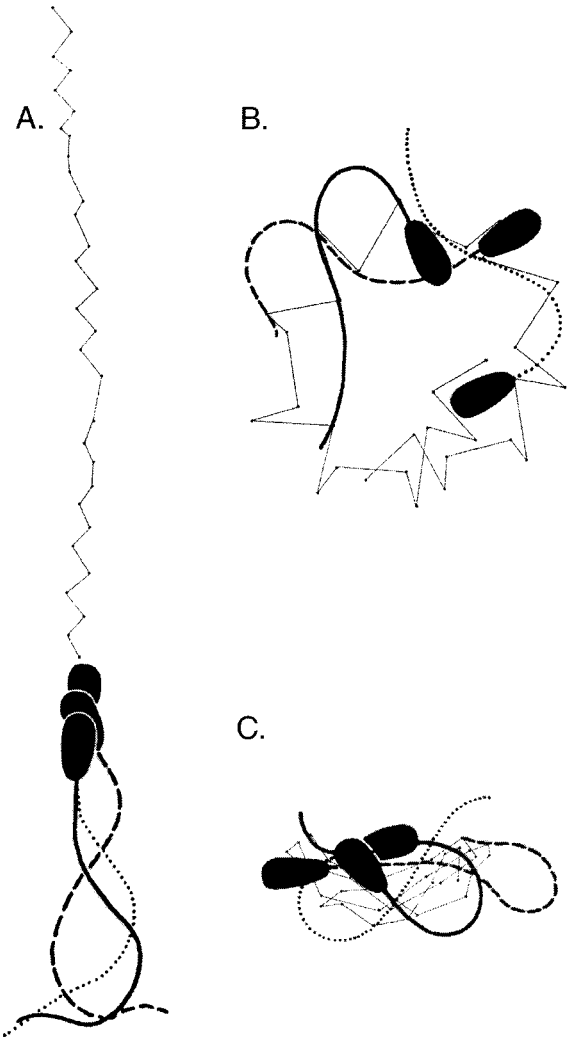

FIG. 1. Bull sperm swimming patterns. Control sperm showing symmetrical flagellar bends and linear trajectory (**A**). Pharmacological agents, thapsigargin, thimerosal, and caffeine induced an increase in bull sperm flagellar bend amplitude and asymmetry, resulting in a circular trajectory (**B**) at low concentrations to a vigorous "figure-8" trajectory (**C**) at high concentrations. Both **B** and **C** were considered as hyperactivated motility. These are tracings from individual videoframes. Successive positions of the head/midpiece junctions at 1/30-sec intervals are indicated by dots joined by straight lines to show sperm trajectories in a 1-sec period.

Ca^{2+} Imaging

Sperm (5 × 10^6/ml) were loaded at 39°C in the dark for 40 min with cell-permeable fluo-3FF/AM (5 μM in the presence of 0.05% Pluronic F-127, diluted in TALP). Extracellular dye was removed by centrifugation for 10 min at 170 × *g*. Sperm were resuspended in medium and incubated for an additional 20 min at 39°C to allow de-esterification of the fluo-3FF/AM to its Ca^{2+}-senstive form fluo-3FF. Dye-loaded sperm were allowed to attach to the glass by their heads in BSA-free TALP in a warm coverslip chamber (PDMI-2 Micro-Incubator, Medical Systems Corp., Greenvale, NY). Ca^{2+}-dependent changes in fluorescence intensity of fluo3-FF were detected with an epifluorescence microscope using an oil immersion 100× Fluar objective (n.a. 1.3, Carl Zeiss Inc.). Stroboscopic illumination was provided by a 75 W xenon arc flash lamp. Light passed through an excitation filter D480/30, a dichroic mirror 505DCLP, and emission filter D530/40 (Chroma Technology Corp., Brattleboro, VT) to a Dage Gen II image intensifier coupled to a Dage CCD 72 video camera. Synchronization of strobe, camera, and image digitization by an Epix Silicon Video Mux board (Northbrook, IL) was controlled by software provided by Elektron Systems (St. Petersburg, FL) and modified by S. Varosi [16]. Consecutive images were digitized at a rate of 2 images/sec. Images were pseudocolored using IPLab spectrum (Signal Analytics, Vienna, VA).

Indirect Immunofluorescence Labeling

Washed ejaculated bull sperm or caudal epididymal hamster sperm were fixed with 1% formaldehyde at room temperature for 15 min. The action of formaldehyde was stopped by adding 0.2 M glycine. Fixative and glycine were discarded after centrifuging the sperm at 500 × *g* for 10 min. The sperm pellet was resuspended in PBS, then 10 μl of sperm suspension were dried down in each well of a Teflon-coated slide (Electron Microscopy Sciences). After rinsing the slides with PBS, 10 μl of 0.1% Triton X-100 were added to each well to permeabilize the sperm membranes for 15 min. Slides were then rinsed and treated with 3% BSA in PBS for 30 min to block nonspecific binding. For IP_3R localization, the permeabilized sperm were probed with affinity-purified polyclonal rabbit antibody, produced against a synthetic peptide consisting of the 15 C-terminal amino acids of rabbit brain type I IP_3R, kindly provided by Drs. R.A. Fissore and J.B. Parys [29]. Controls were incubated with preimmune serum or normal rabbit serum. Antibody binding was visualized using fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Ig) G (Sigma-Aldrich). To detect RyR, permeabilized bull sperm were probed with either BODIPY FL-X ryanodine or monoclonal anti-RyR antibody (clone 34C; Sigma-Aldrich) and the labeling was visualized by tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich). Images were acquired through a SPOT color digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Transmission Electron Microscopy

Washed ejaculated sperm were adjusted to 5×10^6 sperm per 1.8-ml microfuge tube, then centrifuged at 4000 × *g* for 2 min to obtain a pellet. The pellet was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and processed as a tissue block, postfixed with 1% OsO_4, and dehydrated through a graded series of ethanol solutions to 100% acetone. Sperm pellets were embedded in Spurr embedding medium. Thin sections (~80 nm) were cut on a Reichert ultramicrotome and stained with uranyl acetate and lead citrate. All specimens were examined in a Zeiss 902 transmission electron microscope (Carl Zeiss Inc.) at 80 kV.

Scanning Electron Microscopy

Washed ejaculated sperm were fixed with 0.1 M cacodylate buffered 1% OsO_4 at 4°C for 1 h, then macerated with 0.1 M cacodylate buffered 0.1% OsO_4 at 20°C for 18 h to wash out cytoplasmic protein structures [30]. Conductive staining was carried out by immersion of specimens in 2% tannic acid for 2 h, followed by 1% OsO_4 for 1 h, 2% tannic acid for 12 h, and finally immersed in 1% OsO_4 for 1 h. The specimens were dehydrated through a graded series of ethanol solutions and critical point-dried with a Balzer critical point dryer (Technotrade International, Manchester, NH). Specimens were mounted on aluminum stubs, sputter-coated with Au-Pd, and examined using a Hitachi S-4500 field emission scanning electron microscope (Hitachi, Tokyo, Japan).

Statistical Analysis

All data are expressed as means ± SEM. The data were analyzed using Minitab statistical software (Minitab Inc., State College, PA). ANOVA followed by Tukey pairwise comparisons were used to assess differences among treatment groups. Dose dependence of responses to pharmacological agents was tested using linear regression. Values were considered significantly different when $P < 0.05$ in a two-tailed test. Sperm samples from at least three different bulls were used for each set of experiments.

RESULTS

Pharmacological Evidence for a Role of Ca^{2+} Stores in Regulating Hyperactivation

All three pharmacological agents, thapsigargin, caffeine, and thimerosal, immediately induced hyperactivated motility in bull sperm (Fig. 1). The responses were observed to occur within 1 sec when sperm were put in an open chamber that allowed monitoring of sperm motility right after addition of the agents. Sperm treated with thapsigargin, caffeine, and thimerosal all displayed an increased flagellar bend amplitude and asymmetry, resulting in circular trajectories (Fig. 1B) at low concentrations to vigorous ''figure-8'' trajectories (Fig. 1C) at high concentrations. Both swimming patterns (Fig. 1, B and C) were categorized as hyperactivated motility. Control sperm treated with vehicle alone showed the typical swimming pattern of untreated ejaculated sperm, with symmetrical, low-amplitude flagellar bends and linear trajectories.

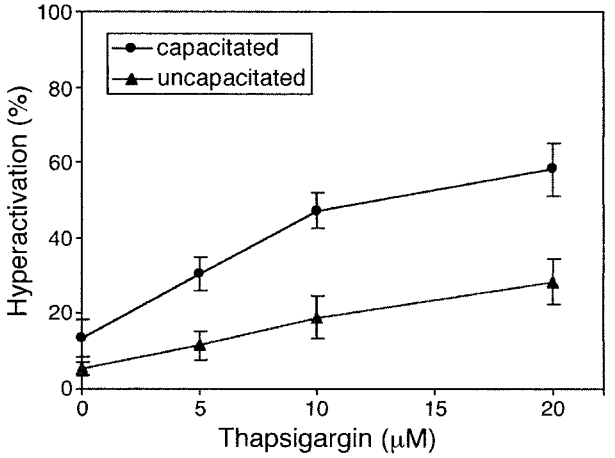

FIG. 2. Dose response of thapsigargin-induced hyperactivation (n = 4 bulls). Sperm were incubated in TALP alone (uncapacitated, ▲) or capacitating medium (capacitated, ●) for 2.5 h before thapsigargin treatment. Thapsigargin (0–20 μM) induced hyperactivation in a dose-dependent manner in both uncapacitated ($R^2 = 0.54$; $P = 0.001$) and capacitated ($R^2 = 0.71$; $P < 0.001$) sperm; however, capacitated sperm responded better than uncapacitated sperm ($P < 0.05$).

Thapsigargin is a potent Ca^{2+}-ATPase inhibitor that depletes intracellular Ca^{2+} stores and thereby increases cytoplasmic Ca^{2+} [24]. Hyperactivation was stimulated by thapsigargin in both uncapacitated and capacitated sperm in a dose-dependent manner (Fig. 2); however, a significant increase of hyperactivation was induced by thapsigargin when sperm were capacitated for 2.5 h with IBMX/db-cAMP before treatment (Fig. 2; $P < 0.05$). Thapsigargin induced hyperactivation even when the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was buffered below sperm resting cytoplasmic Ca^{2+} level [17]. No significant difference was found between sperm incubated in 2 mM $[Ca^{2+}]_o$ and sperm incubated in $[Ca^{2+}]_o$ below 50 nM (Fig. 3; $P = 0.48$). The ability of thapsigargin to induce hyperactivation when external Ca^{2+} was unavailable suggests that release of Ca^{2+} from an intracellular Ca^{2+} store alone is sufficient to initiate hyperactivation.

Caffeine is the most widely used pharmacological agent for inducing intracellular Ca^{2+} release from RyR [31], whereas thimerosal has been shown to release Ca^{2+} from both IP_3R and RyR [32, 33]. Both caffeine and thimerosal stimulated hyperactivated motility in a dose-dependent manner, regardless of whether the sperm were incubated in TALP alone or in TALP with 100 μM IBMX and 1 mM db-cAMP to capacitate them (Fig. 4). Caffeine (2 mM) and 25 μM thimerosal were sufficient to significantly induce hyperactivation (Fig. 5; $P < 0.001$). The responses between capacitated and uncapacitated sperm were not significantly different ($P > 0.3$), indicating that capacitation is not a prerequisite for hyperactivation in these cases. When extracellular Ca^{2+} was buffered below 50 nM by the Ca^{2+} chelator BAPTA to prevent external Ca^{2+} entry, the response to caffeine was significantly reduced (Fig. 6A); however, the response to thimerosal was not affected (Fig. 6B).

The effects of these pharmacological agents on sperm intracellular Ca^{2+} were studied using the fluorescent Ca^{2+}

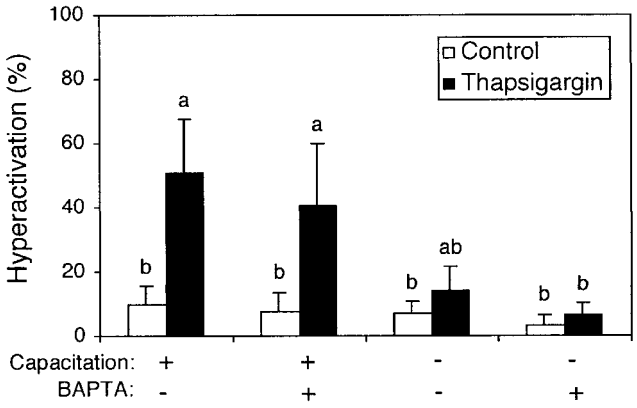

FIG. 3. Effect of extracellular Ca^{2+} on thapsigargin-induced hyperactivation (n = 3 bulls). Sperm were incubated in TALP alone (uncapacitated) or capacitating medium (capacitated) for 2.5 h before thapsigargin treatment. Thapsigargin (10 μM) induced hyperactivation similarly in sperm incubated in media with $[Ca^{2+}]_o$ = 2 mM and $[Ca^{2+}]_o$ below 50 nM (in the presence of BAPTA) (P = 0.48). Control sperm treated with 0.04% DMSO showed no increase in hyperactivation. Bars labeled with different letters indicate significant differences between treatments ($P < 0.05$).

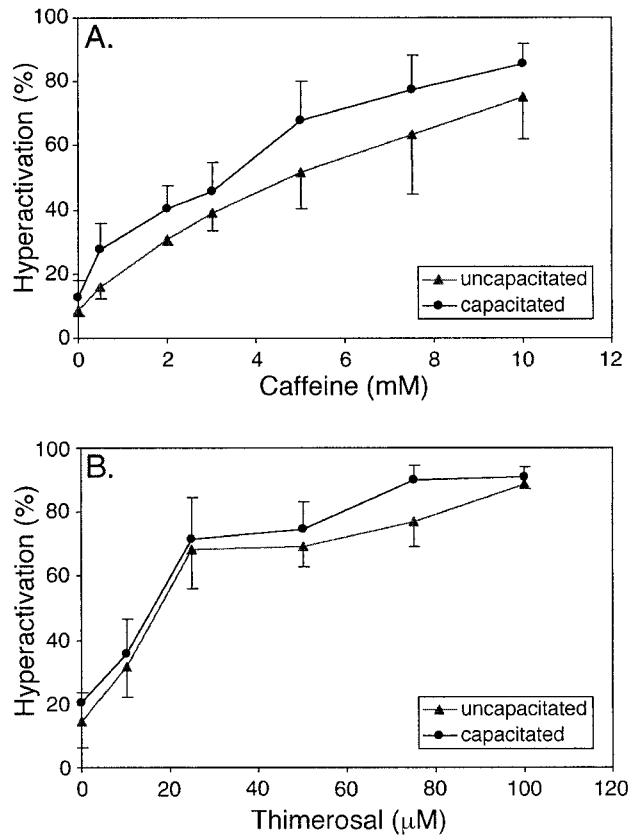

FIG. 4. Dose response of caffeine-induced hyperactivation (**A**) and thimerosal-induced hyperactivation (**B**) (n = 3 bulls). Sperm were resuspended in TALP alone (uncapacitated, ▲) or capacitating medium (capacitated, ●) after washing. Caffeine (0–10 mM) induced hyperactivation in a dose-dependent manner in uncapacitated (R^2 = 0.81; $P < 0.001$) and capacitated (R^2 = 0.83; $P < 0.001$) sperm. Similarly, thimerosal (0–100 μM) induced hyperactivation in a dose-dependent manner in both uncapacitated (R^2 = 0.79; $P < 0.001$) and capacitated (R^2 = 0.78; $P < 0.001$) sperm. No differences were found between uncapacitated and capacitated sperm (**A**, P = 0.20; **B**, P = 0.55).

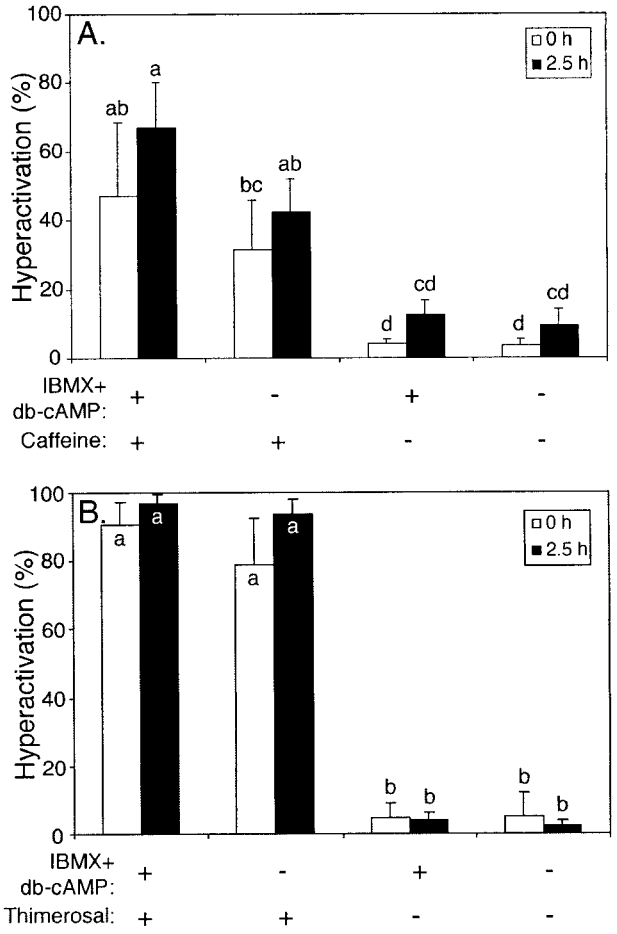

FIG. 5. Effects of capacitation on caffeine-induced hyperactivation (**A**), and thimerosal-induced hyperactivation (**B**) (n = 4 bulls). Sperm were resuspended in TALP with (capacitated) or without (uncapacitated) 100 μM IBMX and 1 mM db-cAMP, and treated with 2 mM caffeine or 25 μM thimerosal immediately (0 h) or after a 2.5-h incubation at 39°C. Incubating sperm in capacitating medium for 2.5 h did not significantly increase the percentage of hyperactivation compared with sperm that had been incubated in the same condition at 0 h. Bars labeled with different letters indicate significant differences between treatments ($P < 0.05$).

indicator fluo-3FF. In consecutive images taken from the same sperm cell before and after treatment, an increase of fluorescence intensity was observed immediately after addition of 10 μM thapsigargin (Fig. 7A), 100 μM thimerosal (Fig. 7B), or 2 mM caffeine (Fig. 7C). The requirement of extracellular Ca^{2+} was observed only when cells were treated with caffeine. The increase of Ca^{2+} was first detected in the neck region of sperm. Addition of TALP alone did not cause an increase in fluorescence intensity (Fig. 7D).

It is well known that the acrosome reaction is also mediated by an increase in intracellular free Ca^{2+}; therefore, we examined the effects of these agents on acrosomes to make sure that the acrosome reaction was not a complicating factor for the results. Acrosomal status was evaluated after addition of pharmacological agents. Sperm with heads that showed a distinct apical ridge were considered intact (Fig. 8A), whereas sperm missing the apical ridge or showing a prominent equatorial segment were considered acrosome-reacted (Fig. 8B) [28]. More than 90% of sperm remained acrosome-intact after thimerosal and caffeine treatments, more than 80% remained acrosome intact after thapsigargin treatment, and no difference was found between treatment groups and controls (Fig. 8). Some sperm sam-

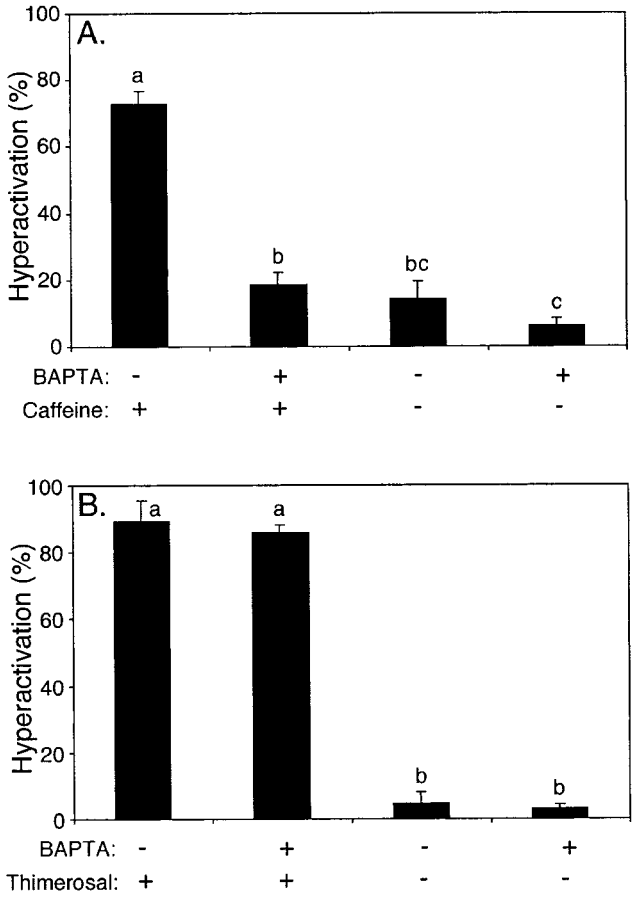

FIG. 6. Effects of extracellular Ca^{2+} on caffeine-induced hyperactivation (**A**) and thimerosal-induced hyperactivation (**B**) (n = 4 bulls). Extracellular Ca^{2+} was buffered by BAPTA to less than 50 nM before treatments. When capacitated sperm were treated with 2 mM caffeine, hyperactivation was significantly increased only when external Ca^{2+} was available ($P < 0.0001$). The response to thimerosal (25 μM) was not affected when external Ca^{2+} was buffered by BAPTA ($P = 0.863$). Bars labeled with different letters indicate significant differences between treatments ($P < 0.05$).

ples were incubated with either 25 μM thimerosal or 2 mM caffeine for 30 min before evaluation, and no significant induction of acrosome reaction was detected (data not shown). It should be noted that in separate experiments, more than 70% of sperm incubated with IBMX and db-cAMP to induce capacitation were acrosome-reacted by the addition of LPC, indicating that these sperm were capacitated.

Evidence of a Unique Intracellular Ca^{2+} Store Located in the Neck Region of Sperm

Indirect immunofluorescence labeling using a polyclonal antibody specific to type I IP_3R showed high densities of IP_3R localized in the acrosome and the neck region of bull sperm (Fig. 9, A and B) and hamster sperm (Fig. 9E). These areas were not labeled by preimmune serum (Fig. 9, C and D) or normal rabbit serum (Fig. 9F). Both monoclonal anti-RyR antibody and BODIPY FL-X ryanodine were used to look for RyR in bull sperm. No labeling was found using anti-RyR antibody (Fig. 9, G and H), whereas BODIPY FL-X ryanodine (10 nM-1 μM) showed nonspecific labeling along the midpiece (Fig. 9I), which could not be blocked by applying 10-fold unlabeled ryanodine (Fig. 9J) [34].

The ultrastructure of the neck region of bull sperm was examined in thin sections. The RNE was located in the neck region and consisted of layers of membrane continuous with the nuclear envelope covering the condensed sperm nucleus (Fig. 10A). The RNE was closely associated with irregularly shaped mitochondria, which usually appeared as long tubular structures oriented lengthwise that extended into the neck. The three-dimensional structure of these irregularly shaped mitochondria could be clearly appreciated under the scanning electron microscope of a specimen with the plasma membrane removed (Fig. 10B). The mitochondria closest to the head were uncoiled from the rest of the helical mitochondrial sheath surrounding the axoneme.

DISCUSSION

The data presented here provide the first evidence that hyperactivated motility may be regulated by an IP_3R-gated intracellular Ca^{2+} store in the neck region of bull sperm. Our primary objective was to investigate the mechanisms regulating hyperactivated motility, especially to understand the source that provides Ca^{2+} to the axoneme in the core of the flagellum. The fact that thapsigargin was able to induce hyperactivation when external Ca^{2+} was unavailable indicates that the release of intracellular Ca^{2+} from a store is sufficient to switch on hyperactivation. Thapsigargin increases intracellular Ca^{2+} by inhibiting Ca^{2+}-ATPase, which is present in the membranes of both IP_3R- and RyR-gated stores [24]. The function of both IP_3R and RyR can be modulated by a variety of agents [35]. The most specific agonist for IP_3R and RyR are inositol 1,4,5-trisphosphate (IP_3) and ryanodine, respectively; however, these agents are not cell permeable. In order to study sperm motility, cell-permeable thimerosal and caffeine were used to understand which type of Ca^{2+} store (IP_3R-gated, RyR-gated, or both) is involved in this process.

The most widely used pharmacological tool for inducing intracellular Ca^{2+} release by activation of RyR is caffeine [31]. Caffeine has also been found to activate Ca^{2+}-permeable cation channels in the plasma membrane to increase intracellular Ca^{2+} [36, 37]. Although caffeine was able to induce hyperactivation in bull sperm, localization experiments indicated that RyR is not present in mature bull sperm. Type 1 and type 3 RyR have been identified in murine testis and in purified populations of spermatocytes and spermatids [38, 39], but only type 3 RyR has been localized in mature mouse sperm [39]. However, type 3 RyR is not sensitive to caffeine [40]. Furthermore, epididymal sperm from a type 3 RyR knockout mouse (provided by Drs. M. Kotlikoff and M. Rishniw, Cornell University) were induced to hyperactivate, indicating that type 3 RyR is not required for switching on hyperactivation (unpublished data). The fact that caffeine could induce hyperactivation only in the presence of extracellular Ca^{2+} indicates that caffeine was probably activating external Ca^{2+} influx pathways instead of releasing Ca^{2+} from RyR stores to induce hyperactivation. Caffeine also produced a different pattern of Ca^{2+} increase in some sperm: Ca^{2+} increase was seen to spread from the neck into the postacrosomal region as well as down the midpiece.

It is well established that initiation of motility in quiescent sperm is coupled with a rise in intracellular cAMP [41, 42]. However, no difference in the percentage of hyperactivated sperm was detected between sperm incubated in TALP alone and those incubated in TALP + db-cAMP + the phosphodiesterase inhibitor IBMX. This indicates that cAMP does not play a role in either caffeine-induced or thimerosal-induced hyperactivation. The immediate re-

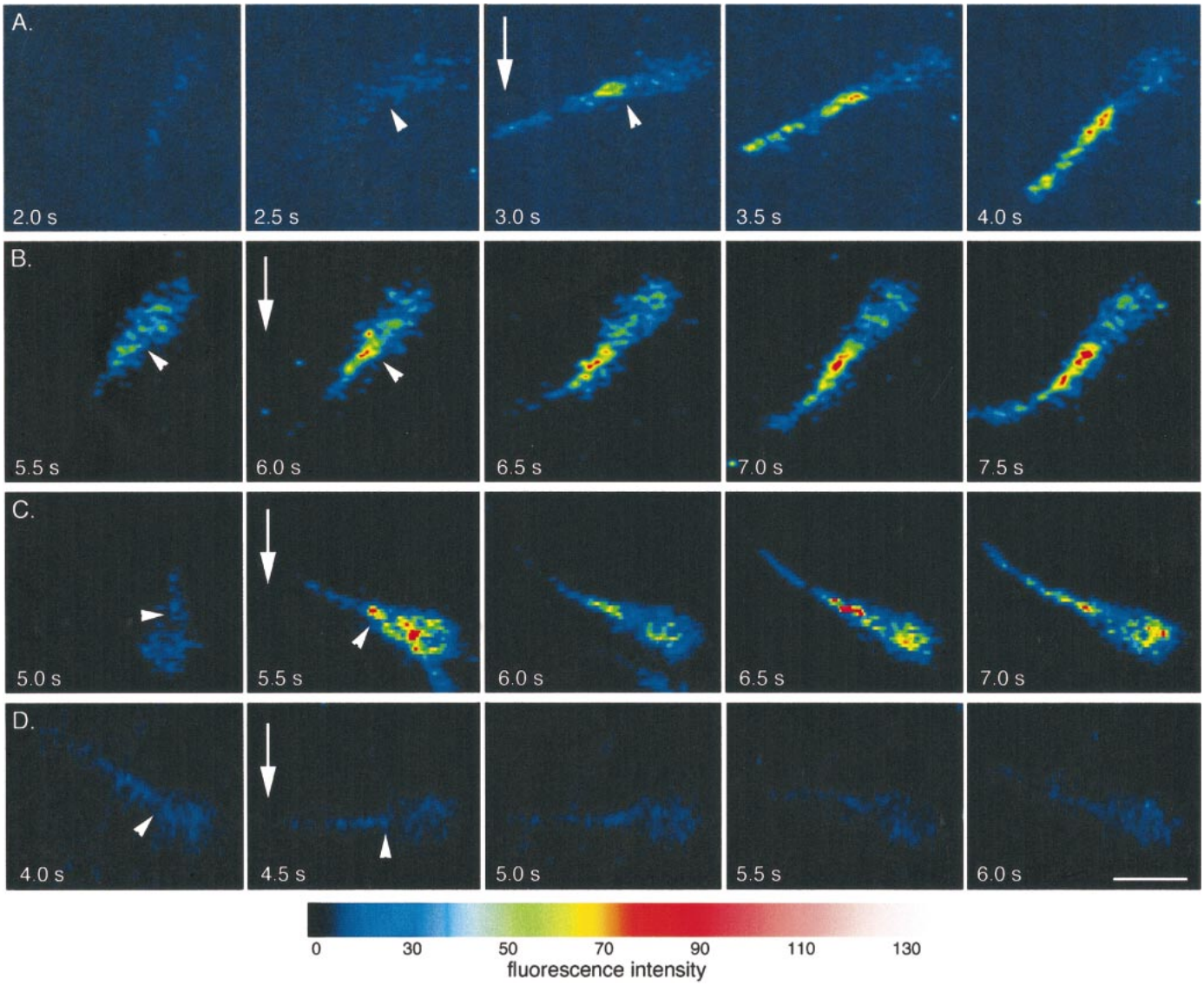

FIG. 7. Ca^{2+} imaging of bull sperm treated with 10 μM thapsigargin (**A**) and 100 μM thimerosal (**B**) in the absence of extracellular Ca^{2+}, 2 mM caffeine in the presence of extracellular Ca^{2+} (**C**), and control with addition of TALP alone (**D**). Addition of thapsigargin, thimerosal, and caffeine caused an immediate Ca^{2+} increase that initiated at the neck region (arrowheads), and the Ca^{2+} increase was seen propagating down the midpiece. Arrows indicate time of addition. No increase in Ca^{2+} could be detected when TALP was added as control. Time lapse is indicated at the lower left corner of each image. Bar = 10 μm.

sponse of sperm to caffeine could result from its ability to increase intracellular Ca^{2+}, rather than its enzymatic activity as a phosphodiesterase inhibitor.

Thimerosal is a sulfhydryl reagent that is able to induce Ca^{2+} release from both IP_3R- and RyR-gated stores [32, 33]. In contrast to caffeine, thimerosal was able to induce hyperactivation when external Ca^{2+} was buffered below the sperm resting Ca^{2+} level by BAPTA. This suggests that thimerosal was working on an intracellular Ca^{2+} store to increase cytoplasmic Ca^{2+}. Thimerosal must have been activating IP_3R rather than RyR, because no RyR was detected in bull sperm. Indirect immunofluorescence localized type I IP_3R to the acrosome and the neck region of bull sperm and hamster sperm. Walensky and Snyder [43] found similar labeling patterns in rat sperm with antibodies to IP_3R.

Thapsigargin has been shown to induce acrosome reactions in several species by increasing intracellular Ca^{2+} [44–46]. However, we did not find a significant induction of acrosome reactions by any of the pharmacological agents tested during the period of our assay. We had specifically developed our protocols for inducing hyperactivation rather than the acrosome reaction. We assessed acrosome reactions immediately after treatments, because hyperactivation was induced immediately; however, examination of sperm 30 min after treatment with thimerosal and caffeine did not reveal significant numbers of acrosome-reacted sperm. We used DIC optics to evaluate acrosomal status in live cells [28] without prolonged procedures used to fix and stain sperm, which might cause acrosome deterioration. Because the acrosome reaction was not significantly increased when hyperactivation was induced by pharmacological agents, it is unlikely that the acrosomal store was involved in inducing hyperactivation in our studies. In addition, external Ca^{2+} entry is required to trigger the acrosome reaction in sperm [44–48], but hyperactivation was induced in our experiments when external Ca^{2+} was not available. Based on studies of sperm stripped of membranes and mitochondria

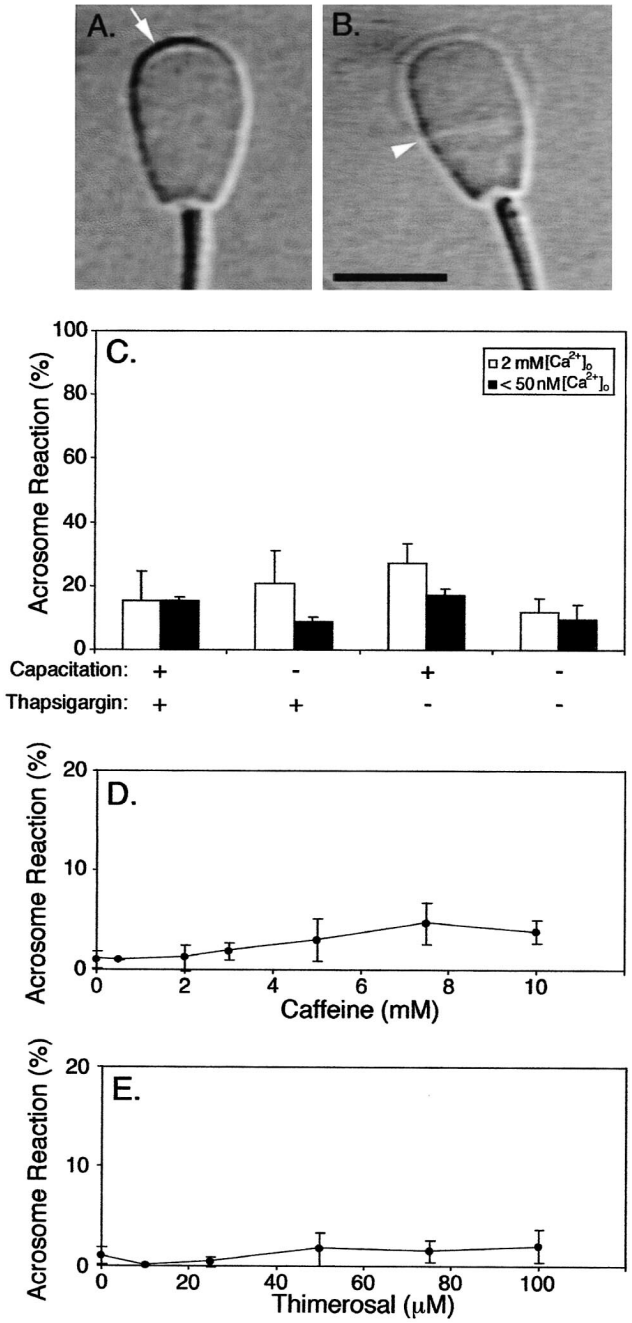

FIG. 8. The acrosomal status was assessed using DIC optics. An intact acrosomal cap is characterized by a distinct apical ridge (arrow) (**A**). An acrosome-reacted sperm lacks the apical ridge and has a prominent equatorial segment (arrowhead); bar = 5 μm (**B**). Acrosome reactions were assessed immediately after treatments. Thapsigargin (**C**), caffeine (**D**), and thimerosal (**E**) did not induce acrosome reactions significantly (n = 3).

by Triton X-100, the crucial site for the action of Ca^{2+} on hyperactivation appears to be the flagellar axoneme [19]. Ca^{2+} ions diffuse slowly and are buffered quickly in cytosol [49]; however, hyperactivation was induced immediately in our studies by thapsigargin, thimerosal, and caffeine. Thus it is unlikely that Ca^{2+} reached the axoneme by diffusing through the cytoplasm from the acrosome.

Unlike the other two agents, thapsigargin produced more hyperactivation when sperm were preincubated in capacitating medium. This could be due to the loss of coating proteins that is known to occur during capacitation. Unlike thimerosal and caffeine, thapsigargin is lipophilic. Loss of

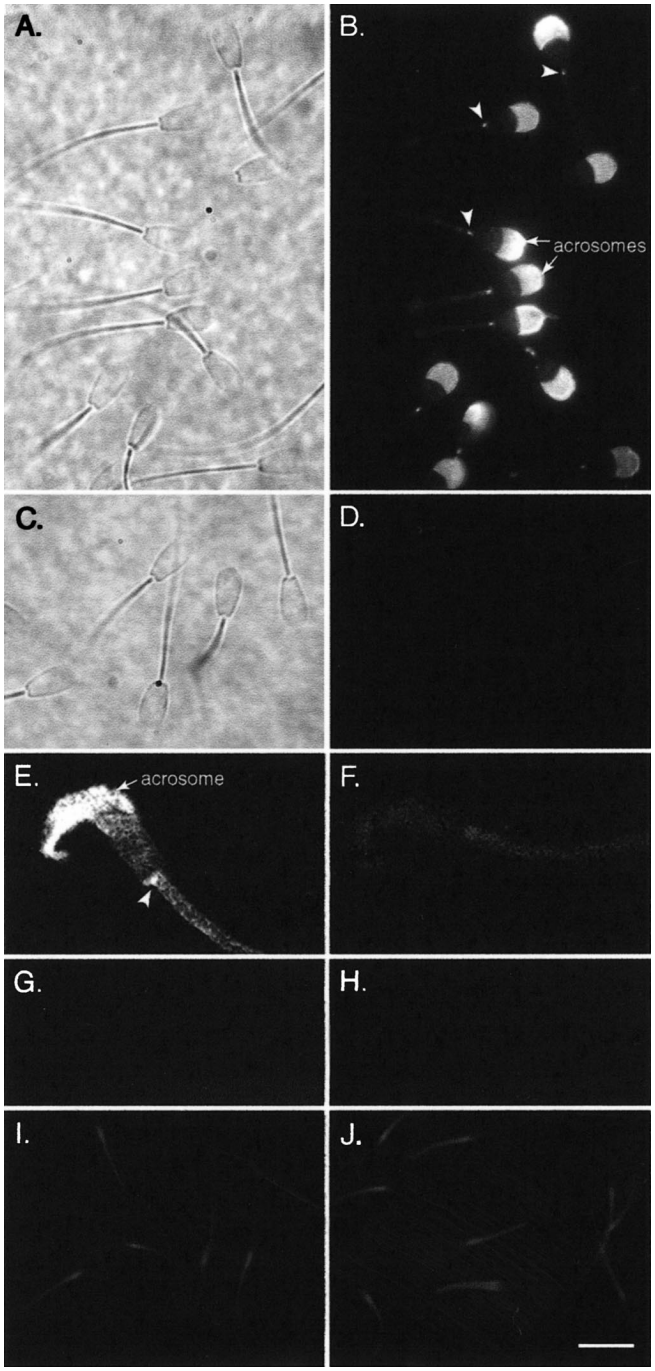

FIG. 9. Localization of IP_3R (**A**–**F**) and RyR (**G**–**J**) in mature sperm. Bull sperm were probed with antibody to IP_3R (**A**, **B**) or preimmune serum (**C**, **D**). Phase-contrast image (**A**) and the corresponding fluorescence staining revealed high densities of IP_3R localized in the acrosomes and neck region (**B**, arrowheads). These areas were not labeled with preimmune serum (**C**, **D**). Hamster sperm also showed IP_3R labeling in the acrosome and neck region (**E**, arrowhead). Incubation of hamster sperm with normal rabbit serum showed no specific labeling (**F**). Bull sperm probed with monoclonal anti-RyR antibody (**G**) or normal mouse serum (**H**) showed no labeling, whereas BODIPY FL-X ryanodine (1 μM) showed faint, nonspecific labeling along the midpiece of flagella (**I**), which could not be blocked by preincubating with 10 μM unlabeled ryanodine for 1 h (**J**). **A**–**D**, **G**–**J**) Bar = 10 μm. **E**, **F**) Bar = 4 μm.

extrinsic, membrane-coating proteins during capacitation could have provided better access for thapsigargin to the lipid bilayer of the plasma membrane and then to the cell's interior.

The intracellular Ca^{2+} source for hyperactivation must be in the neck of the sperm, where a spot of IP_3R was localized. High total calcium was detected in the neck region of human sperm by x-ray microanalysis [50], suggesting the possibility of a localized Ca^{2+} store. The flagellar beat initiates at the base of the flagellum, where the IP_3R was localized. It is here in hamster sperm that we have detected Ca^{2+} oscillations at the frequency of the flagellar beat [16, 17]; and it was the first place showing increased Ca^{2+} after addition of pharmacological agents in the present study (Fig. 7). Calsequestrin, which serves to sequester Ca^{2+} in the sarcoplasmic reticulum, has been localized to this region in human sperm by indirect immunofluorescence labeling [51]. In addition, a high concentration of calmodulin, which has also been detected in the base of the flagellum in several species [52, 53], could respond to Ca^{2+} release from the IP_3R-gated store and regulate sperm motility.

Ca^{2+} stores are membrane-bounded organelles, such as endoplasmic and sarcoplasmic reticula. The only two membrane-bounded organelles found in the neck region of the bull sperm were the RNE and mitochondria. The most likely of these to serve as a Ca^{2+} store is the RNE. The RNE evolves during spermiogenesis as the result of chromatin condensation in the nucleus. Unlike organelles that are useless to mature sperm, the RNE is not shed during sperm maturation, but rather is packaged into the neck region. It was named the ''redundant nuclear envelope,'' because it was believed to be merely leftover membranes. However, the RNE appears to be widespread in mammalian sperm, having been seen in mice [54], hamsters [6], monkeys [55], bats [56], bulls [57], and humans [58]. Why would sperm keep this material when other useless material is so thoroughly discarded? There is physiological evidence that nuclear envelopes serve as Ca^{2+} stores in other cells. Nuclear envelopes in hepatocytes release Ca^{2+} in response to IP_3 [59], while those in starfish oocytes release Ca^{2+} in response to IP_3 and ryanodine [60]. Therefore, it is possible that the RNE in the neck region of mammalian sperm could serve as a Ca^{2+} store and may be involved in regulating sperm motility. It is interesting that the RNE is distributed asymmetrically around the flagellar base and may thus be able to release more Ca^{2+} to one side of the axoneme where it is needed to produce asymmetrical flagellar beats.

The only other membrane-bounded organelles in the region are the mitochondria. In the bull sperm that we examined by transmission electron microscopy, the mitochondria in the proximal midpiece extended into the neck region. These mitochondria were morphologically distinct from the rest of the mitochondria coiled around the midpiece. It is possible that these mitochondria are specialized for Ca^{2+} storage. Nevertheless, IP_3R have never been reported to exist in mitochondria; therefore, the RNE is a better candidate for the Ca^{2+} store.

Capacitation is an incompletely understood sequence of events that prepares sperm for fertilization; more specifically, to undergo the acrosome reaction [6]. Hyperactivation has sometimes been described as a subset of capacitation, because it often occurs in vitro at some point during the capacitation process. It could be possible that loss or alteration of the surface coat of the plasma membrane in the base of the sperm tail during capacitation exposes or activates putative receptors to turn on hyperactivation. Thapsigargin induced a higher percentage of hyperactivation when sperm were capacitated; thus, it could be that thapsigargin had better access into sperm cells after loss of the surface coat. Nevertheless, hyperactivation and capacitation are not inexorably linked. Hamster sperm can be fully capacitated without becoming fully hyperactivated by lowering the bicarbonate concentration in medium [8]. Also, DeMott et al. [61] used modified concentrations of energy substrates and BSA in medium to collect at least 70% hyperactivated hamster sperm an hour or more before

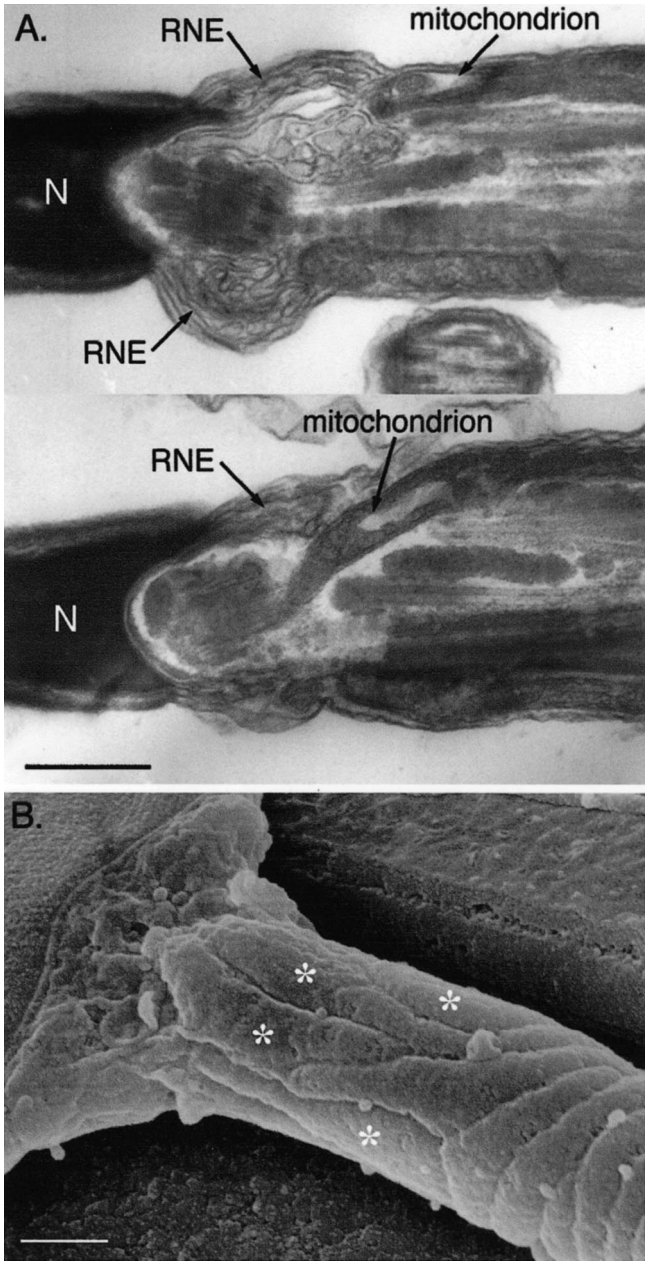

FIG. 10. The ultrastructure of the neck region of bull sperm. **A**) Transmission electron micrographs of two longitudinally sectioned sperm show the RNE in the neck region, closely associated with adjacent mitochondria. N indicates nucleus. **B**) A scanning electron micrograph shows the mitochondria (asterisks) localized in the proximal midpiece as longitudinally oriented tubular structures, irregularly shaped and uncoiled from the rest of the mitochondrial sheath surrounding the axoneme in the midpiece. The plasma membrane and the RNE were not preserved under this condition. **A**, **B**) Bar = 0.3 μm.

the completion of capacitation. The ability for caffeine and thimerosal to induce hyperactivation without the need of capacitation also implies that the pathways to hyperactivation and capacitation are different or that these agents can bypass the initial steps of the pathways.

These results underscored the fact that sperm cells are highly compartmentalized. The IP_3R-gated Ca^{2+} store at the base of flagellum can regulate hyperactivated motility independently from the acrosomal Ca^{2+} store, which induces exocytosis. Acrosomal exocytosis requires extracellular Ca^{2+}, with zona binding initiating a signaling cascade that leads to Ca^{2+} influx [48]. In contrast to the acrosome reaction, little is known about the pathway leading to the Ca^{2+} rise at the axoneme during hyperactivation. We propose that the release of Ca^{2+} from the IP_3R-gated Ca^{2+} store in the neck region raises Ca^{2+} at the axoneme. This proposal is also supported by the findings that Ca^{2+} reaches two different elevated levels in sperm, first with the achievement of hyperactivation, and second with completion of the acrosome reaction [17].

In conclusion, we found that pharmacological agents known to release Ca^{2+} from internal stores immediately induced hyperactivation. Release of Ca^{2+} from internal Ca^{2+} stores was sufficient to initiate hyperactivation, and the rise of Ca^{2+} was initiated in the neck region of sperm. The presence of IP_3R, but not RyR, in mature sperm at the site of flagellar beat initiation indicates the involvement of an IP_3R-gated store in regulating hyperactivation. The distinct regional expression of IP_3R in the neck region confers anatomical and physiological advantages to regulate hyperactivated motility in sperm. The most likely identity of the flagellar Ca^{2+} store is the RNE, which surrounds the axoneme at the base of the flagellum.

ACKNOWLEDGMENTS

We thank Drs. J.B. Parys and R.A. Fissore for the gift of antibodies, and M. Kotlikoff and M. Rishniw for type 3 RyR knockout mouse. We express our gratitude to Genex Cooperative, Inc. for the donation of ejaculated bull sperm. Dr. X.B. Dai provided technical assistance.

REFERENCES

1. Katz DF, Yanagimachi R. Movement characteristics of hamster spermatozoa within the oviduct. Biol Reprod 1980; 22:759–764.
2. Suarez SS, Osman RA. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol Reprod 1987; 36:1191–1198.
3. Cooper GW, Overstreet JW, Katz DF. The motility of rabbit spermatozoa recovered from the female reproductive tract. Gamete Res 1979; 2:35–42.
4. Cummins JM. Hyperactivated motility patterns of ram spermatozoa recovered from oviducts of mated ewes. Gamete Res 1982; 6:53–64.
5. Suarez SS, Katz DF, Overstreet JW. Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. Biol Reprod 1983; 29:1277–1287.
6. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 189–317.
7. Fraser LR, Quinn PJ. A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. J Reprod Fertil 1981; 61:25–35.
8. Boatman DE, Robbins RS. Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. Biol Reprod 1991; 44:806–813.
9. Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in sperm. Biol Reprod 1991; 44:375–381.
10. DeMott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. Biol Reprod 1992; 46:779–785.
11. Suarez SS, Dai X. Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. Biol Reprod 1992; 46:686–691.
12. Stauss CR, Votta TJ, Suarez SS. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. Biol Reprod 1995; 53:1280–1285.
13. Olds-Clarke P. Sperm from $t^{w32}/+$ mice: capacitation is normal, but hyperactivation is premature and nonhyperactivated sperm are slow. Dev Biol 1989; 131:475–482.
14. Olds-Clarke P, Johnson LR. *t* Haplotypes in the mouse compromise sperm flagellar function. Dev Biol 1993; 155:14–25.
15. Yanagimachi R. Requirement of extracellular calcium ions for various stages of fertilization and fertilization-related phenomena in the hamster. Gamete Res 1982; 5:323–344.
16. Suarez SS, Varosi SM, Dai X. Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. Proc Natl Acad Sci U S A 1993; 90:4660–4664.
17. Suarez SS, Dai X. Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. Mol Reprod Dev 1995; 42:325–333.
18. Lindemann CB, Goltz JS. Calcium regulation of flagellar curvature and swimming pattern in Triton X-100 extracted rat sperm. Cell Motil Cytoskeleton 1988; 10:420–431.
19. Lindemann CB, Kanous KS, Gardner TK. The interrelationship of calcium and cAMP mediated effects on reactivated mammalian sperm models. In: Baccetti B (ed.), Comparative Spermatology: 20 Years After. New York: Raven Press; 1991:491–496.
20. Catterall WA. Structure and function of voltage-gated ion channels. Annu Rev Biochem 1995; 64:493–531.
21. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem 1998; 67:653–692.
22. Dutta D. Mechanism of store-operated calcium entry. J Biosci 2000; 25:397–404.
23. Wiesner B, Weiner J, Middendorff R, Hagen V, Kaupp UB, Weyand I. Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. J Cell Biol 1998; 142:473–484.
24. Lytton J, Westlin M, Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J Biol Chem 1991; 266:17067–17071.
25. Bers D, Patton C, Nuccitelli R. A practical guide to the preparation of Ca buffers. Methods Cell Biol 1994; 40:3–29.
26. Galantino-Homer HL, Visconti PE, Kopf GS. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′,4′-monophosphate-dependent pathway. Biol Reprod 1997; 56:707–719.
27. Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.
28. Saacke RG, Marshall CE. Observations on the acrosomal cap of fixed and unfixed bovine spermatozoa. J Reprod Fertil 1968; 16:511–514.
29. Parys JB, Sernett SW, DeLisle S, Snyder PM, Welsh MJ, Campbell KP. Isolation, characterization, and localization of the inositol 1,4,5-trisphosphate receptor protein in *Xenopus laevis* oocytes. J Biol Chem 1992; 267:18776–18782.
30. Tanaka K, Mitsushima A. A preparation method for observing intracellular structures by scanning electron microscopy. J Microsc 1984; 133:213–222.
31. Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol Rev 1997; 49:1–51.
32. Bindoli A, Fleischer S. Induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum by sulfhydryl reagents and chlorpromazine. Arch Biochem Biophys 1983; 221:458–466.
33. Mihai R, Lai T, Schofield G, Farndon JR. Thimerosal increases the responsiveness of the calcium receptor in human parathyroid and rMTC6-23 cells. Cell Calcium 1999; 26:95–101.
34. Zhang X, Wen J, Bidasee KR, Besch HR, Wojcikiewicz RJ, Lee B, Rubin RP. Ryanodine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland. Biochem J 1999; 340:519–527.
35. Ehrlich BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I. The pharmacology of intracellular Ca^{2+}-release channels. Trends Pharmacol Sci 1994; 15:145–149.
36. Guerrero A, Fay FS, Singer JJ. Caffeine activates a Ca^{2+}-permeable, nonselective cation channel in smooth muscle cells. J Gen Physiol 1994; 104:375–394.
37. Schoppe J, Hochstrate P, Schlue WR. Caffeine mediates cation influx

and intracellular Ca^{2+} release in leech P neurones. Cell Calcium 1997; 22:385–397.
38. Giannini G, Conti A, Mammarella S, Scrobogna M, Sorrentino V. The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. J Cell Biol 1995; 128:893–904.
39. Trevino CL, Santi CM, Beltran C, Hernandez-Cruz A, Darszon A, Lomeli H. Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis: possible functional implications. Zygote 1998; 6:159–172.
40. Hakamata Y, Nishimura S, Nakai J, Nakashima Y, Kita T, Imoto K. Involvement of the brain type of ryanodine receptor in T-cell proliferation. FEBS Lett 1994; 352:206–210.
41. Vijayaraghavan S, Hoskins DD. Regulation of bovine sperm motility and cyclic adenosine 3′,5′-monophosphate by adenosine and its analogues. Biol Reprod 1986; 34:468–477.
42. Tash JS. Protein phosphorylation: the second messenger signal transducer of flagellar motility. Cell Motil Cytoskeleton 1989; 14:332–339.
43. Walensky LD, Snyder SH. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. J Cell Biol 1995; 130:857–869.
44. Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. J Exp Zool 1993; 267:350–355.
45. Llanos MN. Thapsigargin stimulates acrosomal exocytosis in hamster spermatozoa. Mol Reprod Dev 1998; 51:84–91.
46. Dragileva E, Rubinstein S, Breitbart H. Intracellular Ca(2+)-Mg(2+)-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. Biol Reprod 1999; 61:1226–1234.
47. Florman HM, Corron ME, Kim TDH, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona-induced acrosomal exocytosis. Dev Biol 1992; 152:304–314.
48. O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 2000; 11:1571–1584.
49. Clapham DE. Calcium signaling. Cell 1995; 80:259–268.
50. Chandler JA, Battersby S. X-ray microanalysis of ultrathin frozen and freeze-dried sections of human sperm cells. J Microsc 1976; 107:55–65.
51. Berruti G, Porzio S. Evidence for a calsequestrin-like calcium-binding protein in human spermatozoa. Eur J Cell Biol 1990; 52:117–122.
52. Jones HP, Lenz RW, Palevitz BA, Cormier MJ. Calmodulin localization in mammalian spermatozoa. Proc Natl Acad Sci U S A 1980; 77: 2772–2776.
53. Feinberg J, Weinman J, Weinman S, Walsh MP, Harricane MC, Gabrion J, Demaille JG. Immunocytochemical and biochemical evidence for the presence of calmodulin in bull sperm flagellum. Biochim Biophys Acta 1981; 673:303–311.
54. Toshimori K, Higashi R, Oura C. Distribution of intramembranous particles and filipin-sterol complexes in mouse sperm membranes: polyene antibiotic filipin treatment. Am J Anat 1985; 174:455–470.
55. Franklin LE. Formation of the redundant nuclear envelope in monkey spermatids. Anat Rec 1968; 161:149–162.
56. Rouse GW, Robson SK. An ultrastructural study of megachiropteran (Mammalia: Chiroptera) spermatozoa: implications for chiropteran phylogeny. J Submicrosc Cytol 1986; 18:137–152.
57. Oko RJ, Costerton JW, Coulter GH. An ultrastructural study of the head region of bovine spermatozoa. Can J Zool 1976; 54:1326–1340.
58. Kerr JB. Ultrastructure of the seminiferous epithelium and intertubular tissue of the human testis. J Electron Microsc Tech 1991; 19:215–240.
59. Gerasimenko OV, Gerasimenko JV, Tepikin AV, Petersen OH. ATP-dependent accumulation and inositol trisphosphate- or cyclic ADP-ribose-mediated release of Ca^{2+} from the nuclear envelope. Cell 1995; 80:439–444.
60. Santella L, Kyozuka K. Effects of 1-methyladenine on nuclear Ca^{2+} transients and meiosis resumption in starfish oocytes are mimicked by the nuclear injection of inositol 1,4,5-trisphosphate and cADP-ribose. Cell Calcium 1997; 22:11–20.
61. DeMott RP, Lefebvre R, Suarez SS. Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. Biol Reprod 1995; 52:1395–1403.