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Progesterone Triggers a Wave of Increased Free

Acrosome Reaction

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The mammalian sperm acrosome reaction (AR), an essential fertilization event, requires an influx of Ca^{2+}. The Ca^{2+} increase occurring in the human sperm head during the AR initiated by progesterone, a putative *in vivo* AR initiator, was investigated using video-image analysis with fura-2, a fluorescent Ca^{2+} probe. Progesterone treatment of capacitated human sperm resulted in a wave-like increase in sperm head cytosolic $[Ca^{2+}]_i$ that appears to increase fastest in a region near the equatorial segment and then spreads throughout the rest of the head. The progesterone-mediated Ca^{2+} increase in the sperm head was strongly inhibited and the wave eliminated by picrotoxin, a blocker of $GABA_A$ receptor/Cl^- channels and an inhibitor of the progesterone-mediated Cl^- efflux and progesterone-initiated AR of human sperm. These results are the first to detect a ligand-mediated Ca^{2+} wave in sperm and to suggest that Cl^- efflux influences Ca^{2+} influx during the AR.

INTRODUCTION

The eutherian mammalian acrosome reaction (AR), a specialized exocytotic event involving the fusion of sperm head membranes (Russell *et al.*, 1979; Yudin *et al.*, 1988), is required for fertilization (Yanagimachi, 1994). The egg zona pellucida is generally thought to be the physiological initiator of the AR of the fertilizing sperm (Kopf and Gerton, 1991), but progesterone, secreted by cells of the cumulus oophorus surrounding the ovulated egg, may also serve in that role (Meizel, 1995; Meizel *et al.*, 1990; Osman *et al.*, 1989). As is true for most exocytotic events, AR initiation usually requires an extracellular Ca^{2+}-dependent influx of Ca^{2+} (Florman *et al.*, 1989; Thomas and Meizel, 1988). In human sperm, progesterone mediates a severalfold transient increase in $[Ca^{2+}]_i$ within seconds of addition (Blackmore *et al.*, 1990; Thomas and Meizel, 1989). While there is strong evidence for the involvement of sperm plasma membrane progesterone receptors in progesterone-mediated sperm events (Blackmore *et al.*, 1991; Meizel and Turner, 1991; Sabeur *et al.*, 1996; Tesarik *et al.*, 1992), the type of Ca^{2+} channel involved in progesterone-mediated sperm events is not yet certain (reviewed in Shi and Roldan, 1995).

In addition to a requirement for Ca^{2+} influx, the mammalian AR requires extracellular Cl^- (Yoshimatsu and Yanagimachi, 1988; Wistrom and Meizel, 1993; Melendrez and Meizel, 1995). Moreover, a Cl^- efflux has been shown to be essential for the progesterone-initiated AR (Turner and Meizel, 1995). Several studies support the involvement of a unique receptor/Cl^- channel complex (resembling a neuronal $GABA_A$ receptor/Cl^- channel) in the progesterone-initiated mammalian AR (Wistrom and Meizel, 1993; Melendrez *et al.*, 1994; Roldan *et al.*, 1994; Shi and Roldan, 1995; Turner and Meizel, 1995). Spectrofluorometric studies of the effects of blockers of neuronal $GABA_A$ receptor/Cl^- channels on $[Ca^{2+}]_i$ in total human sperm populations suggested that the progesterone-mediated increase in $[Ca^{2+}]_i$ essential to the AR is independent of the AR Cl^- requirement (Turner *et al.*, 1994). Those and other results support the view that there are two plasma membrane progesterone receptors involved in the AR: one receptor mediating the increase in $[Ca^{2+}]_i$ and the other, a $GABA_A$ receptor-like/Cl^- channel, involved in increased Cl^- flux (reviewed in Meizel, 1997).

The aims of the present study were to use image analysis with fura-2, a fluorescent Ca^{2+} probe, to (1) examine whether progesterone increases Ca^{2+} in the sperm head during AR initiation, (2) describe the form taken by that increase, and (3) determine the effect on that increase of picrotoxin, a blocker of $GABA_A$ receptor/Cl^- channels, known to inhibit progesterone-mediated Cl^- efflux and the progesterone-initiated AR of human sperm (Turner and Meizel,

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1995; Wistrom and Meizel, 1993). In these studies, we have detected a progesterone-mediated, wave-like increase in the Ca^{2+} of the sperm head during progesterone initiation of the AR. This cytosolic Ca^{2+} wave appears to increase most rapidly in a region near the narrowest part of the acrosome, the equatorial segment, and then spreads rapidly to elevate $[Ca^{2+}]_i$ in the entire head. In addition, picrotoxin inhibits to some extent the progesterone-mediated Ca^{2+} increase in the sperm head and completely eliminates any wave-like characteristics of that increase (i.e., Ca^{2+} increases in a uniform manner throughout the head). To our knowledge, this is the first report of a Ca^{2+} wave accompanying the AR. Moreover, these results combined with earlier studies (Turner and Meizel, 1995) strongly suggest that Cl^- efflux can influence the Ca^{2+} influx involved in the progesterone-initiated AR of human sperm.

MATERIALS AND METHODS

Materials

The following were purchased: progesterone (4-pregnen-3,20-dione), picrotoxin, from Sigma Chemical Co. (St. Louis, MO); Pentex fraction V bovine serum albumin (BSA) from Miles Inc. (Kunkakee, IL); Con A–FITC from EY laboratories, Inc. (San Mateo, CA); fura 2-AM from Molecular Probes Inc. (Eugene, OR); FITC-Guard from Testog Inc. (Chicago, IL); Cell-Tak from Collaborative Biochemical Research (Boston, MA); Falcon Blue Max 15-ml polypropylene tubes from Fisher Scientific Co. (Santa Clara, CA.); microscope slides with 7-mm-diameter wells (No. HTC 10-7) from Cell-line (Newfield, NJ). All other chemicals were reagent grade and were purchased from Sigma Chemical Co., Fisher Scientific Co., or Mallinckrodt Inc. (Paris, KY). Deionized water purified further with a NANO-Pure system, Barnstead/Thermolyne (Dubuque, IA) was used in all of these studies.

Preparation and Capacitation of Sperm

Human semen was obtained from healthy donors by masturbation. A sperm population having >95% motility was obtained by discontinuous Percoll gradient centrifugation as previously described (Thomas and Meizel, 1988). The gradient-prepared and washed sperm were then diluted to 3×10^6/ml in a bicarbonate-buffered medium composed of a modified Tyrode's solution containing lactate, pyruvate, glucose, streptomycin, penicillin G, and 26 mg/ml BSA (Thomas and Meizel, 1988). Mammalian sperm must undergo cellular changes *in vitro* or *in vivo*, collectively called capacitation, before they can respond to initiators of the AR (Yanagimachi, 1994). Here, sperm (aliquots of 500 μl in 15-ml Blue Max tubes) were capacitated by incubation, at 37°C in a humidified, 5% CO_2/95% air atmosphere for 24 hr (Thomas and Meizel, 1989).

Motility Assays

The percentage of motile sperm was determined and a qualitative estimate of motility intensity was made before and after capacitation, fura-2AM loading, and calcium measurements (modified from (Thomas and Meizel, 1988)). These sperm motility determinations were made at room temperature and 500× magnification using a 3.5-μl aliquot of sperm suspension placed into a 7-mm well on a cell-line microscope slide and covered with a coverslip.

Preparation of Fura-2-Loaded Sperm

These procedures have been previously described (Turner *et al.*, 1994). Briefly, capacitated sperm were pooled as 2-ml aliquots in 15-ml polypropylene tubes and loaded with 5 μM fura-2-AM for 30 min. Sperm were then centrifuged on a 40% Percoll gradient, made with Hepes–bicarbonate buffer containing metabolites and 3 mg/ml BSA (FMB-3B), to remove extracellular fura-2AM and dead sperm. After washing with FMB-3B, sperm pellets were pooled and diluted to 2 ml with FMB-3B in preparation for $[Ca^{2+}]_i$ determination. Samples were maintained at 37°C in a water bath for 2–3 hr.

Preparation of Coverslips for $[Ca^{2+}]_i$ Imaging of Human Sperm

Round coverslips, 25 mm in diameter, were first washed with 10% nitric acid, rinsed with nanopure water, and then air dried. A 3×3 grid of dots (1 mm spacing) was drawn on the lower side of each coverslip using a fine-tipped black Sharpie pen (Sanford Corp., Bellwood, IL). Cell-Tak was prepared with sodium carbonate as directed by the manufacturer and applied to the grid area of each coverslip on the upper side. The coverslip was attached, using high vacuum silicone grease, to the bottom of a 6-mm-thick circular plastic ring (o.d., 36 mm; i.d., 12 mm, 1-ml volume) serving as a sample chamber. Each sample chamber had an outlet port attached to a vacuum line for removal of excess solvents and was maintained at 37°C with a stage warmer for all experiments. The chamber was rinsed with nanopure water to activate the Cell-Tak.

$[Ca^{2+}]_i$ Imaging Technique

Sperm were imaged on a Zeiss IM-35 inverted microscope using a 100× objective and 20× occular for a total magnification of 2000×. Fura-2 fluorescence was excited at 350 and 385 nm using a dual monochrometer (Deltascan, Photon Technology International, South Brunswick, NJ) with a 150-W xenon light source and 1.5-nm bandwidth. The fura-2 emission at 450 to 600 nm was detected with an intensified CCD camera (Geniisys manufactured by DAGE-MTI, Michigan City, IN) and a Gould FD5000 image processor (Gould, Inc., Fremont, CA) controlled by a PDP 11/73 host computer (Digital Equipment Corp., Maynard, MA). After averaging four frames at each wavelength, the averaged images were stored on a high-resolution optical disc recorder (Panasonic Model TQ-2028F, Secaucus, NJ). The maximum ratio measured in a 1 mM Ca^{2+} medium was 41.1 and the optimal viscosity correction factor (0.85) and K_d(Sf2/Sb2) (6.3) were determined by calibration using Ca^{2+} standards *in vitro*.

For each experiment, a 100-μl aliquot of sperm suspension was placed on the sample chamber grid coated with activated Cell-Tac and maintained at 37°C for 10 min. FMB-3B was used to remove unattached sperm after loading the sample chamber. For some experiments, sperm were pretreated for 5 min with either 200 μM Picrotoxin or the solvent control (0.05% DMSO). The fura-2-loaded sperm were imaged for 15–30 sec following preincubation and prior to the additions of progesterone (3.18 μM) or solvent control (0.05% DMSO). Only the heads of sperm exhibiting flagellar movement were imaged since these sperm were presumably viable cells.

Assessing AR of Imaged Sperm

Immediately following each experiment, a transmitted light image of the field of sperm was printed using a video printer and the coordinates of the imaged area on the coverslip were determined using the 3 × 3 grid. The coverslip was carefully removed and placed into a vial of 4% formaldehyde in phosphate-buffered saline, pH 7.5 (PBS). The samples were stained the following day using a Con A–FITC method (Meizel and Turner 1993). The staining method was modified to accommodate fixed sperm on a coverslip. The modifications were as follows: Each sample coverslip was removed from the fixative using curved tip forceps with soft tygon pads, placed into a container of 0.2 *M* glycine for 30 sec, removed, and briefly dipped in PBS. The sample coverslip was then placed into a highly humidified chamber (room temperature), 10 μl of Con A–FITC (250 μg/ml) in PBS was added to the grided area, and the chamber was covered to avoid direct light and evaporation. The sample was again rinsed with PBS 25 min later and a 3.5-μl aliquot of FITC-Guard was added to the grid area. A clean microscope slide was placed onto the sample coverslip, the slide was inverted, and the coverslip was sealed to the slide using clear nail polish. The location of the imaged sperm was determined using bright-field microscopy and the coordinates on the grid. The video prints of screen images recorded at the end of imaging were also used to help in locating the imaged sperm. The acrosomal status of imaged sperm was determined by the presence (acrosome-reacted) or absence (not acrosome-reacted) of Con A–FITC fluorescence in the acrosomal region (Meizel and Turner, 1993a).

Analysis of Imaged Sperm

Postacquisitional image analysis was conducted using software developed by R. Tsien in a program named, ''ml 12.'' In order to detect the Ca^{2+} wave in progesterone or picrotoxin plus progesterone experiments, three small circular areas (89 pixels each) in the sperm head were analyzed for $[Ca^{2+}]_i$ (Fig. 1A). One of the circular areas was in the anterior region of the head (anterior head), one included the equatorial segment region of the head (mid head), and one was in the postacrosomal region of the head (posterior head). The small circles allowed more accurate measurements of $[Ca^{2+}]_i$ in the cytoplasm near the equatorial segment region. The approximate location of the equatorial segment region was determined by measurements using phase-contrast micrographs of human sperm heads and micrographs of immunofluorescence localization of the human sperm $GABA_A$ receptor/Cl^- channel (Wistom *et al.*, 1993).

To determine the effect of picrotoxin on the sperm head $[Ca^{2+}]_i$, the average $[Ca^{2+}]_i$ was measured in three larger circles composed of 333 pixels each (Fig. 1B). These circles allow a larger region to be analyzed but do include some regional overlap. Analysis of variance was used to test imaged data for significance. A blocking factor (day of the experiment) was added in the analysis to correct for day to day variability. $[Ca^{2+}]_i$ data were log transformed prior to analysis of variance. An all means Bonferonni post hoc test was used to determine acceptance or rejection of the null hypothesis (SuperANOVA, ABACUS concepts, Berkeley CA) Data were considered significant for $P \leq 0.05$.

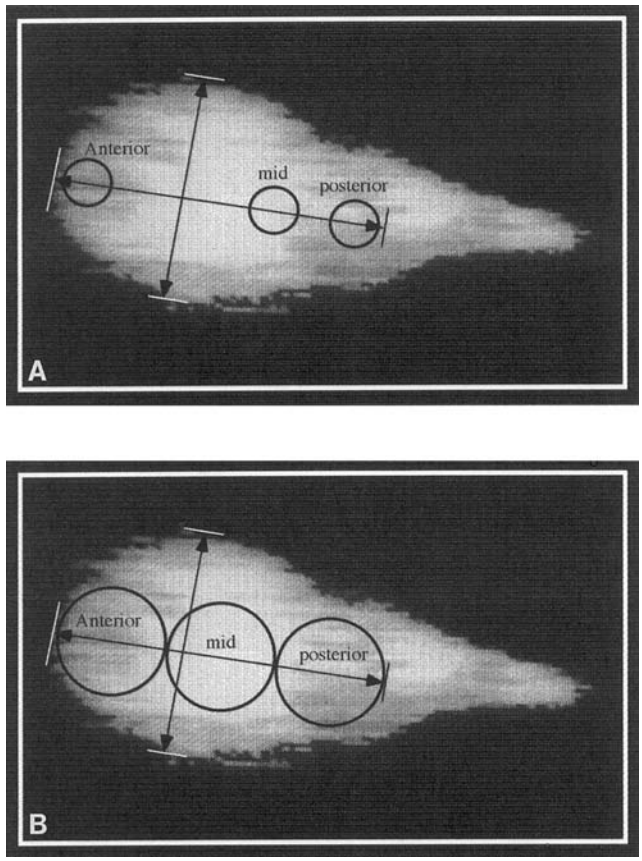

FIG. 1. Representative digitized image of the human sperm head showing regions of interest (ROI) used for detection of the progesterone-mediated Ca^{2+} wave and for determination of $[Ca^{2+}]_i$ in picrotoxin experiments. (A) Detection of Ca^{2+} wave. Small circles enclose the ROI (89 pixels each) assayed for $[Ca^{2+}]_i$. These ROI are in the posterior head, anterior head, and mid head (including the equatorial segment region). The approximate location of the equatorial segment region was determined by measurements using phase-contrast micrographs of human sperm heads and micrographs of immunofluorescence localization of the human sperm $GABA_A$ receptor/Cl^- channel. The length-to-width ratio of the head (black arrows), approximately 1.5 to 1, was used to determine head length on images of fura 2-loaded sperm. The equatorial segment region is approximately two-thirds of the length of the head measured from the anterior end. (B) Larger circles (333 pixels each) enclose the ROI used for determination of $[Ca^{2+}]_i$ in picrotoxin experiments.

RESULTS

Ca^{2+} Wave

We have investigated the changes in free Ca^{2+} in the heads of capacitated human sperm following the addition of 3.18 μM progesterone to stimulate the AR. Progesterone increased sperm-head Ca^{2+} significantly in all of the 62 capacitated sperm analyzed throughout these studies. Moreover, using a time resolution of 2–5 sec, we were able to detect a wave of cytosolic Ca^{2+} that increases fastest in the mid-head region that included the equatorial segment region and then rapidly spreads throughout the entire sperm head. In one study, we investigated this Ca^{2+} response in 35 sperm from 7 different donors (7 different experiments), and 24 of

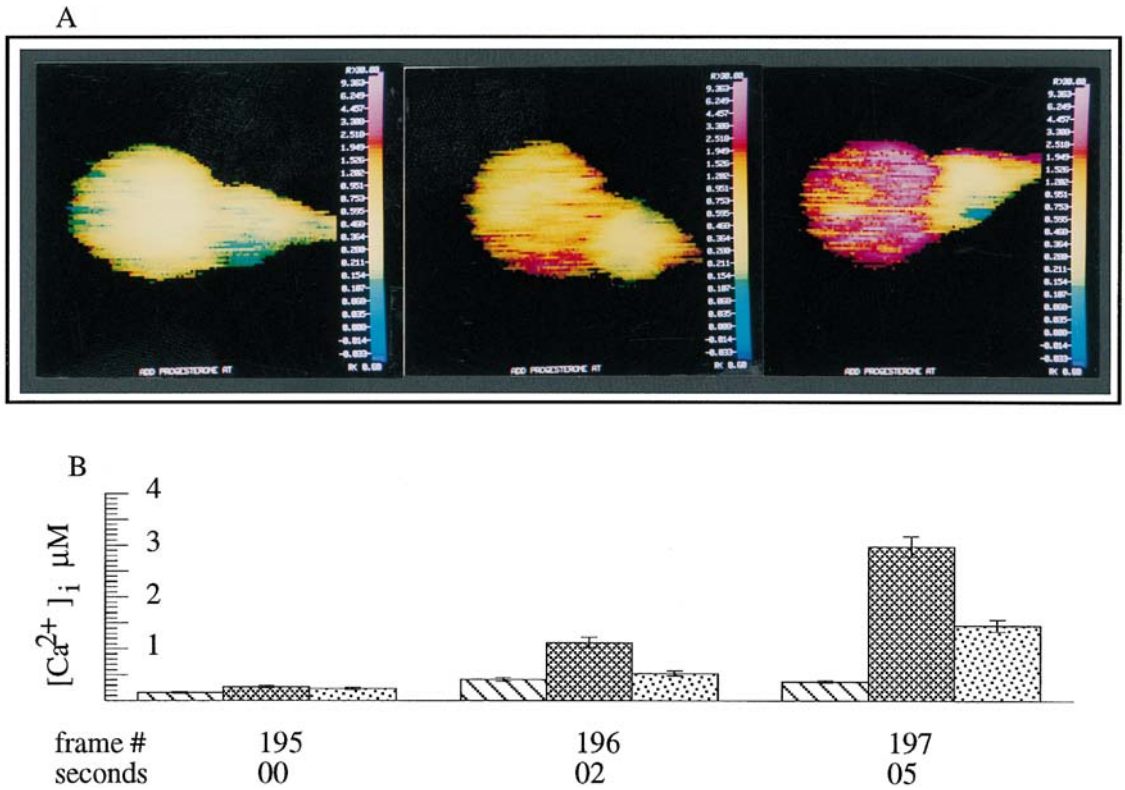

FIG. 2. Fluorescence ratio images of a fura-2-loaded, capacitated human sperm responding to 3.18 μM progesterone. This sperm underwent the acrosome reaction. The anterior end of the head is closest to the top of the figure. (A) Three ratioimages of a single sperm taken at 2- and 3-sec intervals to illustrate the wave of increased $[Ca^{2+}]_i$ beginning in the mid-head region following progesterone exposure. Numbers on color bar indicate $[Ca^{2+}]_i$ in micromoles/liter. (B) Histograms of the average $[Ca^{2+}]_i$ measured in circular regions of 89 pixels centered on the posterior-head ▧, mid-head ▩, and anterior-head ⚃ regions of the sperm image directly above each histogram. Error bars represent the SEM for $[Ca^{2+}]_i$ determined by average pixel values within the circle. Sperm was motile but affixed to the bottom of the image chamber by a biopolymer, Cell-Tak. Original magnification, 2000×.

them exhibited a wave-like increase in which that same mid-head region displayed the greatest rate of increase in $[Ca^{2+}]_i$ (Fig. 2). In those 24 sperm, the next highest rate of increase was in the anterior-head region (6 sperm), the posterior-head region (11 sperm), or both (7 sperm). In all cases, $[Ca^{2+}]_i$ rapidly increases throughout the entire head.

Of the remaining 11 sperm that did not exhibit a wave, 4 were ambiguous due to movement artifacts and 7 did not exhibit such a wave. We conclude that 77% of the capacitated sperm analyzed in that study respond to progesterone with a rapid, wave-like increase in Ca^{2+} which occurs most rapidly in the mid-head region of the sperm (near the equatorial segment). Addition of solvent control only (0.05% DMSO) did not result in any appreciable $[Ca^{2+}]_i$ increase in 16 sperm from 3 donors (Fig. 3).

In many experiments, we were unable to determine the acrosomal status of imaged sperm (i.e., sperm lost from coverslip during treatments, etc.). Of the 35 progesterone-treated sperm studied, we were only able to determine the acrosomal status of 6. Five of those 6 sperm had undergone the progesterone-initiated AR and exhibited a progesterone-mediated Ca^{2+} wave in which the rate of increase in $[Ca^{2+}]_i$ was highest in the mid-head region but was also high in the posterior region. The other sperm was also acrosome-reacted, but $[Ca^{2+}]_i$ increased throughout the head at about the same rate.

Each fura-2 image was acquired by averaging four video frames giving a total data collection time of one-eighth of a second at each wavelength. An additional time of 0.15 sec was required to switch wavelengths, resulting in a total image acquisition time of 0.4 sec. Due to the time required to store these images and compute the ratio, the acquisition of the next image is further delayed by 1.5 sec. However, the image acquisition time of 0.4 sec for each image is sufficiently short to eliminate the possibility that the wave is an artifact of image acquisition since the wave spreads over the sperm head during a period of 2–5 sec. If $[Ca^{2+}]_i$ were changing over the entire sperm head during our collection period, we would have detected those changes in all regions of the sperm head.

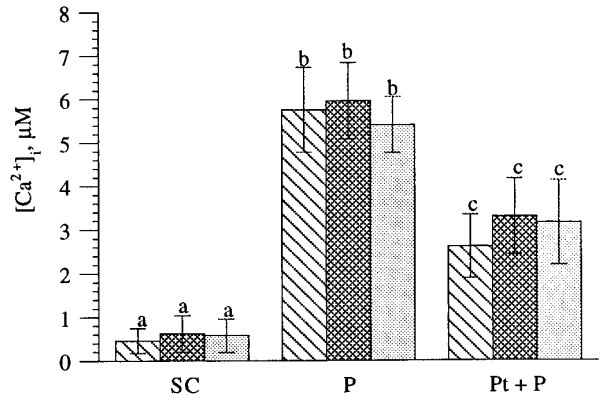

FIG. 3. Average peak $[Ca^{2+}]_i$ in different regions of capacitated human sperm head following progesterone (P) or solvent control (SC) addition with and without picrotoxin (Pt) pretreatment. Picrotoxin is a $GABA_A$ receptor/Cl^- channel antagonist. Regions are posterior head ▧, mid head ▩, and anterior head ▭. Values shown are the mean maximum peak $[Ca^{2+}]_i \pm$ SEM observed within 15 sec following addition. Values with different superscript letters are significantly different ($P < 0.05$). Each of these sperm regions was composed of 333 pixels. SC (5-min pretreatment with solvent control, 0.05% DMSO, followed again by solvent control addition), 16 sperm imaged in three experiments; P (5-min pretreatment with solvent control, followed by addition of 3.18 μM progesterone), 9 sperm imaged in 3 experiments; P + Pt (5-min pretreatment with 200 μM picrotoxin, followed by addition of 3.18 μM progesterone), 15 sperm in 2 experiments. All imaged sperm were motile but affixed to the bottom of the image chamber by a biopolymer, Cell-Tak.

While we cannot rule out the possiblity that immobilization of sperm heads with Cell-Tak had some subtle effect on our results, it is unlikely that any major changes occurred due to Cell-Tak. In these studies, as in all our spectrofluorometric studies of entire sperm populations (e.g., Sabeur *et al.*, 1996), capacitated sperm responded to progesterone but not to solvent control by exhibiting a large increase in $[Ca^{2+}]_i$ and undergoing the AR.

Effect of Picrotoxin

The picrotoxin concentration, 200 μM, in the present studies, is one that is commonly used in electrophysiological studies of neuronal $GABA_A$ receptor/Cl^- channels (e.g., Majewska *et al.*, 1986) and that has been shown to inhibit progesterone-stimulated Cl^- efflux and the AR in human sperm (Turner and Meizel, 1995; Wistrom and Meizel, 1993). We studied 22 sperm from 4 donors (4 separate experiments) from a population that was first preincubated with 200 μM picrotoxin for 5 min. All of them exhibited an increase in sperm head Ca^{2+} following the addition of progesterone; however, the increase reached a lower peak level (46–59% smaller than the normal response) and in 16 of them (73%) did not occur in the normal wave-like manner (Figs. 3 and 4). Surprisingly, two picrotoxin-treated sperm underwent the AR, but these were not sperm that exhibited a normal Ca^{2+} wave. In these same experiments, we examined nine sperm that had been pretreated with solvent (DMSO) instead of picrotoxin and that had undergone the AR after progesterone treatment. Seven of these sperm (78%) displayed the normal Ca^{2+} wave after progesterone addition (i.e., highest rate of $[Ca^{2+}]_i$ increase near equatorial segment and $[Ca^{2+}]_i$ increased throughout the head). We conclude that picrotoxin substantially changed the $[Ca^{2+}]_i$ response following progesterone addition.

In order to compare the time courses of the change in sperm head Ca^{2+} with and without picrotoxin pretreatment, we provide typical responses for the anterior-head region on the same time scale in Fig. 5. The heads of picrotoxin-exposed sperm clearly exhibited a slower increase in free Ca^{2+} than nonexposed sperm and did not exhibit the wave-like increase.

$[Ca^{2+}]_i$ Oscillations

While conducting these experiments, we observed pronounced oscillations in anterior-, mid-, and posterior-head Ca^{2+} after the addition of progesterone. Oscillations occurred during and after the Ca^{2+} transient (Fig. 6). We usually stopped recording the $[Ca^{2+}]_i$ within 5 min after progesterone addition but observed oscillations during that entire period.

DISCUSSION

These studies demonstrate that during AR initiation, progesterone mediates a rapid, transient increase in the free Ca^{2+} of the human sperm head. Moreover, progesterone increased sperm head Ca^{2+} in all of the capacitated sperm analyzed. These results from individual sperm heads agree with single sperm imaging results obtained with progesterone-treated uncapacitated human sperm embedded in gelatin (Plant *et al.*, 1995) except for a delay (approximately 1 min) in the start of the transient seen in the latter study. However, Tesarik and co-workers carried out single cell analysis by confocal microscopy and reported that progesterone addition to capacitated human sperm results in a rapid Ca^{2+} transient (seconds) in 35% of the sperm and very few AR, followed, 2–10 min later, by a second large increase in $[Ca^{2+}]_i$ and the AR in about one-third of those sperm (Tesarik *et al.*, 1996). Although we did detect oscillations in sperm head $[Ca^{2+}]_i$ for up to 5 min after addition of progesterone, we never observed a second large increase in our studies. Furthermore, previous studies have shown that capacitated human sperm should undergo the AR within 30 sec of progesterone addition (Meizel *et al.*, 1990).

Importantly, in the present work, we were able to study the progesterone-mediated spatial $[Ca^{2+}]_i$ changes occurring in the sperm head. Our studies indicated that the single large transient increase observed occurs in a wave-like man-

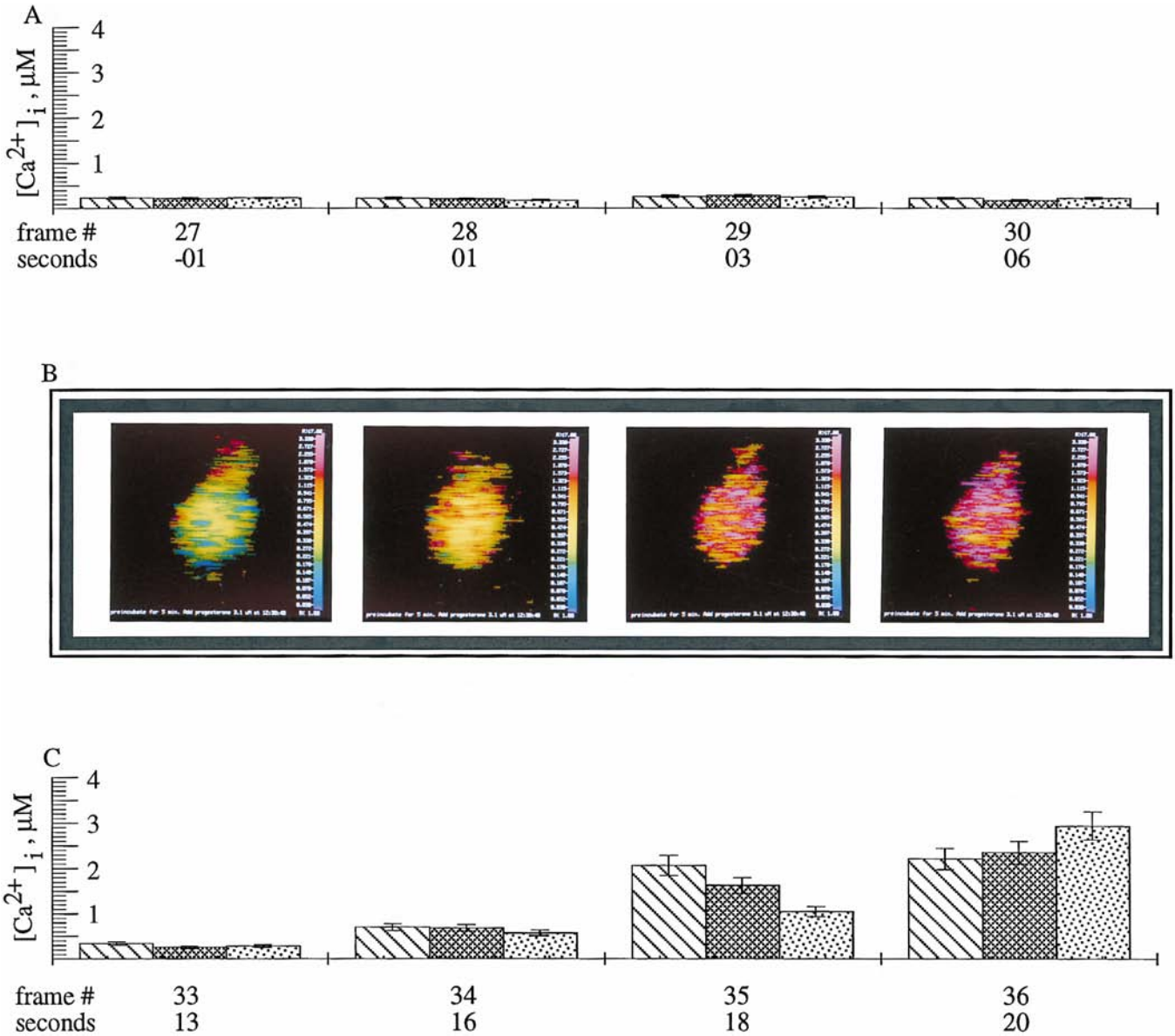

FIG. 4. Fluorescence ratio images of a fura-2-loaded, capacitated human sperm responding to 3.18 μM progesterone after 5-min preincubations with 200 μM picrotoxin, a $GABA_A$ receptor/Cl^- channel antagonist. This sperm did not undergo the acrosome reaction and was motile but affixed to the bottom of the image chamber by a biopolymer, Cell-Tak. (A) Histograms of the average $[Ca^{2+}]_i$ (immediately before and up to 6 sec after progesterone addition) measured in circular regions of 89 pixels centered on the posterior-head ▧, mid-head ▩, and anterior-head ▨ regions (see Fig. 1A). Error bars represent the SEM for $[Ca^{2+}]_i$ determined by average pixel values within the circle. The localized initial $[Ca^{2+}]_i$ increase observed in the absence of picrotoxin (see Figs. 2 and 3) is not detected here. (B) Four ratio images of the same single sperm beginning at 13 sec after addition of progesterone. The anterior end of the head is closest to the bottom of the figure. An increase in $[Ca^{2+}]_i$ occurs throughout the entire head, but no localized initial increase was observed. Original magnification was 2000×. (C) Histograms of the average $[Ca^{2+}]_i$ for the four images shown directly above in B (measurement and symbols as described for A).

ner during which a region near the equatorial segment displayed the greatest rate of increase in $[Ca^{2+}]_i$. Those results, occurring in the majority of capacitated sperm analyzed, could be explained by the presence of a greater density of plasma membrane Ca^{2+} channels near the equatorial segment region. Although the early wave did not always go in the same direction in each sperm, ultimately $[Ca^{2+}]_i$ did increase throughout the sperm head. The former result may

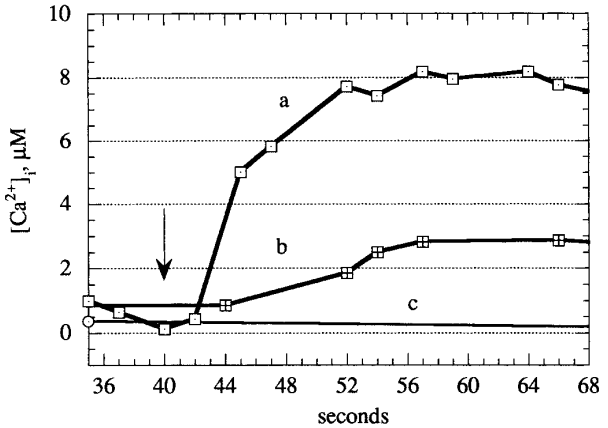

FIG. 5. Time course of the $[Ca^{2+}]_i$ change in the anterior-head region of three separately imaged capacitated human sperm following progesterone or solvent control addition with and without picrotoxin pretreatment. (a) Sperm pretreated with solvent control (0.05% DMSO) for 5 min followed by the addition of progesterone (3.18 μM) underwent an acrosome reaction. (b) Sperm pretreated with picrotoxin (200 μM) for 5 min followed by the addition of progesterone did not undergo the acrosome reaction. (c) Sperm pretreated with solvent control (0.05% DMSO) for 5 min followed by the addition of solvent control did not undergo the acrosome reaction. Progesterone or the solvent control was added at 40 sec (arrow). Note the much slower rate of the $[Ca^{2+}]_i$ increase and lower peak concentration in the picrotoxin-treated sperm. Sperm were motile but affixed to the bottom of the image chamber using the biopolymer, Cell-Tak.

be due to the fact that not all sperm were at the same stage of capacitation.

In the present studies, picrotoxin, a $GABA_A$ receptor/Cl channel blocker that inhibits progesterone-mediated Cl^- efflux in human sperm (Turner and Meizel, 1995), inhibited the progesterone-initiated human sperm AR, as it had in previous studies (Turner *et al.,* 1994; Turner and Meizel, 1995; Wistrom and Meizel, 1993). We also found that picrotoxin significantly reduced sperm head Ca^{2+} influx and eliminated the progesterone-mediated Ca^{2+} wave, producing instead a uniform, slower increase in the sperm head. These results with picrotoxin, a proven inhibitor of sperm Cl^- efflux, suggest that there is an effect of Cl^- efflux on Ca^{2+} influx. The present studies utilizing image analysis and focusing on individual sperm heads were able to detect significant effects of picrotoxin on sperm $[Ca^{2+}]$ that were missed in previous spectrofluorometric studies of the total cellular changes in entire sperm populations (Baldi *et al.,* 1991; Blackmore *et al.,* 1994; Turner *et al.,* 1994). In those previous studies, other Ca^{2+} flux changes occurring throughout the sperm probably ''diluted'' the sperm head changes resulting in no apparent overall change in sperm population $[Ca^{2+}]_i$.

Progesterone causes a depolarization of the sperm plasma membrane (Calzada *et al.,* 1991; Foresta *et al.,* 1993) due partially to Na^+ influx through an anion channel that is also permeable to Ca^{2+} (Foresta *et al.,* 1993). Perhaps Cl^- efflux via $GABA_A$-receptor/Cl^- channels is also responsible for the membrane depolarization (Turner and Meizel, 1995). Activation of $GABA_A$-receptor/Cl^- channels resulting in a membrane depolarization-mediated increase in $[Ca^{2+}]_i$ plays a role in exocytotic secretion by other cells (El-Etr *et al.,* 1995; Hales *et al.,* 1994; Gonzalez *et al.,* 1992). Voltage-sensitive Ca^{2+} channels are involved in Ca^{2+} influx essential to the zona-initated AR (Florman, 1994; Florman *et al.,* 1992). However, experiments on progesterone-mediated Ca^{2+} influx and the AR with antagonists of voltage-sensitive Ca^{2+} channels have yielded conflicting results (reviewed in Shi and Roldan, 1995). If such Ca^{2+} channels are involved in the progesterone-initiated AR, membrane depolarization due to Cl^- flux might be important for their activation. This would be true whether the Ca^{2+} channels were activated solely by membrane depolarization or if a putative ligand-gated, voltage-sensitive Ca^{2+} channel were involved. There is at least one known example, in somatic cells, of a ligand-gated Ca^{2+} channel displaying voltage sensitivity, the neuronal NMDA receptor (McBain and Mayer, 1994).

''Tight spatial coupling'' between incompletely characterized ''low-affinity'' $GABA_A$ receptors and voltage-sensitive Ca^{2+} channels has been shown to exist in cerebellar granule neurons (Hansen *et al.,* 1992). Interestingly, the location of the sperm $GABA_A$-like receptor/Cl^- channel (Wistrom and Meizel, 1993) and a protein containing the binding site for progesterone (Sabeur *et al.,* 1996) appears to be at or near the initial site of Ca^{2+} influx reported here.

Picrotoxin would inhibit sperm head membrane depolarization by blocking the $GABA_A$-like receptor/Cl^- channel. This would delay the opening of the sperm head Ca^{2+} channels and/or result in fewer Ca^{2+} channels activating. The

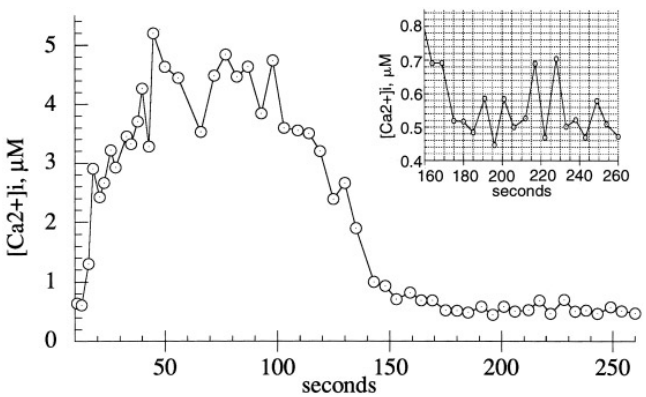

FIG. 6. Ca^{2+} oscillations in the anterior-head region of a single capacitated human sperm following addition of 3.18 μM progesterone at 0 sec. Oscillations not only appear after the Ca^{2+} transient but may also occur during its onset. Inset uses a smaller $[Ca^{2+}]_i$ scale to show smaller oscillations occurring at later time points. Sperm was motile but affixed to the bottom of the image chamber by the biopolymer, Cell-Tak.

46–59% reduction in sperm Ca^{2+} influx detected in the presence of picrotoxin was probably the cause of the observed inhibition in AR. However, we cannot rule out the possiblity that the wave-like nature of the Ca^{2+} influx is also important to the AR. Most of the picrotoxin-treated capacitated sperm examined remained acrosome-intact when treated with progesterone and did not exhibit the progesterone-mediated Ca^{2+} wave.

It should be pointed out that since the AR is inhibited by picrotoxin, we could not say in these channel blocker studies whether we were observing a decreased Ca^{2+} peak or inhibited wave in the head of a capacitated sperm with inhibited AR or an inhibited peak and inhibited or normal wave in the head of an uncapacitated, acrosome-intact sperm. However, our preliminary studies with uncapacitated sperm indicate that: (1) while only about one-third of uncapacitated sperm observed exhibited a progesterone-mediated Ca^{2+} wave in the sperm head, picrotoxin inhibited that wave in nearly all of those sperm; (2) picrotoxin decreased the peak of the progesterone-mediated Ca^{2+} increase in the sperm head by about 25%. Therefore, picrotoxin appears to inhibit progesterone-mediated changes in uncapacitated sperm as well as in those sperm that actually become capacitated. The occurrence of a wave in some uncapacitated sperm does not detract from its possible importance to the AR since it can be considered only one of several events required for the AR.

Based on earlier studies, it is clear that the progesterone-mediated increase in sperm $[Ca^{2+}]_i$ (and therefore the Ca^{2+} wave) requires an influx of that ion (Thomas and Meizel, 1989; Blackmore *et al.*, 1990). However, thapsigargin, a mobilizer of IP_3-sensitive intracellular Ca^{2+} stores, has been reported to initiate the human and mouse AR (Meizel and Turner, 1993b; Walensky and Snyder, 1995) and to potentiate the ability of progesterone to increase intracellular Ca^{2+} in human sperm (Blackmore, 1993). Moreover, IP_3 receptors have been detected in the sperm of several mammalian species and have been localized to the outer acrosomal membrane of mouse sperm (Walensky and Snyder, 1995). Intracellular Ca^{2+} stores have been proposed to be responsible for the propagation of waves occurring in other cells (Berridge and Dupont, 1994), and the present studies do not rule out such a role for sperm Ca^{2+} stores in the progesterone-mediated Ca^{2+} wave. The periodic filling and emptying of such intracellular stores might also account for the oscillations of sperm head Ca^{2+} observed in the present studies (Berridge and Dupont, 1994). Rapid oscillations of sperm flagellar and, in some cases, sperm head Ca^{2+} have been previously reported in acrosome-intact and acrosome-reacted hamster sperm (Suarez and Dai, 1995; Suarez *et al.*, 1993).

In summary, the present results are the first to demonstrate the occurrence of a ligand-mediated Ca^{2+} wave in sperm and to suggest Cl^- efflux may play a role in controlling the influx of Ca^{2+} important to the AR. Further studies will be required to determine whether the spatial–temporal aspect of the progesterone-mediated Ca^{2+} influx is an essential component of the AR mechanism and to elucidate the relationship between Ca^{2+} and Cl^- and their respective receptor/channels during the AR.

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Note added in proof. Aitken and coworkers have recently carried out image analysis studies of human sperm (Aitken, R. J., Buckingham, D. W., and Irvine, D. S., 1996, The extragenomic action of progesterone on human spermatozoa: evidence for a ubiquitous response that is rapidly down-regulated. *Endocrinology* **137,** 3999–4009). Those authors reported that progesterone, but not GABA, mediated a rapid calcium transient in >90% of the sperm in the imaged populations and that fluorescence microscopy detected the calcium transient in the ''acrosomal domain.''

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