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Intracellular Ca^{2+}-Mg^{2+}-ATPase Regulates Calcium Influx and Acrosomal Exocytosis in Bull and Ram Spermatozoa[1]

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ABSTRACT

Calcium influx is required for the mammalian sperm acrosome reaction (AR), an exocytotic event occurring in the sperm head prior to fertilization. We show here that thapsigargin, a highly specific inhibitor of the microsomal Ca^{2+}-Mg^{2+}-ATPase (Ca^{2+} pump), can initiate acrosomal exocytosis in capacitated bovine and ram spermatozoa. Initiation of acrosomal exocytosis by thapsigargin requires an influx of Ca^{2+}, since incubation of cells in the absence of added Ca^{2+} or in the presence of the calcium channel blocker, La^{3+}, completely inhibited thapsigargin-induced acrosomal exocytosis. ATP-Dependent calcium accumulation into nonmitochondrial stores was detected in permeabilized sperm in the presence of ATP and mitochondrial uncoupler. This activity was inhibited by thapsigargin. Thapsigargin elevated the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and this increase was inhibited when extracellular Ca^{2+} was chelated by EGTA, indicating that this rise in Ca^{2+} is derived from the external medium. This rise of $[Ca^{2+}]_i$ took place first in the head and later in the midpiece of the spermatozoon. However, immunostaining using a polyclonal antibody directed against the purified inositol 1,4,5-tris-phosphate receptor (IP_3-R) identified specific staining in the acrosome region, in the postacrosome, and along the tail, but not in the midpiece region. No staining in the acrosome region was observed in sperm without acrosome, indicating that the acrosome cap was stained in intact sperm. The presence of IP_3-R in the anterior acrosomal region as well as the induction, by thapsigargin, of intracellular Ca^{2+} elevation in the acrosomal region and acrosomal exocytosis, implicates the acrosome as a potential cellular Ca^{2+} store. We suggest here that the cytosolic Ca^{2+} is actively transported into the acrosome by an ATP-dependent, thapsigargin-sensitive Ca^{2+} pump and that the accumulated Ca^{2+} is released from the acrosome via an IP_3-gated calcium channel. The ability of thapsigargin to increase $[Ca^{2+}]_i$ could be due to depletion of Ca^{2+} in the acrosome, resulting in the opening of a capacitative calcium entry channel in the plasma membrane. The effect of thapsigargin on elevated $[Ca^{2+}]_i$ in capacitated cells was 2-fold higher than that in noncapacitated sperm, suggesting that the intracellular Ca pump is active during capacitation and that this pump may have a role in regulating $[Ca^{2+}]_i$ during capacitation and the AR.

INTRODUCTION

Intracellular Ca^{2+} has a regulatory role in the control of sperm motility, capacitation, and the acrosome reaction. The use of Ca^{2+} by the cell as an intracellular messenger requires precise regulation of its intracellular concentration [1]. Proposed regulatory sites of sperm intracellular calcium include both the plasma membrane [2] and the mitochondria [3–5]. The fact that sperm intracellular Ca^{2+} is maintained at a very low level (< 0.1 μM) in a medium containing millimolar Ca^{2+} supports the concept that the plasma membrane is the primary regulatory site. However, though several groups have described systems involved in sperm Ca^{2+} secretion [6, 7] and influx [2], little is known about the role of intracellular membranes in regulating Ca^{2+} in spermatozoa.

It has been shown that thapsigargin, a highly specific inhibitor of the microsomal Ca^{2+} pump, can induce elevation of intracellular free calcium $[Ca^{2+}]_i$ [8, 9] and initiate the acrosome reaction (AR) [9, 10] in human and bovine spermatozoa. Putative sites for thapsigargin-sensitive intracellular Ca^{2+} stores include the cytoplasmic droplet, the sperm nucleus, and the acrosome. In a more recent study, it was shown that inositol 1,4,5-tris-phosphate receptors (IP_3-R) are selectively localized to the acrosomes of rat, hamster, mouse, and dog sperm, suggesting that the acrosome is an intracellular Ca^{2+} store [11]. Moreover, working with isolated acrosomal membranes, we recently observed that these membranes possess an ATP-dependent Ca^{2+} pump that is inhibited by thapsigargin [12].

Walensky and Snyder [11] studied permeabilized rat sperm treated with sodium azide to follow ATP-dependent Ca^{2+} loading into nonmitochondrial Ca^{2+} stores. However, using azide to block the mitochondrial electron transport chain does not prevent the buildup of a proton gradient in the mitochondria due to ATP hydrolysis, resulting in active Ca^{2+} transport into the mitochondria.

In the present study, we used a mitochondrial uncoupler to prevent the buildup of a proton gradient; therefore no active accumulation of Ca^{2+} in the mitochondria occurred. Thus, this is the first definitive demonstration of the presence of an intracellular nonmitochondrial ATP-dependent Ca^{2+} pump in mammalian spermatozoa. A role for this internal calcium store in capacitation and in the AR is suggested.

MATERIALS AND METHODS

Materials

Thapsigargin, rabbit anti-IP_3-R polyclonal antibody, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibodies were purchased from Calbiochem (San Diego, CA); $^{45}CaCl_2$ from New England Nuclear (Boston, MA); and Fura-2/AM and Fluo-3/AM from Molecular Probes (Eugene, OR). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Sperm Preparation

Ejaculated bull and ram spermatozoa were obtained using an artificial vagina. The semen was washed three times by centrifugation (780 × *g*, 10 min at 25°C) in NKM buffer containing 110 mM NaCl, 5 mM KCl, and 10 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.4. The washed cells were suspended in NKM buffer to a concentration of 10^9 cells/ml and were maintained at room temperature until use.

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Capacitation and AR

In vitro capacitation of bovine sperm was accomplished by the method of Parrish et al. [13]. Briefly, sperm pellets were resuspended to a final concentration of 10^8 cells/ml in glucose-free TALP medium containing (in mM) 100 NaCl, 3.1 KCl, 1.5 $MgCl_2$, 25 $NaHCO_3$, 0.29 KH_2PO_4, 21.6 sodium lactate, 0.1 sodium pyruvate, 2 $CaCl_2$, 20 Hepes (pH 7.4) and 50 μg/ml BSA, 10 U/ml penicillin, and 20 μg/ml heparin. The cells were incubated in this capacitation medium for 4 h at 39°C with 5% CO_2. At the end of the capacitation, the AR inducer thapsigargin or the Ca^{2+} ionophore A23187 was added for an additional 20 min of incubation. The occurrence of AR was determined using two methods: first, assay of the release of acrosin from acrosome-reacted cells [14]; second, staining of cells by *Pisum sativum* agglutinin (PSA), whereby intact cells are stained and acrosome reacted cells are not [15]. For determination of acrosin release from the cells, the cells were pelleted by centrifugation (7500 × *g*, 5 min 4°C), and the supernatant was adjusted to pH 3.0 with HCl; acrosin activity was determined by the esterolytic assay using benzoylarginine ethyl ester as substrate and recording the optical density increase at 259 nm with time. The molar absorption coefficient was taken as 1150. All the values are given after subtraction of the spontaneous acrosin release.

Staining with PSA was performed using a modification of the method described by Mendoza et al. [15]. Samples of cells treated to induce the AR were smeared on microscope slides. After air drying, sperm smears were dipped in absolute methanol for 30 sec and allowed to dry rapidly. Methanol-fixed smears were incubated with blocking solution (PBS containing 1% BSA) for 10 min, then with biotin-conjugated PSA (50 μg/ml) in PBS containing 1% BSA for 30 min, and finally with peroxidase-conjugated extravidin (1:400) for 10 min. All incubations were performed in a humid chamber. The slides were washed between incubations by dipping in PBS for 5 min. The substrate (AEC from Histostain-SP kit; Zymed Laboratories, South San Francisco, CA) was then added for 10 min; this was followed by washing with distilled water. Hematoxylin was usually used for counterstaining (3 min). The slides were mounted with GVA mounting medium (Zymed) and examined under a brightfield microscope. Cells with red staining over the acrosomal cap were considered acrosome intact, and cells with equatorial red staining or no staining at all were considered acrosome reacted; 200 cells were counted per slide.

$^{45}Ca^{2+}$ Loading Into Permeabilized Cells

Washed spermatozoa in NKM buffer (4 × 10^8 cells/ml) were incubated with 10 μM digitonin for 2 min. These permeabilized cells were washed twice at 4°C in buffer M comprising 250 mM mannitol, 70 mM sucrose, and 10 mM Hepes, pH 7.4 (the pH was adjusted by tetraethylammonium) and kept on ice until use. For the determination of ATP-dependent Ca^{2+} loading, the permeabilized cells were resuspended in buffer B containing 75 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 20 mM Hepes, pH 7.4 (with KOH), 10 mM dithiothreitol, 3% polyethylene glycol (average M_r 8000), 10 μM carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazine (FCCP), 0.05 mM $CaCl_2$ (free Ca^{2+} ~1 μM), and 3 μCi/ml $^{45}CaCl_2$. The cell suspension was incubated at 37°C, and the reaction was started by addition of 2 mM ATP. At appropriate time intervals, a 0.1-ml aliquot was removed and vacuum-filtered on GF/C filters. The cells trapped on filters were washed three times with 5 ml of buffer containing 100 mM KCl, 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA. Radioactivity of dry filters was determined by liquid scintillation in 4 ml of EcoLume (ICN, Costa Mesa, CA).

$^{45}Ca^{2+}$ Loading Into Isolated Plasma Membrane Vesicles

Preparation of sperm plasma membrane vesicles and ATP-dependent Ca^{2+} uptake into these vesicles were performed as described by us previously [6].

Determination of Intracellular Calcium

The intracellular concentration of free Ca^{2+} was assessed using the fluorescent calcium indicator Fura-2 [16]. Washed cells (4 × 10^8/ml) were incubated in buffer A for 60 min, at 37°C, with 4 μM Fura-2/AM. The loaded cells were then washed by centrifugation at 780 × *g* for 10 min to remove extracellular Fura-2, incubated for another 30 min, and washed twice as described above. The cells were used immediately for fluorescence measurements using a Shimadzu (Columbia, MD) RF-5000 spectrofluorophotometer, with the dual excitation wavelength set of 340 nm and 380 nm and emission of 500 nm. During fluorescence measurements, sperm suspensions were stirred at 37°C. The concentration of $[Ca^{2+}]_i$ was calculated using an equation from Tsien et al. [17], $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} = F)$, where $K_d = 224$ nM. F_{max} and F_{min} were recorded at the end of each incubation. F_{max} was determined after the addition of 1% Triton X-100. F_{min} was determined in the presence of 2 mM $MnCl_2$. All values were corrected for autofluorescence of the cells.

The spatial distribution of intracellular calcium was determined using a fluorescence microscopy imaging system as described by us recently [18]. Sperm cells were incubated on 25-mm glass coverslips covered with poly-L-lysine with Fluo-3/AM (2 μM) and pluronic acid (1.5 μM) for 30 min in glucose-enriched PBS in 37°C, 5% CO_2. After incubation, each dish was rinsed twice with glucose-enriched PBS, and the coverslip was placed in the microscope holder. Cells were covered with 1 ml glucose-enriched PBS. Intracellular calcium measurements were performed using a fluorescence microscopic system consisting of an inverted epifluorescence microscope (Axiovert 135M; Zeiss, Oberkochen, Germany), an intensified charge-coupled device C2400 camera (Hamamatsu, Hamamatsu City, Japan), and frame-grabbing software (Galai, Migdal-Haemeck, Israel). A 75-W xenon lamp served as the source for excitation. A 510 long-pass emission filter was used to select fluorescence emission. The first pictures were taken to estimate basal levels of fluorescence. Afterward, cells were irradiated in situ and the fluorescence was measured every 30 sec for 10 min. Analysis of intracellular calcium levels was performed employing Scan Array 2 software (Galai).

Immunocytochemistry and Indirect Immunofluorescence

Sperm cells were collected on glass slides in a cytocentrifuge (1000 rpm, 5 min). The cells were fixed and permeabilized with cold acetone and methanol (10 min each). The slides were then washed in TBS (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) three times, 5 min each, and nonspecific sites were blocked for 30 min with 1% BSA in TBS. For indirect immunofluorescence staining, slides were incubated with polyclonal anti-IP_3-R antibody (rabbit) at concentration of 1 μg/ml for 2 h at room temperature. Con-

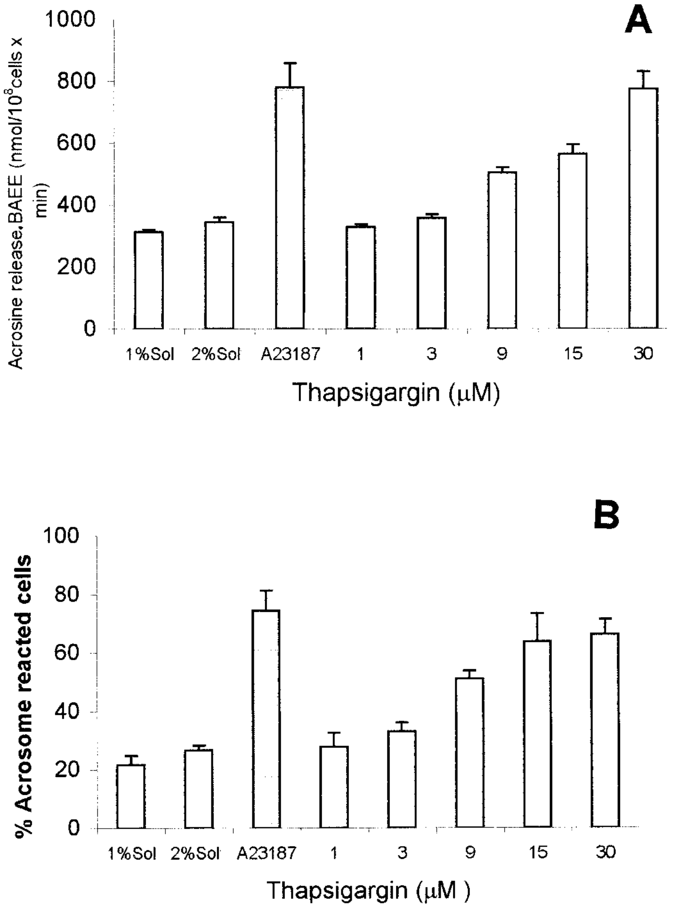

FIG. 1. Dose response for the effect of thapsigargin on the AR. Bovine sperm (10^8 cells/ml) were capacitated for 4 h in TALP medium containing heparin, followed by 20 min of incubation in the presence of increased concentrations of thapsigargin. The thapsigargin was dissolved in dimethylformamide:ethanol (1:3), and the final solvent concentration was 1% except in 30 μM thapsigargin, in which its concentration was 2%. Values are mean ± SD of duplicates from 3 different males. **A**) AR determined by assaying acrosin release from the cells. The results with 9, 15, and 30 μM thapsigargin were significantly different from the control values ($P <$ 0.001). **B**) AR determined by PSA staining. The results with 9, 15, and 30 μM thapsigargin were significantly different from the control values ($P <$ 0.001).

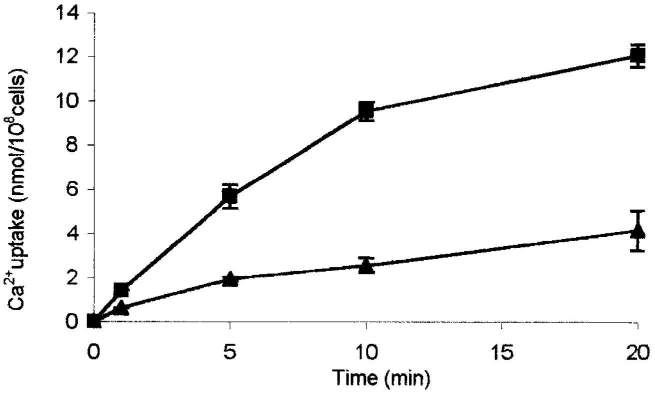

FIG. 2. Effect of the uncoupler FCCP on ATP-dependent Ca^{2+} uptake in permeabilized sperm. Washed bovine spermatozoa (10^8 cells/ml) were permeabilized, washed, and resuspended in buffer B (see *Materials and Methods*) in the presence (triangles) or absence (squares) of FCCP (10 μM). The reaction was started by adding 2 mM ATP, and at various time intervals, 0.1 ml was removed, washed, and counted for radioactivity. The values shown represent the mean ± SD of duplicates from 3 different males.

trols for IP_3-R staining were performed using normal rabbit serum. Slides were washed in TBS three times (5 min each), air dried, and incubated for 10 min in the dark with 1:10 000-diluted FITC-conjugated goat anti-rabbit IgG secondary antibody; they were then washed again in TBS three times, 5 min each. Coverslips were placed with fluoroguard antifade reagent, and the slides were examined by Venox (Olympus, Melville, NY) AHBT3 immunofluorescence microscopy.

TABLE 1. Dose response for the effect of thapsigargin and La^{3+} on the acrosome reaction in ram sperm (% acrosome-reacted cells).*

	Thapsigargin (μM)				
Inhibitor	0	1	5	15	30
None	25 ± 3.0	2.7 ± 3.0	35 ± 4.0	44 ± 5.0	60 ± 6.3
$LaCl_3$ (0.25 mM)	19 ± 4.0	—	19 ± 3.0	—	25 ± 4.0

* Ram sperm (10^8 cells/ml) were incubated for 4 h in NKM buffer containing 2 mM $CaCl_2$ followed by 20 min of incubation in the presence of the indicated concentration of thapsigargin; $LaCl_3$ (0.25 mM) was added 5 min before adding thapsigargin; reaction was determined by PSA staining; values are means ± S.D. of duplicates from 3 independent experiments.

RESULTS

Induction of the AR by Thapsigargin

Thapsigargin selectively inhibits the Ca^{2+}-Mg^{2+} ATPase (Ca^{2+} pump) of intracellular membranes and does not affect the Ca^{2+} pump of the plasma membrane [19]. To determine whether the AR is induced by mobilization of internal calcium stores, we determined the ability of thapsigargin to induce this reaction. Addition of increased concentrations of thapsigargin to capacitated bovine sperm at a concentration range of 1–30 μM significantly enhanced the AR (Fig. 1). This effect of thapsigargin could not be found in noncapacitated bovine sperm. The occurrence of AR was determined by two different assays, morphological and biochemical, and similar results were obtained. At 15 μM thapsigargin, a maximal effect was seen in which 58% of the cells were acrosome-reacted. A similar effect of thapsigargin on the AR in ram sperm was observed, but here the maximal effect on AR was seen at 30 μM thapsigargin (Table 1). Preincubation of spermatozoa with the calcium channel blocker La^{3+} (250 μM) completely inhibited thapsigargin-stimulated AR (Table 1).

Determination of Intracellular ATP-Dependent Ca^{2+} Pump Activity

The stimulation of AR induced by thapsigargin suggests that spermatozoa contain an intracellular, membrane-associated ATP-dependent Ca^{2+} pump. We used digitonin-permeabilized ram and bull sperm to follow ATP-dependent accumulation of Ca^{2+} in intracellular nonmitochondrial Ca^{2+} stores. Added ATP can be hydrolyzed by the sperm mitochondria to create membrane potential via the H^+-ATPase, resulting in active accumulation of Ca^{2+} in the mitochondria. In order to eliminate this effect, it is not sufficient to block the respiratory chain as performed by Walensky and Snyder [11], since under these conditions the mitochondrial H^+-ATPase can still create membrane potential. Therefore, in this study we used the mitochondrial un-

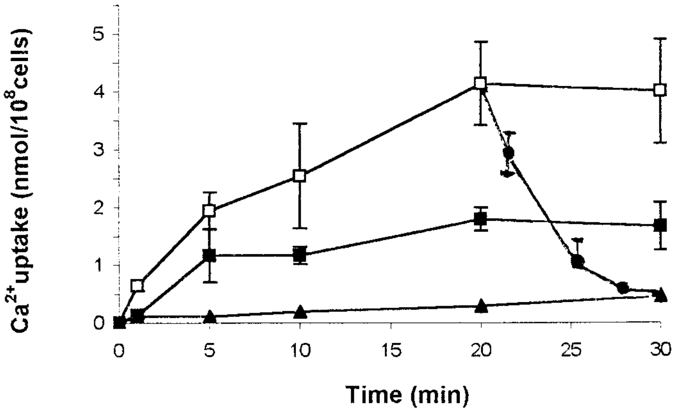

FIG. 3. ATP-dependent Ca^{2+} uptake into nonmitochondrial calcium stores in permeabilized sperm. Bovine spermatozoa (10^8 cells/ml) were permeabilized, washed, and resuspended in buffer B containing 10 μM FCCP. The reaction was initiated by adding 2 mM ATP or buffer (control without ATP). Thapsigargin (10 μM) was added 5 min before addition of ATP. At various time intervals, 0.1 ml cell suspension was removed, washed, and counted for radioactivity. The values are the mean ± SD of duplicates from 3 different males. Control: open squares; thapsigargin: solid squares; no ATP or ATP plus 10 μM A23187: triangles; 10 μM A23187 added at 20 min to control: circles.

coupler FCCP, which dissipates the membrane potential, preventing active transport of Ca^{2+} into the mitochondria.

It can be seen in Figure 2 that ATP-dependent Ca^{2+} uptake into permeabilized sperm was strongly inhibited by FCCP, indicating that ATP-dependent Ca^{2+} uptake by the sperm mitochondria occurs in this system. After 20 min of incubation, about 11 nmol $Ca^{2+}/10^8$ cells was taken up in absence of FCCP, whereas in the presence of the uncoupler, only about 4 nmol $Ca^{2+}/10^8$ cells was taken up. These results indicate that 64% of the ATP-dependent Ca^{2+} uptake was accumulated in the mitochondria and only 36% in nonmitochondrial Ca^{2+} stores.

A time-dependent increase in ATP-dependent Ca^{2+} uptake into nonmitochondrial Ca^{2+} stores in permeabilized spermatozoa is seen in Figure 3. Very little Ca^{2+} uptake was observed in the absence of ATP or in the presence of the Ca^{2+} ionophore A23187, and the ATP-dependent Ca^{2+} uptake was highly inhibited by thapsigargin.

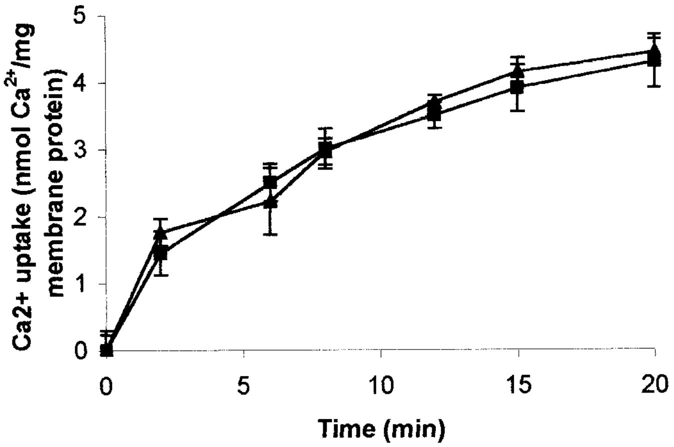

FIG. 4. ATP-dependent Ca^{2+} uptake into isolated plasma membrane vesicles from bovine sperm, Ca^{2+} uptake activity was assayed as described by us previously [6]. The reaction was started by adding 2 mM ATP, and thapsigargin (10 μM) was added 5 min before the ATP. Each point represents the mean ± SD of duplicate sample determination from three membrane preparations from 3 different bulls. Control: triangles; thapsigargin: squares.

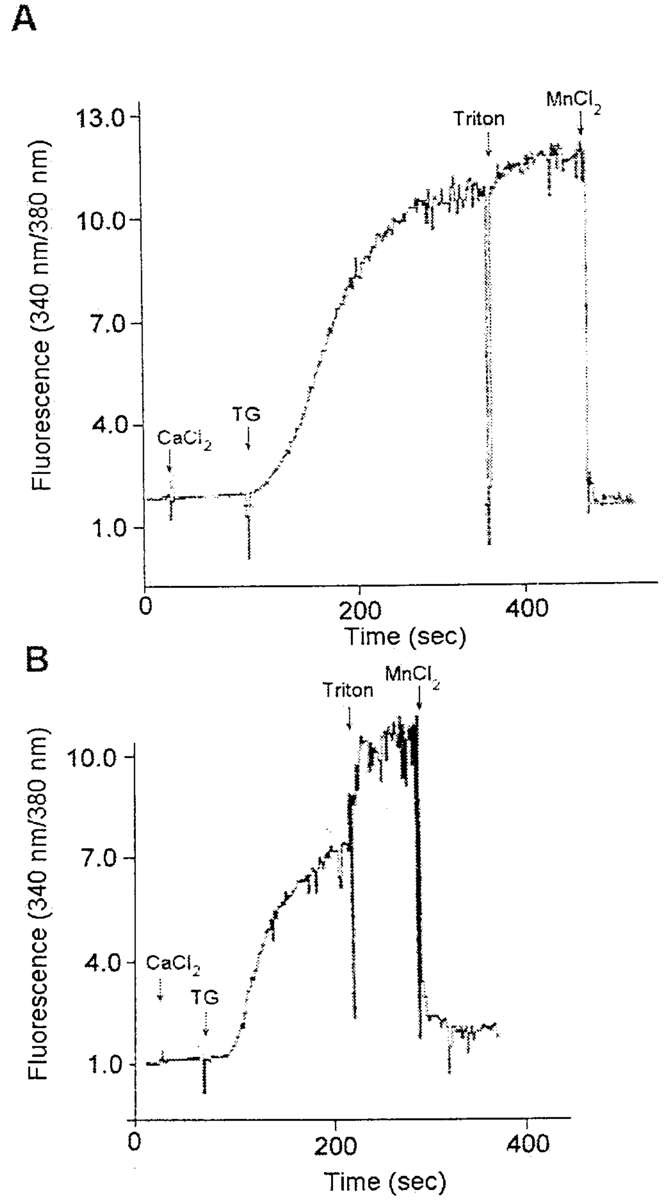

FIG. 5. Effect of thapsigargin on sperm $[Ca^{2+}]_i$. Intact sperm cells (5 × 10^6 cells/ml) incubated in NKM buffer were loaded with Fura-2/AM, and the changes in $[Ca^{2+}]_i$ were recorded using the spectrofluorophotometer (see *Materials and Methods*). At the times indicated by the arrows, the following substances were added (final concentration): 2 mM $CaCl_2$, 3 μM thapsigargin (TG), 0.1% Triton X-100, and 2 mM $MnCl_2$. The data represent one typical experiment out of four repetitions performed with 4 different males. **A**) Ram sperm: $[Ca^{2+}]_i$ was 35 ± 12 nM after addition of $CaCl_2$ and 1600 ± 200 nM after addition of thapsigargin. **B**) Bull sperm: $[Ca^{2+}]_i$ was 25 ± 10 nM after addition of $CaCl_2$ and 600 ± 100 nM after addition of thapsigargin.

The addition of the Ca^{2+} ionophore A23187 to Ca^{2+}-loaded cells after 20-min incubation caused fast release of the accumulated Ca^{2+}, suggesting that Ca^{2+} is moved into nonmitochondrial Ca^{2+} stores by an active transport process. Thapsigargin did not inhibit ATP-dependent Ca^{2+} uptake in isolated plasma membrane vesicles from bovine sperm (Fig. 4), which indicates its specificity for intracellular Ca^{2+} pumps.

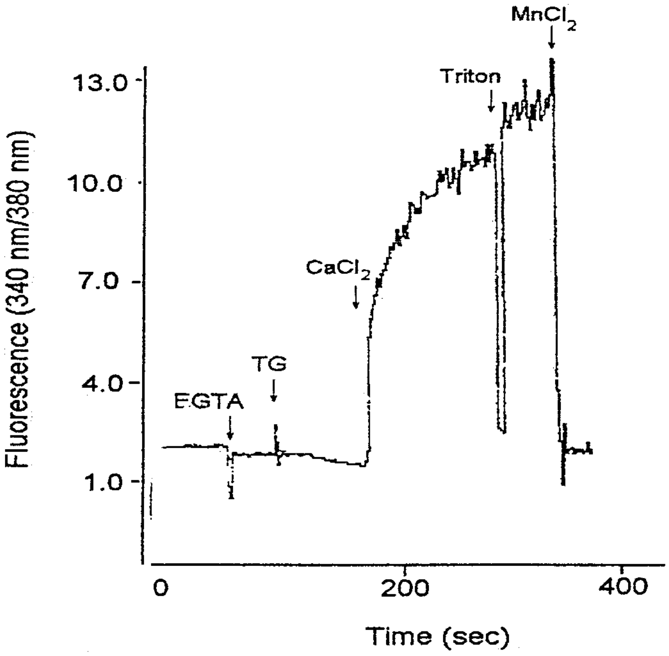

FIG. 6. Effect of thapsigargin on $[Ca^{2+}]_i$ in absence or presence of extracellular Ca^{2+}. Intact ram sperm (5×10^6 cells/ml) were loaded with Fura-2/AM, and changes in $[Ca^{2+}]_i$ were measured. See legend to Figure 5 for more details. At the times indicated by the arrows, the following substances were added (final concentration): 1.5 mM EGTA, 3 μM thapsigargin (TG), 2 mM $CaCl_2$, 0.1% Triton X-100, and 2 mM $MnCl_2$. The data represent one typical experiment out of three repetitions performed with 3 different males.

Elevation of $[Ca^{2+}]_i$ by Thapsigargin

Intracellular Ca^{2+} concentrations were determined in Fura-2-loaded noncapacitated sperm. Low concentrations of thapsigargin (3 μM) caused a fast and significant increase in $[Ca^{2+}]_i$ in ram and bull spermatozoa (Fig. 5). This increase in $[Ca^{2+}]_i$ could not be seen without addition of Ca^{2+} in the presence of the Ca^{2+} chelator EGTA (Fig. 6). These findings indicate that thapsigargin induces Ca^{2+} influx into the cells from the surrounding medium.

The effect of thapsigargin on the spatial distribution of intracellular Ca^{2+} was evaluated in Fluo-3-loaded intact, noncapacitated sperm using a fluorescence microscopy imaging system (Fig. 7). It can be seen that 150 sec after addition of Ca^{2+}, there was a significant increase of intracellular Ca^{2+} in the sperm head. The Ca^{2+} concentration started to increase in the midpiece where the sperm mitochondria are located only after 160 sec. After 200 sec, the $[Ca^{2+}]_i$ in the head and the midpiece reached its maximal value (Fig. 7, see red color at 400 sec), which did not change during the next 40 sec. The increase in $[Ca^{2+}]_i$ in the head under these conditions reflects the induction of AR by thapsigargin. The measurements of changes in $[Ca^{2+}]_i$ in these experiments are summarized graphically in Figure 8.

Localization of the IP_3-R in the Sperm

Localization of IP_3-R in the spermatozoon should provide information concerning the organelle that accumulates Ca^{2+} via the ATP-dependent Ca^{2+} pump described here. Immunocytochemical analysis using polyclonal antibodies that recognize the cytoplasmatic C-terminal of IP_3-R revealed extensive staining in the acrosomal and postacrosomal regions of the head and along the tail, but not in the midpiece of the sperm (Fig. 9). The acrosomal region was not stained in sperm without acrosome (see arrowhead in Fig. 9), indicating that the antibody specifically stained the acrosome. The localization of IP_3-R in the acrosome cap was previously demonstrated in hamster, rat, mouse, and dog sperm [11].

Changes in $[Ca^{2+}]_i$ in Capacitated Sperm

Spectrofluorometric measurements of $[Ca^{2+}]_i$ using Fura-2-loaded capacitated or noncapacitated bovine sperm revealed an increase in $[Ca^{2+}]_i$ after the addition of thapsigargin (Fig. 10). In noncapacitated cells, the addition of Ca^{2+} caused a very slight increase in $[Ca^{2+}]_i$; and the addition of thapsigargin was followed by a lag time of about 30 sec and then a fast increase in $[Ca^{2+}]_i$ (Fig. 10A). In capacitated cells, the initial concentration of Ca^{2+} was higher than that in noncapacitated cells (60 ± 20 vs. 25 ± 10 nM); addition of Ca^{2+} to the medium induced an almost 2.5-fold increase in $[Ca^{2+}]_i$ (160 ± 40 nM), followed by a 7-fold increase after addition of thapsigargin (1100 ± 250 nM) (Fig. 10B). The response to thapsigargin was different between noncapacitated and capacitated sperm. In capacitated sperm there was no lag time, and the increase in Ca^{2+} was 3.9 times slower; but the final $[Ca^{2+}]_i$ reached was twice as high (1100 ± 250 vs. 500 ± 150 nM). These data clearly demonstrate that significant changes in the mechanisms involved in Ca^{2+} regulation occur during sperm capacitation.

DISCUSSION

In this study, we demonstrate three aspects of the role of intracellular Ca^{2+} stores in controlling the AR. 1) We show the existence of intracellular nonmitochondrial Ca^{2+} stores that actively accumulate Ca^{2+} by an ATP-dependent Ca^{2+} pump; 2) we demonstrate that inhibition of this pump by thapsigargin causes fast elevation of $[Ca^{2+}]_i$, first in the sperm head and later in the midpiece, and only in the presence of extracellular Ca^{2+}; 3) we show that inhibition of this Ca^{2+} pump in capacitated sperm causes a much higher (2-fold) increase in $[Ca^{2+}]_i$ than in noncapacitated cells and results in acrosomal exocytosis. For many years, it was believed that since a spermatozoon does not contain an endoplasmic reticulum, there is no intracellular nonmitochondrial Ca^{2+} store in this cell. Recently, it was shown that thapsigargin can cause an increase in sperm $[Ca^{2+}]_i$ [8, 9] and that it induces the AR [9, 10, 12] in capacitated spermatozoa. Thapsigargin is a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump [20], and its effect on sperm cells was the first indication of the existence of intracellular Ca^{2+} stores. Concerning the specificity of thapsigargin in spermatozoa, we showed here that the plasma membrane Ca^{2+} pump is not sensitive to thapsigargin (Fig. 4) while the Ca^{2+} pump of acrosomal membranes is highly inhibited by thapsigargin [12].

The first evidence for the existence of an intracellular ATP-dependent Ca^{2+} pump in rat sperm was suggested by Walensky and Snyder [11]. They followed ATP-dependent Ca^{2+} uptake in permeabilized sperm and attempted to prevent Ca^{2+} accumulation in the mitochondria by blocking mitochondrial respiration with sodium azide. However, under these conditions, the added ATP can be hydrolyzed by the mitochondrial H^+-ATPase, resulting in the creation of membrane potential and active accumulation of Ca^{2+} into the mitochondria. In the present study, we used the mitochondrial uncoupler FCCP and showed that under these

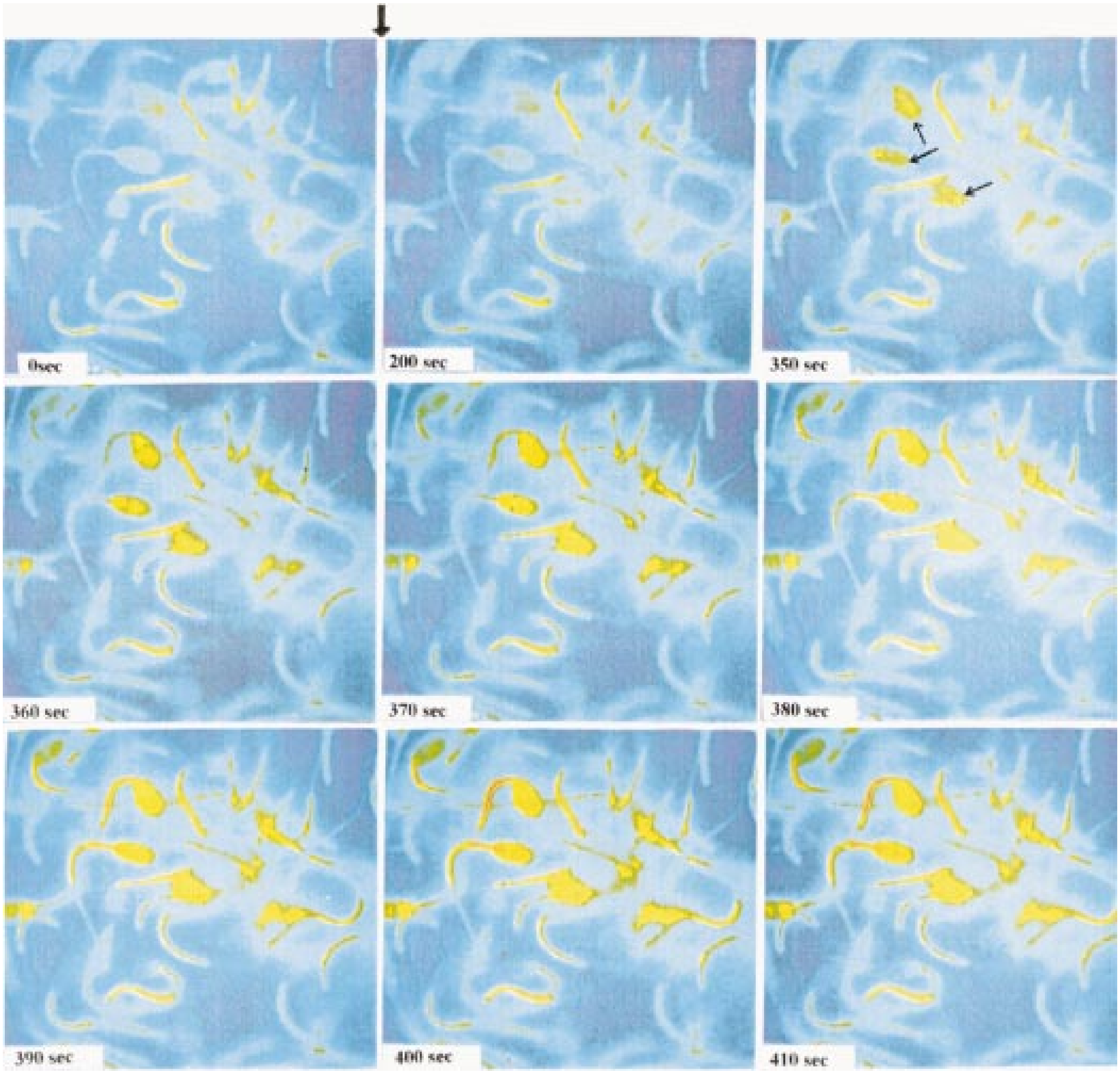

FIG. 7. Effect of thapsigargin on the spatial distribution of $[Ca^{2+}]_i$: intact noncapacitated bovine sperm in TALP medium without Ca^{2+} were loaded with Fluo-3/AM and photographed successively at the indicated times (see *Materials and Methods*). Thapsigargin (3 μM) was added at the beginning, and after 200 sec, 2 mM $CaCl_2$ was added (see arrow). The arrows in the picture after 350 sec indicate an increase in $[Ca^{2+}]_i$ in the sperm head. ×1000 (published at 90%).

conditions, the Ca^{2+} uptake into the permeabilized cells is much lower (Fig. 2). It is well known that in the presence of uncoupler, the mitochondrial membrane potential is dissipated—conditions under which no ATP synthesis or active Ca^{2+} uptake can take place. Thus the remaining Ca^{2+} uptake is highly suggestive of nonmitochondrial Ca^{2+} stores. The ATP-dependent Ca^{2+} uptake in permeabilized sperm is strongly inhibited by thapsigargin, and very little uptake can be seen in the presence of the Ca^{2+} ionophore A23187 (Fig. 3), indicating that Ca^{2+} is accumulated in intracellular nonmitochondrial Ca^{2+} stores.

In intact bull and ram spermatozoa, thapsigargin induces the acrosomal reaction (Fig. 1) as well as an increase in $[Ca^{2+}]_i$ (Fig. 5). These effects are found only in the presence of extracellular Ca^{2+}; they could not be seen when Ca^{2+} influx was blocked by the calcium blocker La^{3+} (Table 1) or when extracellular Ca^{2+} was chelated by EGTA (Fig. 6). Since the ATP-dependent Ca^{2+} pump of the plasma membrane is not affected by thapsigargin (Fig. 4), we suggest that the effect of thapsigargin on AR and $[Ca^{2+}]_i$ is due to the inhibition of an intracellular Ca^{2+} pump. It is known that AR is a calcium-dependent process that requires extracellular Ca^{2+} [21]. In a wide range of somatic cells, release of calcium from internal stores also promotes extracellular calcium influx across the plasma membrane [22]. This phenomenon is called capacitative calcium entry. The

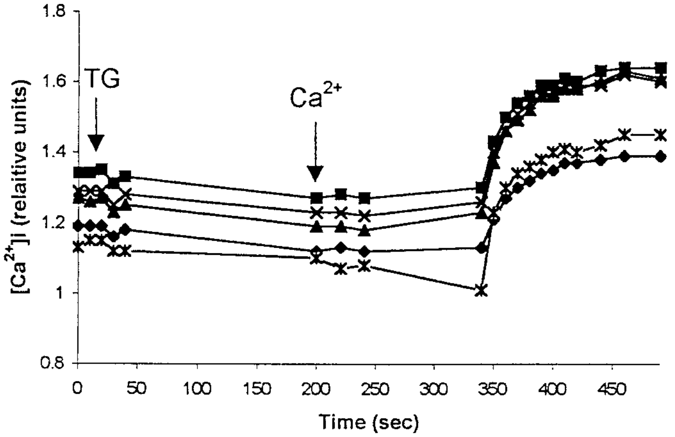

FIG. 8. Graphic presentation of $[Ca^{2+}]_i$ in 5 randomly chosen noncapacitated bovine sperm cells loaded with Fluo-3/AM. The changes in Ca^{2+} were measured after addition of 3 μM thapsigargin (TG) and 2 mM Ca^{2+}. The Ca^{2+} concentration scale is expressed in relative units.

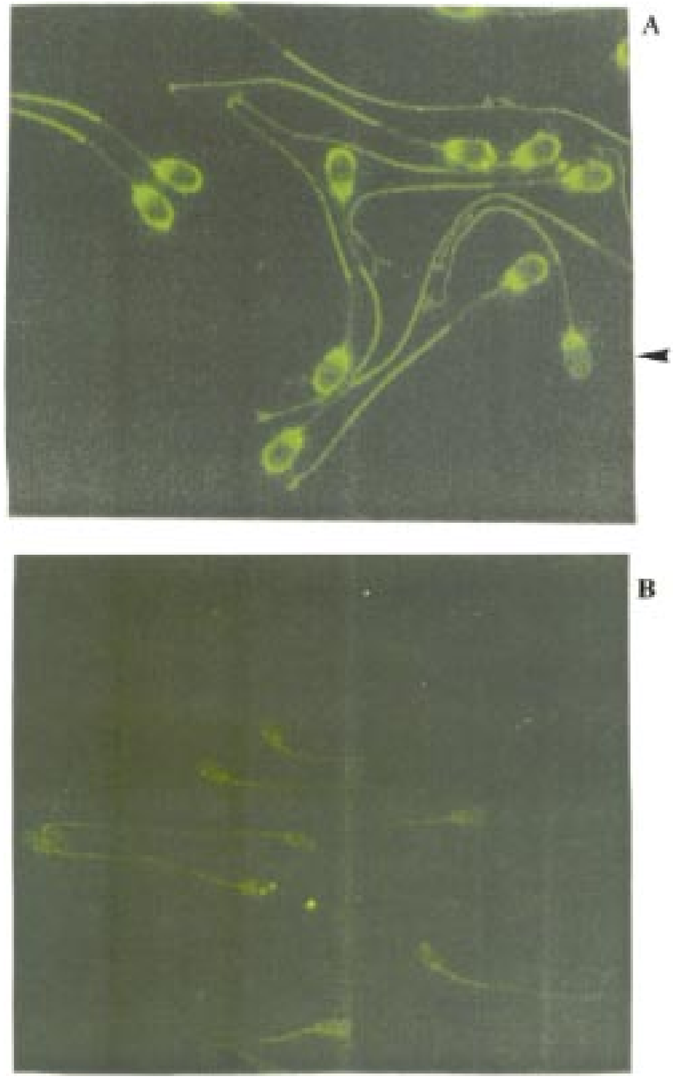

FIG. 9. Localization of IP_3-R in spermatozoa. **A**) Visualization of bovine sperm IP_3-R using rabbit polyclonal anti-IP_3-R antibody and FITC-conjugated second antibody. Arrowhead: acrosome-reacted cell. **B**) Control using normal rabbit serum instead of rabbit anti-IP_3-R antibody.

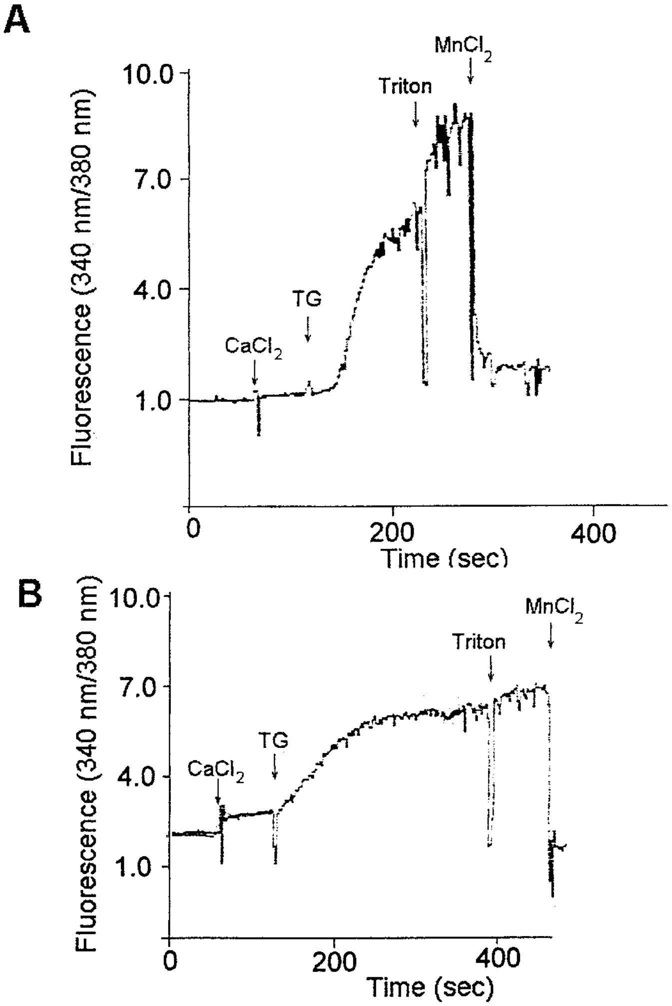

FIG. 10. Effect of capacitation on thapsigargin-induced changes in bovine sperm $[Ca^{2+}]_i$. Sperm (5×10^6 cells/ml) were capacitated for 3 h in TALP medium containing heparin, followed by 1-h incubation in the presence of Fura-2/AM. At the end of the capacitation time, the cells were washed and resuspended in TALP without Ca^{2+}, and changes in fluorescence were recorded. At the arrows, the following substances were added (final concentration): 2 mM $CaCl_2$, 3 μM thapsigargin (TG), 0.1% Triton X-100, and 2 mM $MnCl_2$. The data represent one experiment, typical of three repetitions performed with 3 different males. **A**) Noncapacitated sperm, incubated for 4 h in TALP without heparin. $[Ca^{2+}]_i$ was 25 ± 10 nM before addition of $CaCl_2$, 35 ± 15 nM after addition of $CaCl_2$, and 500 ± 150 nM after addition of thapsigargin. **B**) Capacitated sperm: $[Ca^{2+}]_i$ was 60 ± 20 nM before addition of $CaCl_2$, 160 ± 40 nM after addition of $CaCl_2$, and 1100 ± 250 nM after addition of thapsigargin.

mechanism for this phenomenon is not completely understood, although it is accepted that the opening of a calcium channel in the plasma membrane is due to reduction of Ca^{2+} in the internal stores [23]. Thus, the inhibition of internal Ca^{2+} pump by thapsigargin in the sperm will cause reduction of Ca^{2+} in this store, resulting in the opening of Ca^{2+} channel in the plasma membrane.

In many secretory cells, the initial focal release of Ca^{2+} from internal stores has a priming effect on exocytosis [24, 25]. Since acrosomal exocytosis can be induced by thapsigargin, it is likely that the internal Ca^{2+} store in sperm would be localized in close proximity to the region in which acrosomal exocytosis occurs, i.e., in the acrosomal region of the sperm head. In many somatic cell types, calcium stored in internal membranes is released via an IP_3-gated Ca^{2+} channel [22]. Thus, the localization of IP_3-R in the acrosome region of bull and ram sperm (Fig. 9) suggests that this organelle may function as a calcium store. This IP_3-R was clearly localized in the acrosome of rat, hamster, mouse, and dog sperm, suggesting that the acrosome is the internal calcium store [11]. These authors also showed that ATP-dependent Ca^{2+} uptake in permeabilized rat sperm is inhibited approximately 50% by inclusion of IP_3 in the incubation medium, suggesting that Ca^{2+} is released from the store by IP_3. The fact that they found only 50% inhibition by IP_3 suggests that under the Ca^{2+} uptake conditions used, Ca^{2+} is also accumulated in IP_3-insensitive stores, possibly in the mitochondria, as suggested earlier in this discussion.

Working with isolated bull sperm acrosomes, we found that these membranes possess an ATP-dependent Ca^{2+} uptake activity, which is blocked by thapsigargin [12]. In addition, these membranes possess a cAMP/protein kinase A (PKA)-dependent Ca^{2+} channel, suggesting the existence on the acrosomal membrane of either a cAMP-gated calcium channel [26] or a channel opened upon phosphorylation by PKA [27]. Moreover, sperm acrosomes are rich in calreticulin, a high-capacity Ca^{2+}-binding storage protein [28] that has been reported to copurify with an IP_3-sensitive Ca^{2+} store [29]. This information further supports our notion concerning the function of the acrosome as an intraspermatozoal calcium store.

During sperm capacitation, $[Ca^{2+}]_i$ is enhanced in the acrosomal region of the head and in the tail [30]. In capacitated cells, thapsigargin induces a large increase in $[Ca^{2+}]_i$ (Fig. 10), leading to acrosomal exocytosis. Thus, the data suggest that the internal Ca^{2+} pump is active during the capacitation process.

The kinetics of Ca^{2+} influx and the maximal $[Ca^{2+}]_i$ induced by thapsigargin in capacitated cells differ from those found in noncapacitated sperm (Fig. 10). In capacitated cells, the maximal $[Ca^{2+}]_i$ induced by thapsigargin is 1100 ± 100 nM, while in noncapacitated sperm the $[Ca^{2+}]_i$ reached only 500 ± 60 nM. Since $[Ca^{2+}]_i$ is higher in capacitated cells, and it accumulated in the acrosomal region, it is possible that a more significant reduction of acrosomal Ca^{2+} induced in capacitated cells by thapsigargin causes greater influx of Ca^{2+}. It is not clear why, after thapsigargin is added to noncapacitated cells, there is a lag time followed by a fast increase in $[Ca^{2+}]_i$, whereas in capacitated cells there is no lag time and the rate of Ca^{2+} influx is much slower (Fig. 10). Similar results were obtained by Parrish et al. [9], who determined $[Ca^{2+}]_i$ in individual bovine sperm using the imaging technique. Since we suggest that Ca^{2+} in the acrosome of capacitated cells is higher than in noncapacitated cells, it takes longer to deplete the stores sufficiently to trigger calcium entry from the extracellular medium, and this is the rate-limiting step for activating the capacitative calcium entry mechanism.

Our suggestion concerning the possible accumulation of Ca^{2+} in the acrosome during capacitation is further supported by the findings that phospholipase C (PLC), which hydrolyzes phosphoinositides to produce IP_3, is activated by the egg zona pellucida (ZP_3), indicating that IP_3-dependent Ca^{2+} release takes place only at the end of the capacitation process [31]. It was also shown that PLCγ is translocated to the sperm plasma membrane during capacitation [31, 32] prior to its activation by the egg zona pellucida. Thus, the formation of IP_3 just before the occurrence of AR would keep the acrosomal IP_3-gated Ca^{2+} channel inactive during capacitation, allowing the accumulation of Ca^{2+} in the acrosome during the capacitation process.

Recently it was suggested that binding of sperm to zona pellucida may activate adenylyl cyclase to form cAMP that activates PKA to open a Ca^{2+} channel in the acrosomal membrane [1]. As a result, the $[Ca^{2+}]_i$ in the sperm cytosol is increased and PLC is activated to produce IP_3 and diacylglycerol. IP_3 would cause further release of acrosomal Ca^{2+} via the IP_3-gated Ca^{2+} channel, resulting in Ca^{2+} depletion in the acrosome and activating the capacitative calcium entry mechanism. This final step would cause a relatively high elevation of $[Ca^{2+}]_i$ thereby activating actin-severing proteins, leading to dispersion of the F-actin barrier intervening between the outer acrosomal and the overlying plasma membrane [32]. The primed membranes would then be able to come into contact and fuse, releasing the acrosomal contents and completing the AR.

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