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Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis: possible functional implications

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Summary

During spermatogenesis the activity of intracellular Ca^{2+}-release channels is likely to play an important role in different specific cellular functions. Accordingly, messenger RNAs for the three inositol 1,4,5-trisphosphate receptor (IP_3R) subtypes were found to be present throughout spermatogenesis. Immunocytochemical analysis revealed distinct distribution patterns of the mature IP_3Rs during sperm differentiation. At early stages, IP_3Rs are distributed throughout the cytoplasm, and as differentiation proceeds they become selectively localised to the Golgi complex. Consistently, spermatogonia underwent large intracellular Ca^{2+} release in response to thapsigargin (TG), while smaller responses were detected in late spermatocytes and spermatids. The distribution of IP_3Rs and the larger Ca^{2+}-release responses found in spermatogonia, suggest that IP_3Rs may be involved in cell proliferation at this stage. This notion is supported by our observations in a spermatogenic cell line that depletion of intracellular Ca^{2+} pools using TG inhibits cell division, and that incubation with an IP_3R-I antisense oligonucleotide completely inhibited proliferation. Furthermore, the three genes encoding ryanodine receptor proteins (RyRs) are expressed at all stages of spermatogenesis. However, immunocytochemical studies with specific antibodies against each of the RyR subtypes detected types 1 and 3 in spermatogenic cells and only type 3 in mature sperm. In contrast to IP_3Rs, RyRs remain scattered in the cytoplasm throughout differentiation. Functional responses to caffeine and ryanodine were absent in spermatogenic cells and in mature sperm. These findings suggest that IP_3Rs have significantly more important roles in spermatogenesis than RyRs, and that one of these roles is crucial for cell proliferation.

Keywords: Cell proliferation, Intracellular calcium channels, IP_3 receptor, Ryanodine receptor, Spermatogenesis

Introduction

Ca^{2+} is a ubiquitous signal transduction element in eukaryotic cells. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) are tightly regulated through a balance between Ca^{2+} release from intracellular stores and entry across the plasma membrane. Ca^{2+} release is mediated by two classes of distinct, yet related channels: the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the ryanodine receptors (RyRs). Both classes are responsible for the release of Ca^{2+} from intracellular stores leading to rises in $[Ca^{2+}]_i$ in response to diverse stimuli. These proteins belong to two different receptor families encoded by several genes. The three known RyR genes are RyR-1, RyR-2 and RyR-3, also known as skeletal, cardiac and brain types, respectively. Three different genes were initially reported to encode IP_3R subtypes, designated type I, II and III (Berridge, 1993); more recently, a fourth and a fifth subtype were identified, although only partial sequence information is available (Suresh, 1996). Alternatively spliced isoforms have been also reported for both proteins (Danoff *et al.*, 1991;

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Iida & Bourguignon, 1994). Each of these proteins associates in tetrameric complexes to form Ca^{2+} release units. It has been shown that the same cell can express both IP_3Rs and RyRs, and more than one subtype of each class. There is evidence that IP_3Rs can form either homo- or heterotetramers (Monkawa *et al.*, 1995), and it is possible that the same applies to RyRs. This diversity could account for the functional differences reported for these channels.

In addition to its well-characterised involvement in muscle contraction, secretion and fertilisation, Ca^{2+} plays a central role during more general cellular processes such as cell growth, proliferation and death. Although the mechanisms responsible for generating Ca^{2+} signals are diverse, Ca^{2+} release channels have been repeatedly implicated, at least in some cell types, in the regulation of the above-mentioned cellular processes. For example, depletion of the IP_3-sensitive Ca^{2+} pools results in cell cycle arrest in a smooth muscle cell line (DDT1MF-2) (Gosh *et al.*, 1991). Ca^{2+} signals also participate in cell differentiation (Holliday *et al.*, 1991; Gu & Spitzer, 1997). It is unclear, however, to what extent $[Ca^{2+}]_i$ affects differentiation in other non-excitable cells.

Spermatogenesis is a complex and highly coordinated process by which spermatogonia proliferate and differentiate to produce mature sperm. This process depends on the simultaneous capacity of spermatogonia to undergo efficient proliferation and to enter into a finely controlled differentiation programme that includes a meiotic cycle. Ca^{2+} signals are expected to participate in this interesting process, but very little is known about $[Ca^{2+}]_i$ regulation in spermatogenic cells. Indirect evidence implicates the participation of $[Ca^{2+}]_i$ signalling during sperm differentiation. For example, Santi *et al.* (1996) suggested that Ca^{2+} influx mediated by the activation of T-type Ca^{2+} channels could represent a signal for male germ cell differentiation. This suggestion was made on the basis that $[Ca^{2+}]_i$ increases mediated by T-type Ca^{2+} channels encode different aspects of neuronal differentiation (Gu & Spitzer, 1993, 1995). It turns out that pachytene spermatocytes have the lowest resting $[Ca^{2+}]_i$ (~50 nM) among the population of germ cells, and that this value increases gradually in subsequent stages of germ cell differentiation (Santi *et al.*, 1997). In addition, the importance of $[Ca^{2+}]_i$ for the physiology of mature sperm has been clearly demonstrated. Specifically, it is known that during the acrosome reaction, an essential exocytotic event that precedes sperm–egg fusion, an elevation of $[Ca^{2+}]_i$ is required (Darszon *et al.*, 1996). A role for Ca^{2+} release channels in sperm physiology has been suggested by Walensky & Snyder (1995), who identified IP_3Rs in the acrosome of mature sperm.

Our group has been interested in studying the participation of $[Ca^{2+}]_i$ in sperm physiology. The identity and functional properties of voltage-gated Ca^{2+} channels present in spermatogenic cells were analysed previously (Santi *et al.*, 1996). We also found that spermatogenic cells mainly contain transcripts for the α1e gene and to a minor extent α1a (Liévano *et al.*, 1996). These results are in agreement with the observation that only T-type Ca^{2+} currents are present in spermatogenic cells (Arnoult *et al.*, 1996; Liévano *et al.*, 1996; Santi *et al.*, 1996).

The present work extends the characterisation of elements involved in male germ cell Ca^{2+} signalling by analysing the identity, subunit composition and subcellular distribution of the intracellular Ca^{2+} release channels in different stages of spermatogenic cell differentiation and in mature spermatozoa. These data are complemented with experiments designed to assess the possible functional role of intracellular Ca^{2+} release channels during different stages of spermatogenesis involving proliferation, meiosis and differentiation. We also provide data on the effects of proliferation and survival of a spermatocyte cell line with antisense oligonucleotides used to arrest the synthesis of functional Ca^{2+} release channels.

Materials and methods

Spermatogenic cells

Testes from 10 adult CD1 mice were used for each separation. Each testis was decapsulated and subjected to collagenase and trypsin digestion in EKRB (in mM: 120 NaCl, 25.2 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 11 glucose; 1× essential amino acids, 1× non-essential amino acids (Sigma) as previously described (Bellvé, 1993)). Cells were resuspended in 0.5% bovine serum albumin (BSA)/EKRB, filtered through 80 μm mesh Nytex nylon and sedimented at unit gravity through a 2–4% BSA/EKRB linear gradient generated on a Staput chamber at 4 °C (total volume 1.1 l). After 2.5 h of sedimentation, 10 ml fractions were collected and viewed under a light microscope to determine cell morphology and purity of the cell fractions. Similar fractions were pooled. We used purified populations of cells at four different stages of differentiation: pachytene spermatocytes (PS), round spermatids (RS), condensing spermatids (CS) and residual bodies (RB). Type A and B spermatogonia were directly isolated by enzymatic digestion of testes of 7-day-old-mice. For immunocytochemistry experiments, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min.

Sperm

Mouse sperm were collected from caput epididymis. Sperm were allowed to disperse freely in PBS for

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10–15 min at room temperature. An aliquot from this cell suspension was immediately fixed with paraformaldehyde (5% final concentration) for at least 10 min, and used as the non-acrosome-reacted sperm. The remaining sperm were capacitated at 37 °C, 5% CO_2 for 30 min and the acrosome reaction was induced by incubation under the same conditions as capacitation with 400 μM progesterone. This progesterone concentration has been observed to produce the acrosome reaction in about 90% of sperm (J.L. De la Vega, unpublished observations). Similar immunocytochemical results were obtained when the acrosome reaction was induced with 15 μM A23187. Finally, cells were fixed with paraformaldehyde. The percentage of acrosome-reacted sperm was determined by staining with coomassie blue as previously described (Miller *et al.*, 1992).

RNA isolation and RT-PCR experiments

Total RNA from isolated spermatogenic cells or total tissue was extracted using RNAzol (Life Technologies) according to the manufacturer's instructions. The Superscript system (Life Technologies) was used for reverse transcription polymerase chain reaction (RT-PCR). Total RNA was digested with RNAse-free DNAse and 5 μg were reverse transcribed using random hexamer primers (2.5 ng/ml). The resulting cDNA was used for amplification of RyR and IP_3R cDNA fragments by PCR using the following primers RyR: R1 5' GCGAATTCTAYTGGGAYAARTTYGTNAA 3' forward (YWDKFVK), R2 5' GCGAATTCAYTAYAAYAAYTTYTTYTT 3' forward (HYNNFFF), R3 5' GCGGTACCRAARAARAARAADGTDATRTC 3' reverse (DITFFFF); IP_3R: I1 5' GCGGATCCYTTNCKYTGYTCNGTCATYTG 3' reverse (QMTEQRK), I2 5' GCGAATTCATGGARCARATHGTNTTYCC 3' forward (MEQIVFP), I3 5' GCGAATTCGARCAYAAYATGTGGMAYTA 3' forward (EHNMWNY). The primers were directed against the carboxyl termini of both proteins. PCR reactions were done using standard conditions. The cDNA fragments amplified by PCR were gel purified and cloned into M13mp18, M13mp19 or Bluescript SK^+, and subsequently sequenced using Sequenase Version 2.0 DNA sequencing kit (USB, Cleveland, OH).

Immunocytochemistry

After fixation, spermatogenic or sperm cells were attached to slides. Cells were blocked with either 2% BSA or 5% non-fat milk for at least half an hour. Sperm were permeabilised with 0.1% Triton for 10 min, followed by 3 × 5 min washes with PBS. Primary antibodies (1/200 dilution) were applied and incubated overnight at room temperature, then cells were washed 3 × 5 min with PBS. Fluorescein isothiocyanate (FITC)- or rhodamine-conjugated secondary antibodies (1/80, 1/300 dilution, respectively) were added and incubated for 1 h at room temperature. Cells were then washed 3 × 5 min with PBS. Preparations were mounted with 50% glycerol/PBS and sealed with nail varnish. Fluorescence images were acquired using a BioRad MRC-600 Kr-Ar confocal microscope attached to a Zeiss Axioskop, ×100 objective with 1.3 numerical aperture (NA). Controls used for the antibodies raised against RyR included incubation with the secondary antibody alone. Controls for the antibody raised against IP_3R were done using the synthetic peptide used to generate the antibody (GGVGDVLRKPS) and with an unrelated peptide (GFDLQGGGVG). The following primary antibodies were used: goat anti-rabbit brain RyR and sheep anti-rabbit skeletal RyR (Upstate Biotechnology, cat. nos. 06–416 and 06–410) and rabbit anti-IP_3R synthetic (Calbiochem, cat. no. 407143). Secondary antibodies used were: anti-goat-FITC and anti-sheep-FITC (Calbiochem, cat. nos. 401514 and 402104), and anti-rabbit rhodamine-conjugated (Boehringer Mannheim, cat. no. 1238841). Figures showing immunological results are representative of at least five independent experiments in which more than 50 cells were examined.

Brefeldin A experiments

The immunocytochemical procedure was identical except that the spermatogenic cells were previously treated with 20 μg/ml of brefeldin A for 1.5 h at room temperature before fixation with 4% paraformaldehyde.

Cell dissociation for physiological experiments

Spermatogenic cells were obtained as described in Santi *et al.* (1996). Briefly, decapsulated testes of adult male mice (anaesthetised with ether and killed by cervical dislocation) were washed with ice-cold saline and incubated for 15 min at 28 °C in Ca^{2+}-free saline containing 1 mg/ml collagenase type I (Sigma, St Louis, MO) plus 0.4 mg/ml DNAse type I (Sigma); pH 7.35. Washed seminiferous tubules were then incubated for 10 min in Ca^{2+}-free saline containing 0.4 mg/ml trypsin (Worthington Biochem, Freehold, NJ). Thereafter, tubules were washed twice with Ca^{2+}-free solution supplemented with 1% bovine BSA and mechanically dissociated. The resulting cell suspension was stored at 4 °C. An aliquot of this suspension was seeded in a Plexiglas recording chamber (Warner Instruments, mod. RC-25, Handem, CT) placed on the stage of an inverted microscope equipped with phase-contrast optics (Nikon Diaphot TMD, Nikon, Japan). The cells remained undisturbed until they attached to the bottom of the recording chamber formed by a no. 1 glass

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coverslip previously coated with either poly-L-lysine (Sigma) or Cell-Tack (Collaborative Biomedical Products, Bedford, MA).

Intracellular calcium measurements

The methods of intracellular calcium measurement are described in detail elsewhere (Hernández-Cruz *et al.*, 1995). Briefly, a coverslip containing freshly dissociated spermatogenic cells was mounted in a recording chamber (Warner Instruments, mod. RC-25, Hamden, CT) on an inverted microscope (Nikon Diaphot TMD, Nikon, Japan). Cells were loaded for 30–45 min at room temperature with fura-2 by incubation with the acetoxymethyl (AM) ester form of the dye (fura-2/AM; Molecular Probes, Eugene, OR), at a final concentration of 1 μM, with no dispersing agents added. Cells were then rinsed continuously for another 15 min before the beginning of the experiments.

Ca^{2+} levels were determined by recording pairs of images using alternating illumination with 340 and 380 nm excitation provided by two nitrogen pulsed lasers. These lasers were alternately triggered at frequencies ranging from 2 to 15 Hz under computer control (Biolase Imaging System, Newton, MA). Background images were obtained from an area of the coverslip free of cells and used for on-line background subtraction. The key elements of the fluorescence Ca^{2+} imaging system were a high numerical aperture UV objective (Nikon UV-F 100X, 1.3 NA), an intensified charge-coupled device camera (c2400–87, Hamamatsu, Bridgewater, NJ) and the Biolase Imaging System running under their FL-2 software. The system allows real-time simultaneous acquisition of fluorescence measurements from multiple areas of interest placed on individual cells or within a single cell. All Ca^{2+} determinations in this study were obtained from entire cells. Ca^{2+} concentrations were calculated from fluorescence measurements at 340 and 380 nm excitation wavelengths using the formula:

$$[Ca^{2+}] = K_D(F_f/F_b)(R - R_{min})/(R_{max} - R)$$

where the fura-2 calculated dissociation constant (K_D) for CA^{2+} is 300 nM, F_f/F_b is the ratio of fluorescence values for Ca^{2+}-free/ Ca^{2+}-bound indicator at 380 nm excitation, R is the ratio of fluorescence at 340/380 nm for the unknown [Ca^{2+}], and R_{min} and R_{max} are the ratio of fura-2 fluorescences at 340/380 nm of Ca^{2+}-free and Ca^{2+}-bound fura-2. The values of F_f/F_b, R_{min} and R_{max} for Ca^{2+} were determined by measuring under identical conditions the fluorescence of a glass capillary 100 μM in diameter containing calibration solutions with 50–100 μM fura-2 (pentapotassium salt; Molecular Probes, and known Ca^{2+} concentrations in the range from 10 nM to 40 μM.

Solutions

For physiological experiments, cells were continuously superfused with a recording solution containing (in mM: 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 HEPES-Na, and 5 glucose, pH 7.4). Test solutions containing drugs (ryanodine, caffeine, thapsigargin, cyclopiazonic acid) were pressure-applied (10 p.s.i.) via a puffer pipette located within 100 μm of the cells under examination. Application of test solutions was controlled by the solenoid valve of a Picospritzer II device (General Valve, Fairfield, NJ). Control experiments showed that, with this procedure, the external medium surrounding the cell is replaced within less than 100 μs. Experiments were carried out at 22–23 °C.

Spermatogenic cell line

Experiments were carried out using the GC2spd(ts) spermatogenic cell line (a kind gift from Dr J.L. Millan). This cell line was obtained by co-transfection of Adenovirus Large T antigen and $p53^{ts}$ into isolated preleptotene spermatocytes (Hoffman *et al.*, 1994). The latest report on these cells show that they are arrested in a premeiotic stage, are unable to differentiate and have similarities with primary germ cells according to a PCR analysis (Wolkowicz *et al.*, 1996). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin–streptomycin solution, 1% non-essential amino acids (100×, Gibco BRL), 1 mM sodium pyruvate (Sigma) and 12% fetal bovine serum (Gibco BRL) in a 5% CO_2: 95% air incubator (Forma Scientific) at 37 °C.

Thapsigargin treatment

Cells were incubated in 35 mm dishes with or without 10 nM thapsigargin and proliferation rate was followed over time. Dishes (one control, one treated) were removed from the incubator approximately every 24 h and the total number of cells was determined using a haemacytometer. Cell viability was established by trypan blue exclusion. Control experiments included incubation of cells with dimethyl sulphoxide (DMSO; thapsigargin vehicle), which showed no significant difference compared with cells cultured in the absence of DMSO.

Antisense experiments

Cells were incubated in the presence of 10 μM of one of the following oligonucleotides: antisense IP_3-I(GCGGTACCGTAGTTGGTCTTATTATTTTG), RyR-1 (TCATGTCATCACACTTCATGTCAGGCTCGTCCTCATCTT), RyR-2 (AGATGATGCGGTAGATCTCATACTCGTCTCCTGCTGGGT), RyR-3 (TCATGTCGTCACAC-

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TTCATATCCGGCTCATCGTCATCTT); sense: IP_3-I (GCGAATTCGAGAAGTCTGAGCTCTGGGT), RyR (GCGAATTCTAYTGGGAYAARTTYGTNAA), and a non-related oligonucleotide (GATCCTGCTTCAACA-GTGCTTGAACG). The oligonucleotide was added every 24 h during 3 consecutive days. After treatment, the total number of cells was determined for each condition.

Results

Expression of RyR and IP_3R messenger RNAs

To determine which subtypes of RyRs and IP_3Rs are expressed in the distinct classes of spermatogenic cells, we investigated the presence of its corresponding mRNAs by RT-PCR in purified populations. For this purpose, degenerate and specific oligonucleotides directed to the pore-forming region, which is the most conserved region of these intracellular Ca^{2+} release channels, were used (see Materials and Methods). Four sets of primers were designed for the IP_3Rs and two for the RyRs. cDNA templates from pure populations of spermatogenic cells from different stages including PS, RS, CS and RB cells (see Materials and Methods) were tested. The RyR primers yielded a unique band visualised in an agarose gel (not shown), when using any of the templates described above.The amplification products were cloned into M13mp18, M13mp19 or Bluescript SK^+, and several individual clones were sequenced. Sequence analyses revealed the presence of the three reported RyR mRNAs in each of the cell types tested. When using IP_3R primers for the same kind of analysis, sequences corresponding to the type I, II and III IP_3R were found throughout the spermatid stages studied. Since spermatogenic cells from young mice cannot be isolated with a high efficiency, reliable cDNA templates from spermatogonia could not be obtained. As an approximation to find out which subtypes are expressed in these highly proliferative diploid cells, we purified RNA from the spermatogenic cell line GC2spd(ts). This cell line has been shown to be composed of diploid spermatogenic cells (see Materials and Methods). RT-PCR experiments done with templates from these cells indicated the presence of all the IP_3R and RyR subtypes (data not shown).

Expression and subtype distribution of RyR and IP_3R proteins

Specific antibodies were used to determine the cellular distribution of the RyRs and IP_3Rs, and to confirm the presence of the mature subunits in the different stages of spermatogenesis as well as in mature sperm. For IP_3Rs, an antibody raised against a synthetic oligopep-

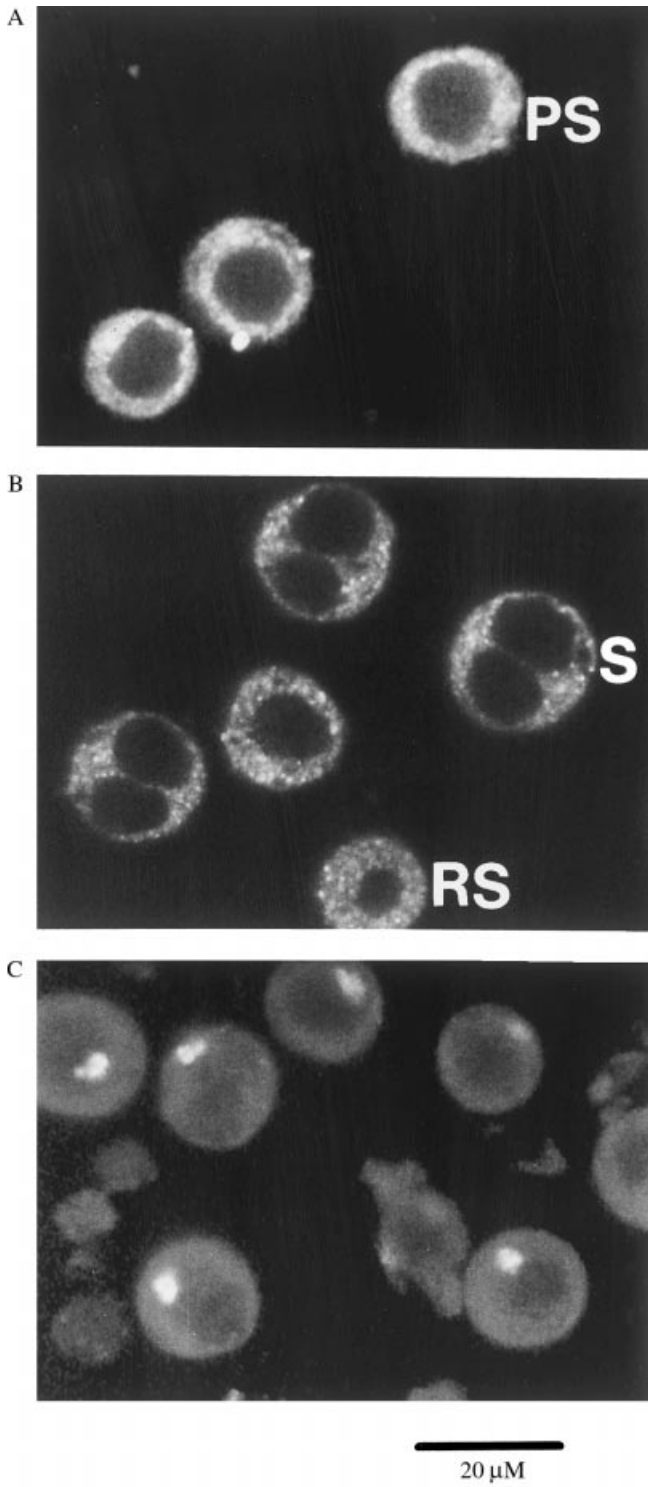


Figure 1 Distribution of ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R) in spermatogenic cells. A purified fraction containing a mixed population of round spermatids (RS), pachytene spermatocytes (PS) and their symplasts (S) were probed as described in Materials and Methods with (*A*) anti-RyR-1, (*B*) anti-RyR-3 and (*C*) anti-IP_3R. Images were acquired using a BioRad MRC-600 Kr-Ar confocal microscope attached to a Zeiss Axioskop, ×100 objective with 1.3 NA. Cell identity was determined by cell morphology and size.

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tide specific for the type I IP_3R subtype, but which in fact recognises all three IP_3R subtypes, was used. For the RyRs we used antibodies specific for each of the three known subtypes. Both mature sperm and dispersed cells from mouse testis were utilised. Purified populations of PS, RS and CS were found to express IP_3R, RyR-1 and RyR-3, but not RyR-2, when probed with the respective antibodies. These results are shown in Fig. 1, in a fraction of a cell purification containing PS, RS and their symplasts, which are normally formed during the dissociation of cells from testes. The different cell types are identified according to the fraction where they come out of the BSA gradient, cell morphology and size. Conventional and confocal fluorescence microscopy revealed that RyR-1 (Fig. 1*A*) and RyR-3 (Fig. 1*B*) were homogeneously distributed in the cytoplasm, as expected of a protein associated with the endoplasmic reticulum (ER) membrane.

In contrast, the IP_3R immunostaining was selectively localised to a crescent or ring-shaped organelle (Fig. 1*C*) that we assumed corresponds to the Golgi apparatus, because of its shape and juxtanuclear localisation, as reported in anatomical studies of spermatogenic cells (Burgos & Gutiérrez, 1986; Suárez-Quian *et al.*, 1991). To test this assumption, we used the drug brefeldin A (BFA), a fungal antibacterial agent that causes selective disassembly of the Golgi complex in many cell types (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). Adult mouse spermatogenic cells (PS, CS) were incubated with 20 μg / ml BFA for 1.5 h before fixation with 4% paraformaldehyde. In BFA-treated cells the immunostaining with antibodies raised against IP_3Rs no longer displayed the distinct labelling of the Golgi complex seen in untreated cells. This result indicates that during these stages of germ cell differentiation (PS, CS), IP_3RS are selectively localised to the Golgi apparatus. Interestingly, when spermatogenic cells were stained with 1 μM BODIPY-FL-thapsigargin (Molecular Probes, Eugene, OR), a fluorescent analogue of the high-affinity inhibitor of the Ca^{2+}-ATPase located in intracellular Ca^{2+} pools, the Golgi apparatus was also prominently stained (Fig. 2). Positive control experiments showed that antibodies raised against IP_3R and RyR subtypes 1 and 3 stained rat sympathetic neurons in culture. This staining was uniformly distributed throughout the cytoplasm (data not shown). To determine the distribution of IP_3Rs at earlier stages of germ cell differentiation, we performed IP_3R immunocytochemistry using dissociated cells from testes of 7-day-old mice. At this age only spermatogonia types A and B, and Sertoli cells, are present in the seminiferous cord (Bellvé, 1993). As shown in Fig. 3, IP_3Rs are evenly distributed through the cytoplasm of immature male germ cells and as differentiation proceeds they become selectively localised to the Golgi; RB showed a homogeneous signal throughout the cell.

Distribution of IP_3Rs and RyRs in mature sperm

In a previous report by Walensky & Snyder (1995), IP_3Rs were found selectively localised to the acrosome of non-reacted sperm of several species. In our experiments, patchy immunostaining with the antibody against IP_3R was seen at the tip of non-reacted sperm heads. Only after the completion of the acrosome reaction, when the acrosome membrane has fused with the plasma membrane, was an acrosome-shaped immunostaining observed (Fig. 4*A*). These results suggest that following fusion of the two membranes, at least a fraction of the IP_3Rs remain at the sperm plasma membrane, where they may still have a function in fertilisation. The discrepancy between our observations and those of Walensky & Snyder (1995) could be due to antibody differences. Additionally, we also observed IP_3R immunostaining in the distal part of the flagellum, which remained unchanged after completion of the acrosome reaction. Control experiments of IP_3R immunostaining were done in the presence of 100× excess of the corresponding synthetic peptide (Fig. 4*C*). Under these conditions the fluorescence signal was negligible. Incubation in the presence of an unrelated peptide of the same size did not block anti-IP_3R immunostaining.

Using antibodies raised against RyRs types 1, 2 and 3, we could only detect type 3 in mature sperm. The staining pattern obtained with the RyR-3 antibody was similar in acrosome-intact and in acrosome-reacted sperm (see Fig. 4*B*). For anti-RyR experiments the controls included incubation with the secondary antibody only. In this case no significant fluorescence was observed.

Intracellular Ca^{2+} measurements in spermatogenic cells

The possible functional role played in intracellular Ca^{2+} stores in spermatogenic cells from adult mice was examined in two ways: (1) by determining the resting Ca^{2+} levels, a measure of the cell's competence to buffer Ca^{2+} loads, and (2) by measuring the magnitude of the Ca^{2+} rises elicited by application of thapsigargin (TG) or cyclopiazonic acid (CPA), two specific inhibitors of the Ca^{2+}-ATPase of the endoplasmic reticulum. These inhibitors assess the importance of this Ca^{2+} pump and the content of the associated Ca^{2+} stores. Resting $[Ca^{2+}]_i$ in the different cell types examined was as follows: PS, 56.5 nM ± 7.2 SE (n = 10); RS, 122.2 nM ± 13.8 SE (n = 19); and CS, 200.2 nM ± 46.3 SE (n = 5). These differences ($p < 0.02$), are likely to be related to differentiation and maturation of male germ cells. As shown in Fig. 5 both TG and CPA elicit slow $[Ca^{2+}]_i$ rises in PS that slowly return to baseline levels. In general, RS have either smaller responses or fail to respond altogether. When the magnitude of the $[Ca^{2+}]_i$

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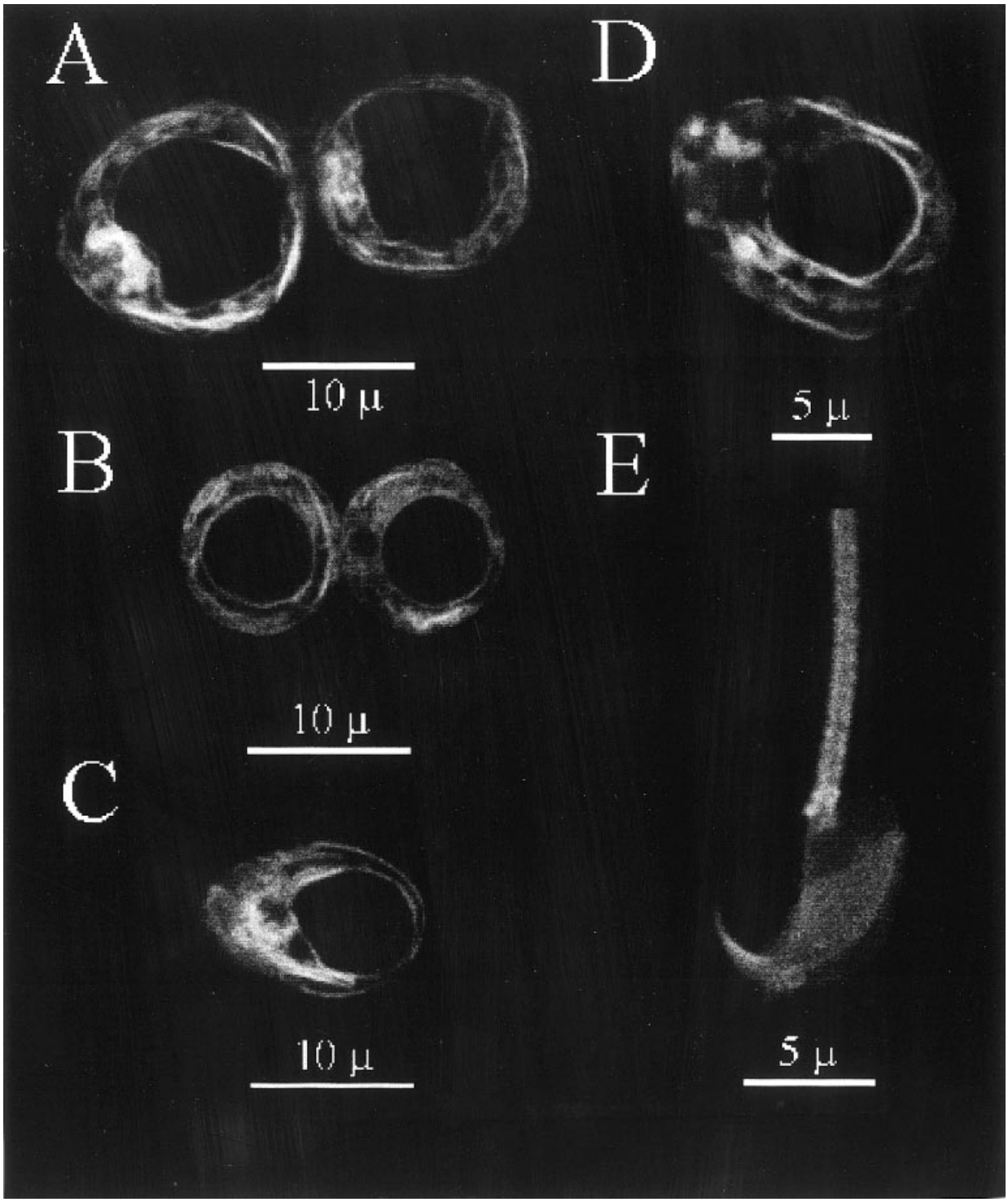


Figure 2 Fluorescence micrographs obtained from spermatogenic and sperm acutely dissociated from adult mouse testis. Cells were examined after 45 min incubation with 1 μM BODIPY-FL-thapsigargin. Cells were imaged with a Bio-Rad MRC 1024 confocal microscope attached to a Nikon TMD300 inverted microscope equipped with a ×100 UV-F glycerol immersion objective 1.3 NA (λ_{ex} = 488 nm and λ_{em} = 520 nm) (*A*) Pair of pachytene spermatocytes. In both cells the Golgi complex is prominently stained, as well as the nuclear membrane and cytoplasmic structures presumably corresponding to endoplasmic reticulum. (*B*) Pair of round spermatids. The Golgi complex staining is less distinct at this stage. (*C*), (*D*) Condensing spermatids, showing staining of the nuclear membrane and developing acrosome vesicle. (*E*) Mature testicular sperm. Staining is more prominent in the hood-like structure covering the distal two-thirds of the sperm head and in the tip of the sperm head. The connecting body, as well as the proximal flagellum, are also prominently stained.

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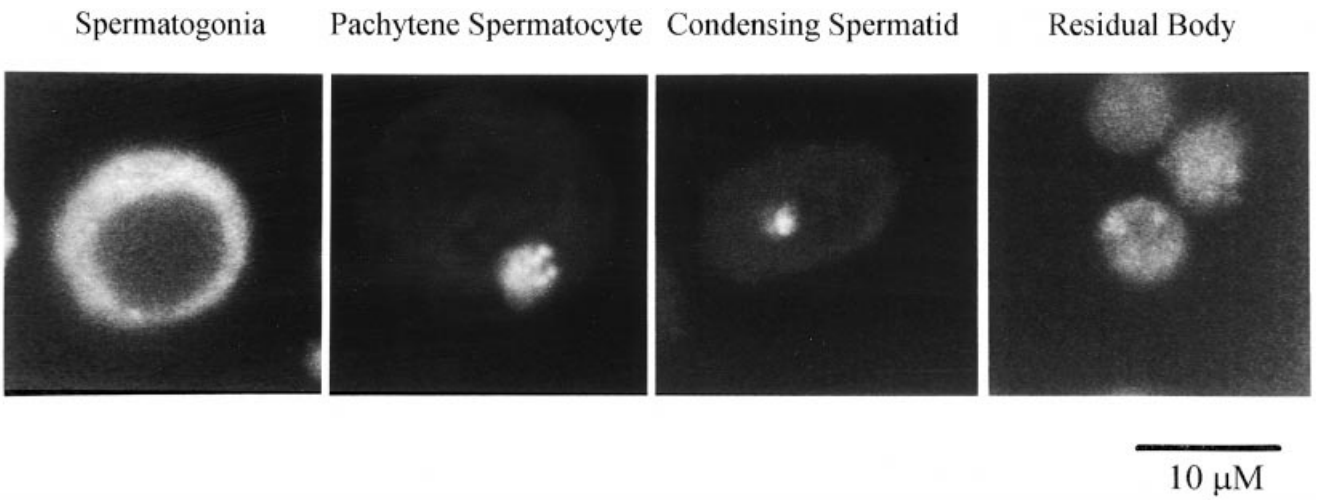


Figure 3 Differential distribution of the IP_3 receptor. The different spermatogenic cell types were labelled as described in Materials and Methods. At earlier stages of differentiation the distribution of the receptor was throughout the cytosol and as differentiation proceeded the IP_3R was selectively localised to the Golgi. Residual bodies are stained throughout the cell.

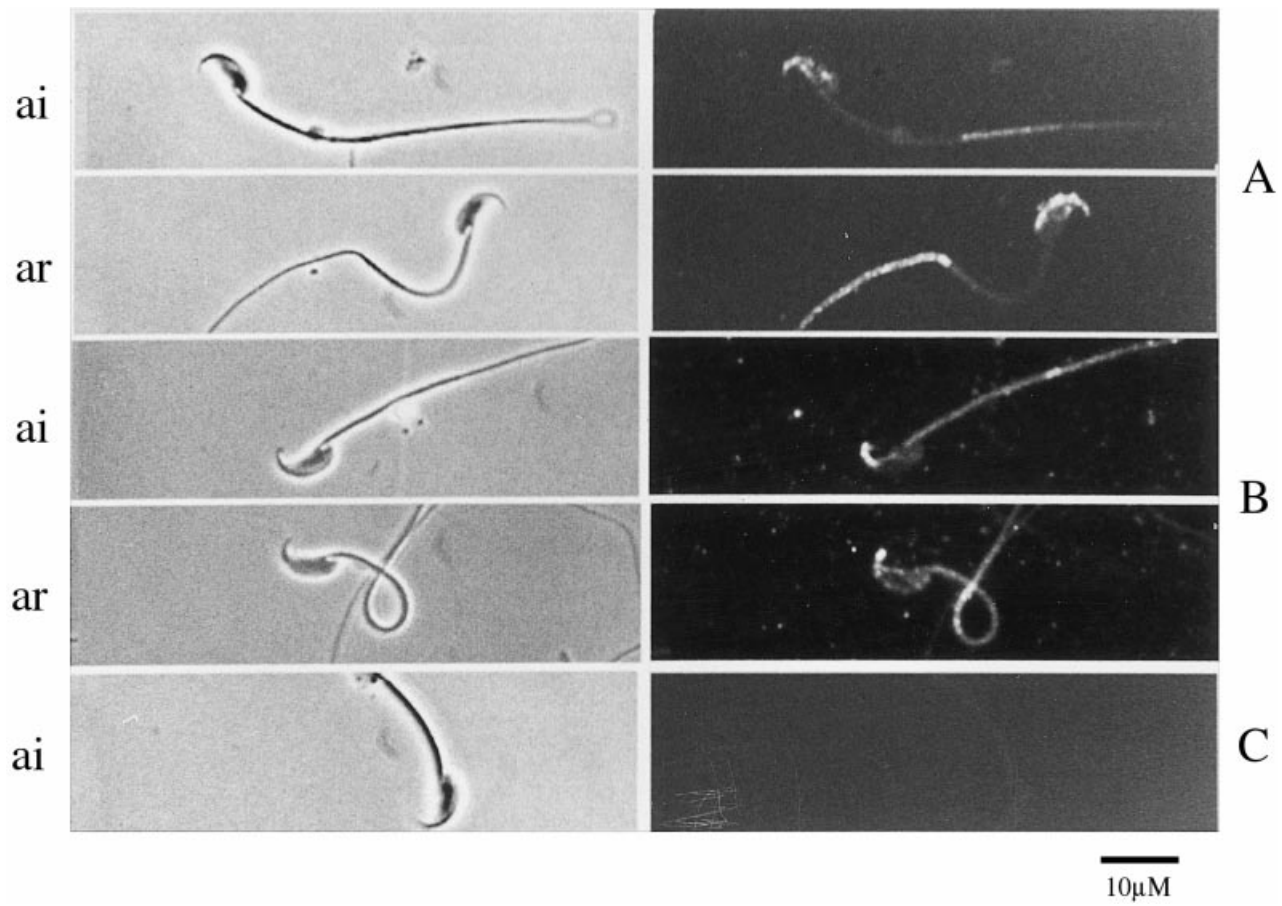


Figure 4 Immunofluorescence staining of mouse sperm. Acrosome-intact (ai) or acrosome-reacted (ar) sperm were probed with (*A*) anti-IP_3R, (*B*) anti-RyR-3 and (*C*) anti-IP_3R preincubated with the corresponding antigen peptide. Left: Phase contrast image. Right: Fluorescence image acquired as described in Materials and Methods.

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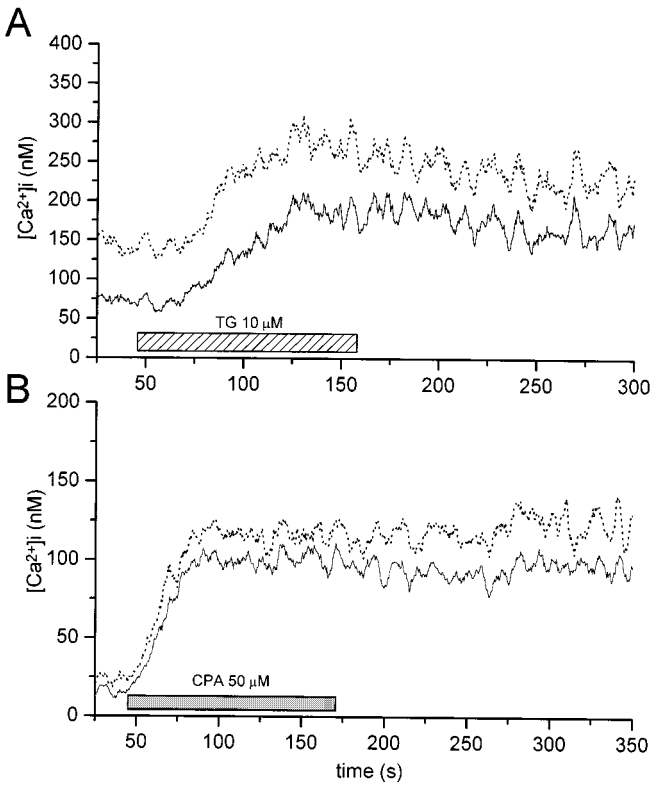


Figure 5 Two specific inhibitors of the endoplasmic reticulum (ER) Ca^{2+}-ATPase: (*A*) thapsigargin (TG; 10 µM) and (*B*) cyclopiazonic acid (CPA; 50 µM) increase the $[Ca^{2+}]_i$ of freshly dissociated mouse spermatogenic cells. Recordings of $[Ca^{2+}]_i$ were obtained from fura-2/AM-loaded pachytene spermatocytes as described in Materials and Methods. The inhibitors were applied with a puffer pipette placed near the cell, and elicited slow $[Ca^{2+}]_i$ rises that slowly returned to baseline values upon wash-out of the drug (not shown). The duration of drug application is represented by the horizontal bars. Each panel shows responses from two individual cells recorded simultaneously.

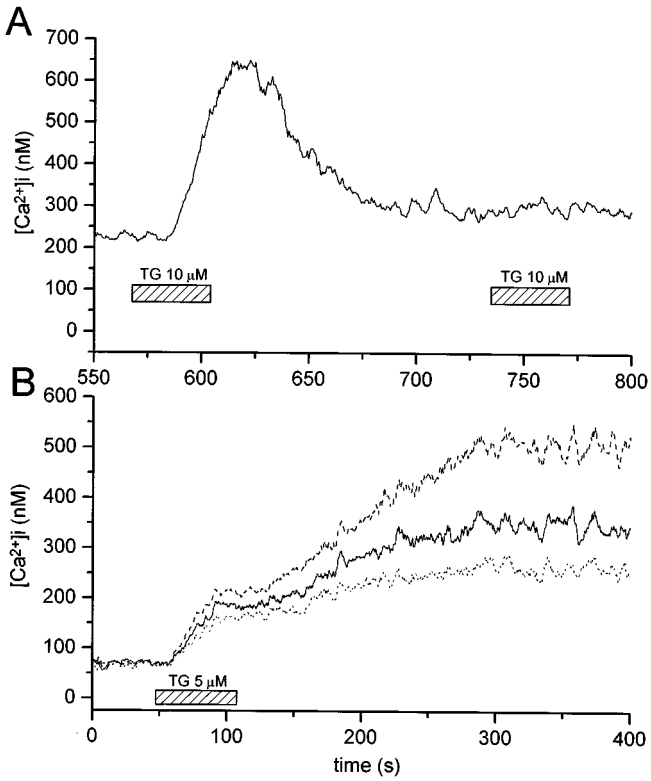


Figure 6 Thapsigargin (TG) elicits two types of responses from freshly dissociated spermatogonia obtained from a 7-day-old mouse. (*A*) Ca^{2+} mobilisation induced in the first class of spermatogonia. The transient response elicited by TG application in these cells (represented by the horizontal bar) is followed by a relatively fast decay. Notice that a subsequent application of TG is ineffective, suggesting the depletion of the TG-sensitive CA^{2+} pool. (*B*) Responses obtained from the second class of spermatogonia. Simultaneous recordings from a group of three spermatogonia of the same type are shown. After an initial response to TG addition, a larger and sustained $[Ca^{2+}]_i$ increase develops. The secondary $[Ca^{2+}]_i$ increase seen after drug exposure usually required more than 10 min to return to resting Ca^{2+} levels. About 50% of isolated spermatogonia exhibited either type of response.

rises induced by TG or CPA (pooled data) were compared, it became clear that they were significantly larger in PS (mean $\Delta[Ca^{2+}]_i$: 102.8 nM ± 12.6 SE; $n = 16$) than in RS (36.7 nM ± 7.5 SE; $n = 16$; $p = 9.3 \times 10^{-5}$). Interestingly, PS showed both low resting Ca^{2+} levels and a larger content of TG/CPA-sensitive Ca^{2+} stores. This suggests that TG-sensitive Ca^{2+}-ATPases of the ER may actively participate in keeping the characteristic low resting Ca^{2+} levels of mammalian spermatocytes.

Resting Ca^{2+} levels were also examined in isolated spermatogonia obtained from 7-day-old mice. These cells had a mean resting $[Ca^{2+}]_i$ of 74.8 nM ± 5.1 SE (n = 19), that is, a significantly higher $[Ca^{2+}]_i$ than adult mouse spermatocytes but lower than spermatids. After TG application, spermatogonia responded with two types of Ca^{2+} signals. Half the cells showed a transient Ca^{2+} rise followed by a fast decay (Fig. 6*A*), while other half, after an initial response closely associated with the stimulus, displayed a larger, sustained $[Ca^{2+}]_i$ increase (Fig. 6*B*). Interestingly spermatogonia display large TG-sensitive Ca^{2+} stores and diffuse IP_3R immunostaining. For comparison, GC2spd(ts) cells had a significantly lower resting $[Ca^{2+}]_i$ (43.3 nM ± 3.4 SE (n = 18) than spermatogonia ($p = 1 \times 10^{-5}$) or even than spermatocytes. GC2spd(ts) cells consistently gave good responses to 10 µM TG, as shown in Fig. 7. The mean amplitude of these responses was 284.21 ± 30.75 nM (n = 6).

Involvement in IP_3Rs in cell proliferation

To analyse more closely the role of intracellular Ca^{2+} pools in the control of cell growth and proliferation during the early stages of spermatogenesis, GC2spd(ts)

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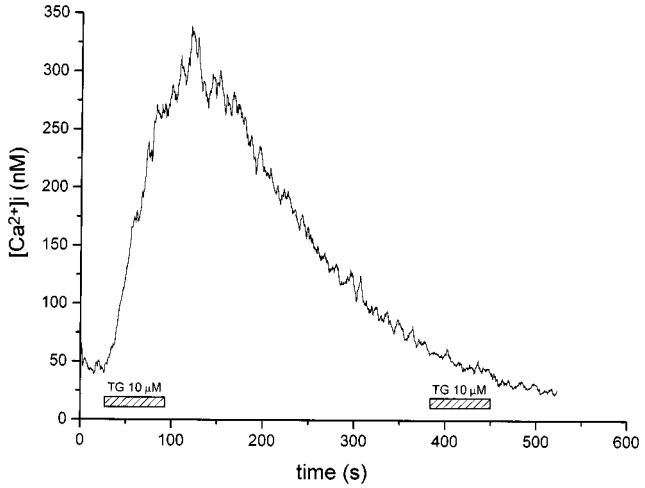


Figure 7 Thapsigargin (TG) readily releases Ca^{2+} in a cell line derived from primary spermatocytes. TG 10 µM was applied to a cell from the GC2spd(ts) cell line as indicated by the horizontal bar. The inhibition of the ER Ca^{2+}-ATPase by the TG consistently elicited a transient $[Ca^{2+}]_i$ elevation. A second application of TG (second horizontal bar) did not release more Ca^{2+}, suggesting depletion of an intracellular store whose Ca^{2+} content is limited.

cells were cultured in the presence of 10 nM TG, and proliferation was followed over time. The GC2spd(ts) cell line, obtained by immortalising an enriched population of primary spermatocytes, has been shown to be composed of diploid spermatogenic cell types (Wolkowicz *et al.*, 1996). TG addition to cultured cells has been used previously to inhibit normal $[Ca^{2+}]_i$ responses (Short *et al.*, 1993; Ntambi & Takoya, 1996). In those studies, it was demonstrated that TG treatment does not cause non-specific effects on cell function. We explored the effect on proliferation of TG in concentrations from 1 nM to 10 µM (data not shown). Fig. 8 shows that 10 nM TG is enough to strongly affect proliferation. In addition, a significant induction of cell death was also observed, which by DAPI (4',6-diamidino-2-phenylindole·2HCl) staining was found to be apoptotic.

GC2spd(ts) cells were cultured for 24 and 72 h in the presence of 10 nM TG to determine $[Ca^{2+}]_i$. Resting $[Ca^{2+}]_i$ measured at the end of the TG treatment was, respectively, 69.5 nM ± 9.4 SE (n = 10) and 97.9 nM ± 5.2 SE (n = 26). These values are significantly higher than those obtained from untreated GC2spd(ts) cells (43.35 ± 3.30, n = 18; $p = 3.9 \times 10^{-3}$ and 7×10^{-10}, respectively). These data suggest that TG-treated cells are unable to maintain low resting $[Ca^{2+}]_i$, perhaps as a result of the lack of functional ER Ca^{2+}-ATPases. The results could explain, at least in part, the effects of TG on proliferation and survival.

To assess more directly the participation of intracellular Ca^{2+} channels in proliferation, we incubated

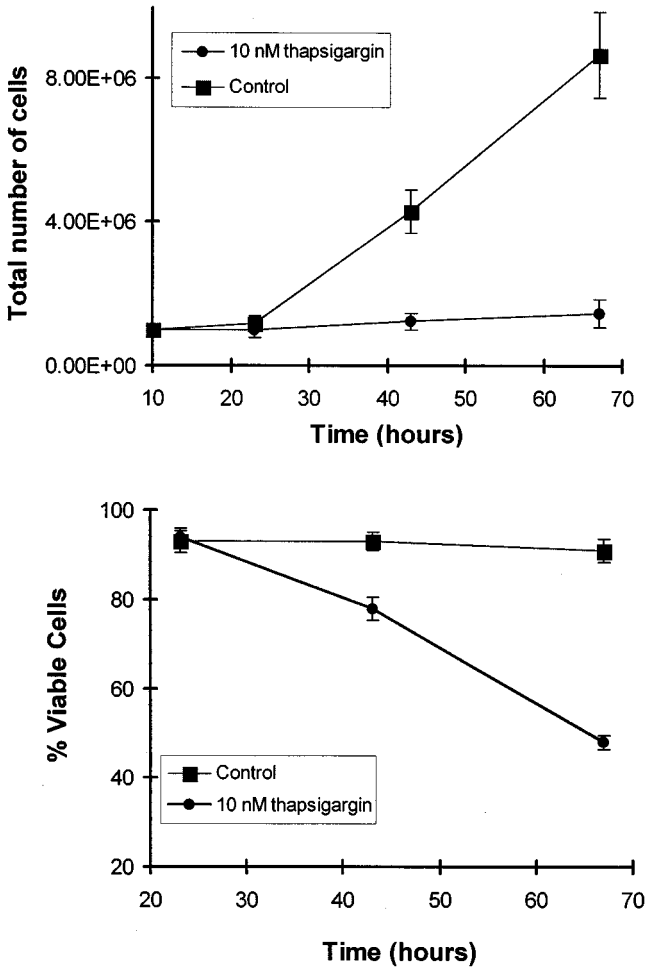


Figure 8 Thapsigargin affects cell proliferation. GC2spd(ts) cells were incubated in the presence or absence of 10 nM thapsigargin. Above: The total number of cells was determined at the indicated times. Below: Cell viability was determined by trypan blue exclusion. Values are the mean of at least four experiments in duplicate. Bars represent the standard error.

GC2spd(ts) cells with an antisense oligonucleotide for the IP_3R-I. Sustained addition of the oligonucleotide during three consecutive days resulted in complete inhibition of cell proliferation. As shown in Fig. 9, incubation of cells with other sense and antisense oligonucleotides did not have any significant effect on proliferation. Additionally, the antisense oligonucleotide did not affect cell survival, indicating that the apoptotic cell death observed with the TG treatment is not mediated by IP_3R-I.

Discussion

This study was aimed at determining the identity and cellular localisation of intracellular Ca^{2+} channels in spermatogenic cells and spermatozoa. We found that

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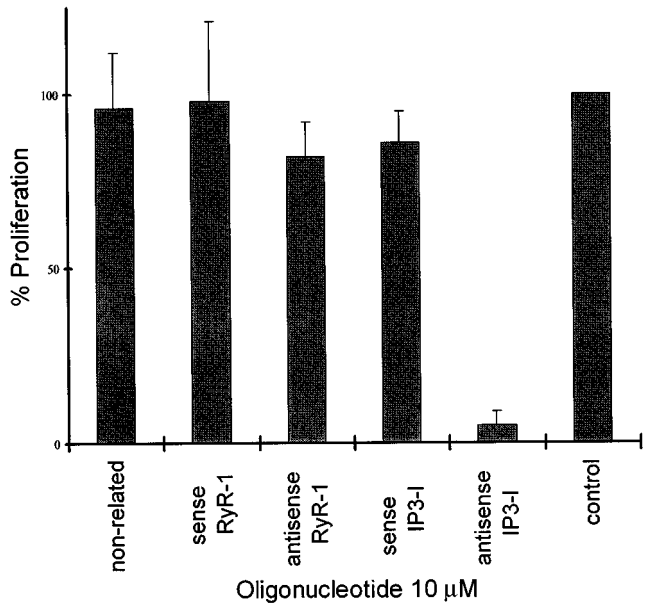


Figure 9 Antisense oligonucleotides for IP_3R-I inhibit cell proliferation. Cell proliferation of GC2spd(ts) was inhibited by an antisense oligonucleotide for the IP_3-I. A total of 1×10^4 cells were incubated in the presence of 10 μM of the indicated oligonucleotide for 3 days. After treatment, the total number of cells was determined. Each value in the figure is the mean of at least three experiments in duplicate. The data were normalised, setting the final number of cells in the control sample to 100%. Bars represent the standard error. The results from the antisense oligonucleotide for RyR-1 are included in this figure; similar results were obtained with antisense oligonucleotides for RyR-2 and RyR-3.

the IP_3R types I, II and III are expressed from spermatogonia up to the last stages of spermatogenesis. Our results also showed that mRNAs encoding for the three known RyR subtypes are present; however, specific antibodies only detected RyR-1 and RyR-3 in spermatogenic cells, and RyR-3 in spermatozoa. Usually IP_3Rs and RyRs are located in the sarcoplasmic/endoplasmic reticulum as well as in other intracellular Ca^{2+} pools such as calciosomes (Rossier & Putney, 1991). Recent evidence suggests the presence of IP_3Rs in the plasma membrane of some cells (Fukimoto *et al.*, 1992; Khan *et al.*, 1992; Cunningham *et al.*, 1993). Here, we show that the IP_3Rs from PS and subsequent stages of differentiation are largely localised to the Golgi complex. From our work it is not clear whether these receptors are active when present in this organelle. However, the fact that at the same cell stages fluorescent TG also localises to the Golgi complex, indicating the presence of the ATPase, suggests the presence of a functional Ca^{2+} store. Previous reports have indicated ATP-dependent Ca^{2+} transport in the Golgi complex (Mughal *et al.*, 1986; Van Corven *et al.*, 1986; Sorin *et al.*, 1997) as well as Ca^{2+} release from the Golgi apparatus (Yoshimoto *et al.*, 1990; Zha & Morrison, 1995). This information suggests a possible role of the Golgi complex in Ca^{2+} homeostasis of spermatogenic cells.

Considering the information stated above, we can assume that at least a fraction of the Ca^{2+} released in response to TG in spermatogenic cells may originate from the Golgi complex operating as a Ca^{2+} pool. Alternatively, immature IP_3Rs could accumulate in the Golgi complex in preparation for their further localisation in the acrosome of mature sperm, where they could become functional, and participate during the acrosome reaction.

Another interesting finding of our work is the dynamic distribution of the IP_3Rs seen during germ cell differentiation. These channels appear homogeneously distributed in the cytoplasm at early stages of development and become concentrated to the Golgi complex only during postmitotic stages. An interesting correlation exists between cell responses to TG and receptor distribution. The TG responses are larger when the channels are distributed fairly homogeneously through the cytoplasm, and smaller when the channels are present in the Golgi complex. Different interpretations to this result can be given: for instance that the Golgi Ca^{2+}-ATPase is less sensitive to TG (Sorin *et al.*, 1997). It can also be speculated that the localisation of the IP_3R in Golgi gives a smaller response due to the fact that low levels of intraluminal Ca^{2+} could be found within this organelle.

The most significant Ca^{2+} release observed in spermatogonia in response to TG, together with the growth arrest induced in cultured spermatogenic cells after TG treatment, strongly suggest that intracellular Ca^{2+} participates in the control of the cell cycle at this stage. The fact that this effect is mimicked by incubation with an IP_3R-I antisense oligonucleotide directly implicates the IP_3R in the control of spermatogonia proliferation. IP_3R has been shown to activate cell division in T lymphocytes after stimulation of the T cell antigen receptor. In that case also the type I subunit was the one involved (Jayaraman *et al.*, 1995). T lymphocyte proliferation, however, is only partially inhibited by blockage of the IP_3R, whereas that of GC2spd(ts) cells depends completely on this receptor's activity. This result suggests that at least in GC2spd(ts) cells, cell growth is only mediated by the IP_3R signalling pathway. Other cell lines are known to cease cell division when intracellular Ca^{2+} pools are depleted (Short *et al.*, 1993). However, in the GC2spd(ts) cell line apoptosis is also induced by TG. Since apoptosis is not observed with the specific inhibition of the IP_3R-I with the antisense oligonucleotide, it is possible that cell death in this cell line is not mediated by changes in intracellular Ca^{2+}, but rather by an extracellular Ca^{2+} influx induced upon TG treatment. Nonetheless, there are different examples of apoptosis mediated by the IP_3R (Marks, 1997).

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In some cases, it is the induction of an extracellular Ca^{2+} influx that provokes cell death, as in the case of immature lymphocytes, in which specifically type III IP_3R is involved (Khan *et al.*, 1996). In other cell types the absence of Ca^{2+} produced by depletion of intracellular Ca^{2+} pools is what causes apoptosis, suggesting that intracellular Ca^{2+} can also protect from cell death (Reynolds & Eastman, 1996).

Walensky had previously detected the presence of the IP_3R in the acrosome of non-reacted mouse and rat sperm (Walensky & Snyder, 1995). We also detected IP_3R in unreacted sperm, but only in acrosome-reacted sperm did we find an acrosome-shaped immuno-signal. This result is not surprising since during the acrosome reaction the acrosome membrane fuses with the plasma membrane. Part of the mixed new membrane remains in the region of the cell that was close to the acrosome, and part generates hybrid vesicles that are lost. Differences in the antibodies used in the two studies could account for the distinct staining patterns observed. Interestingly, we also observed IP_3R staining in the distal region of the flagellum. Although preferentially an intracellular Ca^{2+} channel, there is growing evidence suggesting that the IP_3R is present and active in the plasma membrane of certain cell types (Fujimoto *et al.*, 1992; Khan *et al.*, 1992; Cunningham *et al.*, 1993).

Even though we were unable to detect caffeine-induced Ca^{2+} release in any of the different stages of differentiation studied, we cannot rule out the possible relevance of the RyR subtypes present in these cells. This consideration arises from the fact that the RyR types translated change during spermatogenesis. It is still not clear whether all RyRs are caffeine-sensitive. Significantly, in mature sperm cells the only detectable receptor was the brain subtype, which is believed to be caffeine-unresponsive (Hakamata *et al.*, 1994). The immunosignal for brain RyR in mature sperm is distributed randomly in the head, being stronger at the tip, and was equivalent before and after the acrosome reaction. Previous studies suggested that if present, RyRs are not active in mature sperm since caffeine and ryanodine failed to block $^{45}Ca^{2+}$ uptake in permeabilised sperm (Walensky & Snyder, 1995).

Sperm are very specialised cells responsible for the generation of a new individual. Achieving this goal requires the orchestration of their signalling cascades, perhaps using variations not apparent in other cell types. It is well established that a rise in $[Ca^{2+}]_i$ is required for the acrosome reaction, and thus for successful fertilisation. Several models for the acrosome reaction have been proposed, but the precise order of events and the molecular identity of the participants remain elusive. As in sea urchin sperm (Darszon *et al.*, 1996), two different Ca^{2+} channels are believed to participate in the mammalian sperm acrosome reaction (Florman, 1994). Planar lipid bilayer experiments have revealed the presence of at least two different Ca^{2+} channels (Cox & Peterson, 1989; Beltrán *et al.*, 1994; Labarca *et al.*, 1994; Chan *et al.*, 1996). One of them has properties reminiscent of the RyR (Labarca *et al.*, 1995). Since this receptor is present in mature sperm, we can not rule out its possible participation in the $[Ca^{2+}]_i$ rise required for the mammalian sperm acrosome reaction.

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