

Ryanodine receptors are expressed and functionally active in mouse spermatogenic cells and their inhibition interferes with spermatogonial differentiation

Pieranna Chiarella¹, Rossella Puglisi¹, Vincenzo Sorrentino², Carla Boitani¹ and Mario Stefanini^{1,*}

¹Department of Histology and Medical Embryology and Centro di Eccellenza Biologia e Medicina Molecolare, University of Rome "La Sapienza", Via A. Scarpa 14, 00161 Roma, Italy

²Department of Neuroscience, Section of Molecular Medicine, University of Siena, Via A. Moro, 53100 Siena, Italy

*Author for correspondence (e-mail: mario.stefanini@uniroma1.it)

Accepted 21 April 2004

Journal of Cell Science 117, 4127-4134 Published by The Company of Biologists 2004

doi:10.1242/jcs.01283

Summary

Ryanodine receptors (RyRs) are intracellular calcium release channels that are highly expressed in striated muscle and neurons but are also detected in several non-excitable cells. We have studied the expression of the three RyR isoforms in male germ cells at different stages of maturation by western blot and RT-PCR. RyR1 was expressed in spermatogonia, pachytene spermatocytes and round spermatids whereas RyR2 was found only in 5- to 10-day-old testis but not in germ cells. RyR3 was not revealed at the protein level, although its mRNA was detected in mixed populations of germ cells. Caffeine, a known agonist of RyRs, was able to induce release of Ca²⁺

from intracellular stores in spermatogonia, pachytene spermatocytes and round spermatids, but not spermatozoa. Treatment with high doses of ryanodine, which are known to block RyR channel activity, reduced spermatogonial proliferation and induced meiosis in in vitro organ cultures of testis from 7-day-old mice. In conclusion, the results presented here indicate that RyRs are present in germ cells and that calcium mobilization through RyR channels could participate to the regulation of male germ maturation.

Key words: Ryanodine receptor, Spermatogonia, Meiosis, Spermatogenesis

Introduction

Changes in intracellular Ca²⁺ concentrations result from the opening of Ca²⁺ channels located either on the plasma membrane or on the endoplasmic/sarcoplasmic reticulum. These changes in intracellular Ca²⁺ concentration act as signals that are responsible for controlling many physiological functions (Berridge et al., 2003). Ca²⁺ mobilization from intracellular compartments is operated via two Ca²⁺ release channels families: the first is sensitive to the second messenger inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃] and is known as the Ins(1,4,5)P₃ receptor family [Ins(1,4,5)P₃Rs]; the second is characterized by the ability to bind the plant alkaloid ryanodine, and thus is known as the ryanodine receptor family (RyRs). It has been known for long that Ins(1,4,5)P₃Rs are ubiquitous, as they are expressed in a variety of cell types (Berridge, 1993), while RyR isoforms are preferentially expressed in excitable cell systems, RyR1 and RyR2 being preferentially expressed in striated muscles. However, all three RyR isoforms are detected in many other tissues (Rossi and Sorrentino, 2002). In the past years, data concerning the presence and the possible role of RyRs in non-excitable tissues and cell lines have been published (Ozawa, 2001), suggesting a more widespread involvement of these receptors in controlling intracellular Ca²⁺ signalling (Giannini et al., 1995; Kang et al., 2000; Suzuki et al., 1991; Tunwell and Lai, 1996). Interestingly, it has been proposed that in some cell types,

RyRs can be involved in regulating cell differentiation and developmental processes. In fact, Ca²⁺ transients seem to be necessary for normal differentiation of *Xenopus* myocytes in vivo and in vitro, because blocking RyRs with a high concentration of ryanodine in cultured *Xenopus* myocytes impairs normal myofibril organisation and sarcomere formation (Ferrari et al., 1998). In addition, in the developing *Xenopus* myotome, suppression of Ca²⁺ release through RyRs, again with high doses of ryanodine, affects somite maturation (Ferrari and Spitzer, 1999). A role for RyR-mediated Ca²⁺ signals during skeletal muscle development has been also suggested by recent studies on in vitro differentiation of mouse foetal myoblasts (Pisaniello et al., 2003).

In the testis, spermatogenic cells express several Ca²⁺ channels, including voltage operated Ca²⁺ channels (VOCCs) and Ins(1,4,5)P₃Rs, which might control Ca²⁺ signals involved in sperm capacitation and acrosome reaction (Serrano et al., 1999; Trevino et al., 1998; Walensky and Snyder, 1995). In particular, because VOCC channels are expressed in mouse spermatocytes where they represent the primary pathway for voltage gated Ca²⁺ entry, it has been speculated that they might also be involved in meiotic germ cell division and differentiation (Santi et al., 1996). Because blocking Ca²⁺ currents with nifedipine also inhibits the acrosome reaction, it seems that these channels are maintained in spermatozoa and may contribute to the influx required to trigger the acrosome reaction (Santi et al., 1996).

Initial evidence that RyRs were expressed in adult mouse testis was obtained by RNase protection analysis and by in situ hybridisation, which revealed that both RyR1 and RyR3 were present in regions of the seminiferous epithelium enriched in spermatocytes and spermatids (Giannini et al., 1995). In addition, immunocytochemical studies confirmed RyR1 and RyR3 presence in spermatocytes and early spermatids, whereas in mature spermatozoa only RyR3 was revealed (Trevino et al., 1998). In mouse and bull spermatozoa no RyR expression was detected using anti-RyR antibodies or BODIPY FL-x ryanodine (Ho and Suarez, 2001). However, functional responses to caffeine and ryanodine have never been demonstrated in male germ cells even though they were responsive to Ins(1,4,5) P_3 (Walensky and Snyder, 1995).

In this manuscript we report experiments aimed at verifying RyR expression in total testis and in purified populations of germ cells at various stages of differentiation from spermatogonia to spermatozoa. In addition we investigated whether caffeine, an agonist of RyR channel activity, was effective in activating Ca^{2+} release from internal compartments in male germ cell preparations. Finally, to verify a possible functional role of RyR in the mitotic phase of spermatogenesis, we used in vitro cultures of immature mouse testis to study the effects of RyR inhibition with high doses of ryanodine. Our results demonstrate that RyRs are expressed in male germ cells where they can be activated by caffeine and that a high ryanodine concentration can affect spermatogonial proliferation and differentiation.

Materials and Methods

Animals

CD1 mice were used in all experiments. Animals were housed in accordance with guidelines for animal care of University of Rome 'La Sapienza' and were sacrificed by cervical dislocation. Tissues were taken away and immediately used or frozen in liquid nitrogen and stored at -80°C for further analysis.

Cell preparations

Pachytene spermatocytes and round spermatids were obtained from 30-day-old mouse testis as previously described (Boitani et al., 1980). The cell suspension obtained following enzymatic digestion of testicular tissue was fractionated by velocity sedimentation at unit gravity on 0.5–3% albumin gradient (Staput method). Identity and purity of isolated cell types were assessed by both flow cytometry and microscopy analysis. Cells were washed twice with phosphate buffered saline (PBS) and then processed as needed.

Highly purified type A spermatogonia were obtained from 7-day-old mouse testis as previously described (Morena et al., 1996). Briefly, the cell suspension obtained following enzymatic digestion of testicular tissue was plated for 1 hour on plastic dishes coated with *Datura stramonium* agglutinin (DSA) (Sigma). Cells non-adhering to the lectin were fractionated on a discontinuous percoll density gradient (Pharmacia Biotech, Milan, Italy), giving a cell fraction containing at least 85% type A spermatogonia.

Epididymal spermatozoa were collected from adult mice by squeezing the cauda epididymides in PBS. Cells were centrifuged at 1000 *g* for 15 minutes and washed twice in PBS.

Sertoli cells were isolated from 7-day-old mice as described previously (Scarpino et al., 1998). After three days in culture at 32°C , Sertoli cells were washed with medium and stored at -80°C or treated with ovine follicle stimulating hormone (FSH) (20 ng/ml) to

evaluate cAMP production into the medium, as measured by enzymeimmunoassay (Amersham, Bucks, UK).

Organ culture

In vitro organ culture of 7-day-old mouse testis was performed as previously described (Boitani et al., 1993). Briefly, testicular tissue was cut into approximately 1 mm fragments and arranged on steel grids that had been previously coated with 2% agar. Grids were then placed in organ culture dishes (Falcon) with medium wetting the lower surface of the grid. Ovine FSH (o-FSH-17, NIH, Bethesda, MD), ryanodine (Sigma, St Louis, MD) and 8-Bromide-cyclicADP-ribose (8-Br-cADPR) (Sigma) were added to the culture medium at the concentrations indicated in figure legends. Testis fragments were cultured for up to 72 hours at 32°C in a humidified atmosphere of 5% CO_2 in air. During the last 5 hours of culture, testicular fragments were labelled with 5-bromo-2'-deoxyuridine (BrdU) diluted 1:400 according to cell proliferation kit instructions (Amersham, Bucks, UK). Samples were washed twice, fixed in Bouin's fluid, and processed for light microscopy analysis.

Analysis of germ cell proliferation and differentiation

We cut 5 μm thick serial sections of cultured testicular fragments for immunocytochemical staining. An anti-BrdU monoclonal antibody (diluted 1:10) (Amersham) and an antimouse peroxidase-conjugated secondary antibody (diluted 1:80) (Dako) were used to reveal labelled cells on sections counterstained with carmalum.

Spermatogonia and spermatocytes were identified on the basis of their morphological features. Spermatogonia proliferation was assessed by counting at least 100 tubules containing more than five BrdU-labelled spermatogonia. Spermatogonia progression into meiotic compartment was assessed by counting at least 100 tubules containing more than five meiotic cells so as to determine the tubular differentiation index (TDI), which is defined as the percentage of seminiferous tubules displaying cells undergoing meiosis (Meistrich and van Beek, 1993).

TUNEL assay

Evaluation of apoptotic cells was performed using in situ cell death detection kit (Boehringer). Histological sections were treated with 15 $\mu\text{g/ml}$ proteinase K in Tris pH 7.5 at room temperature, rinsed with PBS and incubated in 0.3% H_2O_2 in methanol for 30 minutes. After permeabilization in 0.1% Triton X-100 in sodium citrate, sections were incubated with TUNEL reaction mixture and then with anti-fluorescein peroxidase-conjugated antibody. Positive control was prepared by adding 1 U/ μl DNase for 10 minutes and negative control was performed by omitting DNase.

RT-PCR analysis

Total RNA was extracted from tissues or isolated cell populations using the guanidinium thiocyanate-caesium chloride ultracentrifugation method (Chirgwin et al., 1979). 5 μg of total RNA from tissues and cells were reverse transcribed (RT) with the Superscript II RT (Gibco Brl) in a total reaction volume of 20 μl , according to the manufacturer's instructions. cDNA was amplified using the following sets of primer sequences: RyR1 sense 5' GAAGGTTCTGGACAAACACGGG 3' and antisense 5' TGCTCT-TGTTGTAGAATTTGCGG 3'; RyR2 sense 5' GAATCAGTGAG-TTACTGGGCATGG 3' and antisense 5' CTGGTCTCTGAGTTCT-CCAAAAGC 3'; RyR3 sense 5' CTTGCTATCAACTTCATCCTGC 3' and antisense 5' TCTTCTACTGGGCTAAAGTCAAGG 3'. These primers amplify a 435 bp region of RyR1, a 635 bp region of RyR2 and a 505 bp region of RyR3 (Fitzsimmons et al., 2000). Amplification conditions were: 94°C for 45 seconds, 60°C for 1 minute and 72°C for 1.5 minutes, for 36 cycles. Negative controls

were performed by omitting the cDNA template from the PCR reactions and by performing a reverse transcription omitting the reverse transcriptase enzyme (RT minus).

Protein extraction and western Blot analysis

Microsomal membranes from testis, heart, skeletal muscle and diaphragm were prepared as previously described (Giannini et al., 1995). Briefly, tissues were homogenized in a buffer containing 0.32 M sucrose, 5 mM Hepes pH 7.4, 0.1 mM PMSF, 10 μ M leupeptin, 10 μ M pepstatin A. Microsomal membranes were obtained as a pellet by centrifugation at 100,000 g for 1 hour at 4°C. Spermatozoa were lysed in RIPA buffer pH 7.6 (154 mM NaCl, 1 \times PBS, 1% Triton X-100, 12 mM NaDOC, 0.2% NaN₃, 2% SDS, protease inhibitors cocktail), sonicated 3 minutes and centrifuged for 10 minutes at 10,000 g. Supernatant was stored at -80°C. Protein concentration of the microsomal fraction was determined using the bicinonic acid assay kit (Pierce).

For western blot analysis, microsomal proteins were resolved on 5% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C, Amersham). Membranes were probed with antisera specific for each of the three RyR isoforms diluted 1:1000 in blocking buffer. Polyclonal rabbit antisera able to distinguish the three RyRs were developed against purified GST fusion proteins corresponding to the region of low homology situated between the transmembrane domains 4 and 5 (divergent region 1, or D1) of the RyR1, RyR2 and RyR3 proteins, as previously described (Giannini et al., 1995). These antibodies have been shown not to cross-react with each other (Tarroni et al., 1997). Antigen detection was performed using an anti-rabbit biotinylated secondary antibody (Zymed) amplifying the signal with streptavidin-biotin-AP system (Biorad). The blotted membranes were processed using the CDP star detection reagent (NEN). For competition experiments 16 μ g of the GST-RyR3 fusion protein were incubated with antiserum anti-RyR3 diluted 1:1000 for 3 hours at room temperature before membrane immunoblotting.

Ca²⁺ measurements

Spermatogonia, spermatocytes, spermatids and a mixed germ cell suspension, immediately after isolation, were incubated for 40 minutes at 32°C in plain culture medium (MEM) or MEM containing 10 mM CaCl₂ and subsequently plated onto glass coverslips pre-coated with 100 μ g/ml poly-L-lysine at a final concentration of 4 \times 10⁵ cells per coverslip. After adhesion, cells were incubated in 1.5 ml MEM containing 1 μ M Fura 2-AM (Calbiochem) for 1 hour at 32°C, washed for 30 minutes and then treated with caffeine or ryanodine. The fura-2 fluorescence was recorded on a Nikon inverted microscope using 40 \times objective. Recordings were performed at 340-380 nm excitation wavelengths. Calibration of the signal was obtained with 5 μ M ionomycin, following by recording minimal fluorescence upon addition of 3 mM EGTA and 25 mM Tris-HCl pH 10.5. Ca²⁺ concentration was determined by formula: $[Ca^{2+}]_i = Kd(F_0/F_s)(R - R_{min}/R_{max} - R)$ (Grynkiewicz et al., 1985). The ability to respond to other Ca²⁺ release inducers was assessed by stimulating cells with ATP 100 μ M in the absence of CaCl₂ preloading treatment.

Statistical analysis

Comparisons of cell numbers between different treatments were performed by ANOVA.

Results

Detection of RyRs in germ cells by western blot and RT-PCR

To characterize the pattern of expression of RyRs in the

germinal compartment of the testis, western blot and RT-PCR experiments were performed in whole testis extracts prepared at different ages of development and in purified populations of germ cells at various stages of differentiation. RyR1 was detected in microsomal membranes at all ages during testis development (Fig. 1A). Among testicular cells its presence was revealed in highly purified spermatogonia, pachytene spermatocytes and round spermatids but not in Sertoli cells (Fig. 1B). Epididymal spermatozoa were also negative. In adult skeletal muscle (positive control) and in testicular microsomal fractions, RyR1 generally appeared as two bands of different molecular weights, both specific for the receptor, where the lower one corresponds to a degradation product of the upper band. Considering the different amount of microsomal proteins used for skeletal muscle and testicular germ cells, it can be concluded that RyR1 levels in the germinal compartment are considerably low.

To support these data further, we performed RT-PCR analysis of RyR1 in germ cells. RyR1 PCR product was detected in whole testis preparation, in a mixed population of germ cells isolated from 30-day-old testis, and in highly purified spermatogonia (Fig. 1C). A faint RT-PCR product corresponding to RyR1 was obtained in Sertoli cells, but this is probably due to the presence of contaminating spermatogonial mRNA in these preparations.

Western blot analysis with antibodies specific to RyR2

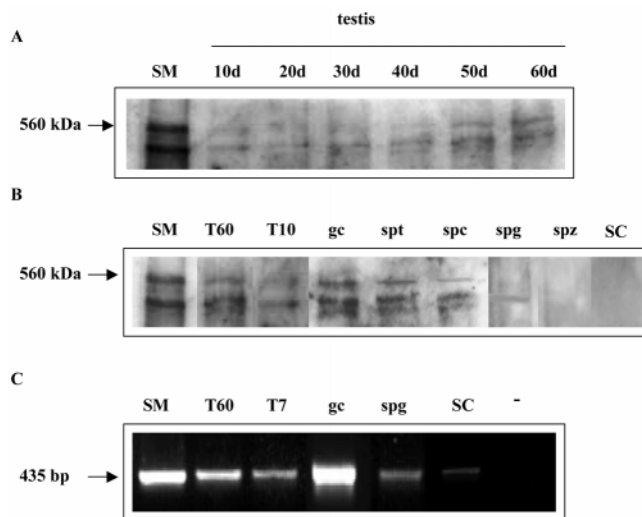


Fig. 1. Analysis of RyR1 expression by immunoblot and RT-PCR. (A) RyR1 detection in microsomal proteins prepared from adult mouse skeletal muscle (SM) (10 μ g), and mouse testis (100 μ g) at different ages of postnatal development between 10 and 60 days. (B) Immunoblot of microsomal proteins prepared from adult mouse skeletal muscle (SM), 60-day-old testis (T60), 10-day-old testis (T10), mixed populations of germ cells (gc), round spermatids (spt), pachytene spermatocytes (spc), highly purified spermatogonia (spg), epididymal spermatozoa (spz), Sertoli cells (SC). 10 μ g of proteins were loaded for SM, whereas 100 μ g for all other samples. (C) RT-PCR analysis of RyR1 expression in adult skeletal muscle (SM), 60-day-old testis (T60), 7-day-old testis (T7), mixed germ cells (gc), highly purified spermatogonia (spg) and Sertoli cells (SC). Using isoform-specific primers, a single 435 bp product was identified. Lane marked '-' is the negative control, where cDNA has been omitted in the PCR reaction.

identified a positive band in microsomes prepared from 7-10-day-old testis, but not in microsomes from 20- to 60-day-old mouse testis (Fig. 2A). This result was confirmed by RT-PCR analysis because a RyR2 specific fragment was amplified in 7-day-old testis (Fig. 2B). RyR2 signal was detected, although to less extent, also in RNA from cells enriched in spermatogonia. However it was not found in highly purified spermatogonia nor in Sertoli cells, which, together with peritubular smooth muscle cells, represent the main cell components of 7-day-old testis. This suggests that RyR2 detection in 7-day-old testis is more likely due to the presence of peritubular smooth muscle cells, which are known to express this isoform (Barone et al., 2002) and whose frequency in relation to germ cells undergoes a rapid decrease during spermatogenic cell development. Western blot with anti RyR3 antibodies did not detect a specific protein either in adult testis microsomes or in mixed germ cells (Fig. 3A). A protein of lower molecular weight found in testicular and germinal microsomes in Fig. 3A is not specific because it does not disappear when the immunoblot is probed with anti-RyR3 antibodies that have been pre-incubated with the recombinant GST-RyR3 protein (data not shown). Because the RyR3 protein could not be detected in western blot, we investigated whether germ cells express RyR3 mRNA. RyR3 mRNA was found in RNA prepared from total testis and a mixed population of germ cells enriched in primary spermatocytes and round spermatids suggesting that meiotic and post-meiotic cells, but not spermatogonia, are likely to express RyR3 mRNA (Fig. 3B). These results are in agreement with previous data (Giannini et al., 1995).

Caffeine-induced calcium responses in germ cells

To verify whether germinal RyRs were effectively functional in evoking Ca^{2+} release from intracellular stores we performed microfluorometric analysis of cytoplasmic Ca^{2+} levels in germ cells loaded with Fura 2-AM after stimulation with caffeine, a known agonist of RyR channel.

Measurements were carried out on isolated spermatogonia,

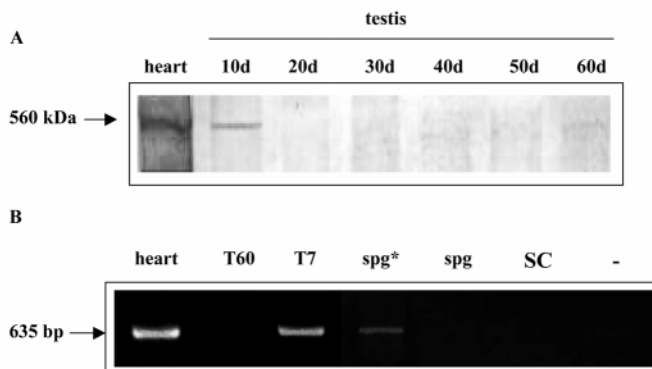


Fig. 2. RyR2 expression by western blotting and RT-PCR in the developing testis and isolated germ cells. (A) Immunoblot of microsomal proteins prepared from adult heart (10 μ g), and total testis (100 μ g) at different ages of postnatal development between 10 and 60 days. (B) RT-PCR analysis of RyR2. Using isoform-specific primers, a single 635 bp product was identified in 7-day-old testis (T7) and in an enriched (approximately 60%) population of spermatogonia (spg*), but not in highly purified spermatogonia (spg) and in Sertoli cells (SC). Lane marked '-' is the negative control.

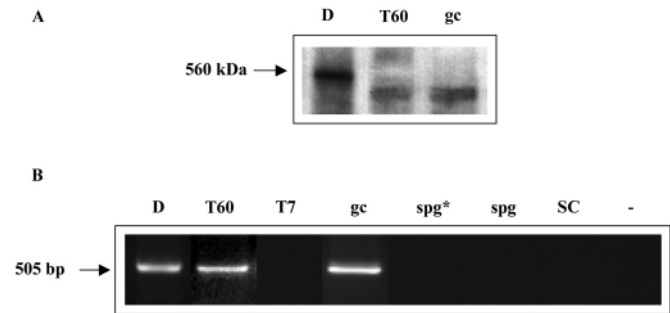


Fig. 3. Analysis of RyR3 expression by western blotting and RT-PCR. (A) Immunoblot of 100 μ g microsomal proteins extracted from diaphragm (D), 60-day-old testis (T60), mixed germ cells (gc) probed with antibody against RyR3. α -RyR3 recognized a single specific band in diaphragm and a lower molecular weight band in testis and germ cells, which was proven to be nonspecific. (B) RyR3 detection by RT-PCR using isoform-specific primers that gives a single 505 bp product. Lane marked '-' is the negative control.

pachytene spermatocytes and round spermatids immediately after purification. Stimulation with caffeine (10 mM) did not result in any detectable change in Ca^{2+} levels in spermatogonia, spermatocytes and spermatids (Fig. 4A-C). However, when cells were preincubated for 1 hour in 10 mM $CaCl_2$ -containing medium, to increase the Ca^{2+} concentration of intracellular stores, application of caffeine (10 mM) clearly evoked Ca^{2+} release in spermatogonia, pachytene spermatocytes and round spermatids (Fig. 4D-F). The response to caffeine was dose-dependent as demonstrated in a mixed population of germ cells, obtained from 30-day-old mice, and stimulated with caffeine at different concentrations from 1 to 10 mM (Fig. 5). Similar results were obtained using ryanodine as agonist at concentrations known to activate the channels (4-10 μ M) indicating that the observed Ca^{2+} release undoubtedly occurred through RyRs (data not shown). Moreover, to answer the question whether any Ca^{2+} mobilising stimulus requires an increase of extracellular Ca^{2+} , we stimulated non pre-loaded germ cells with ATP (100 μ M) and we did observe calcium transients (data not shown). Overall these data are in line with the notion that RyRs can be activated under conditions of increased Ca^{2+} loading of intracellular stores.

Inhibition of RyRs by ryanodine affects spermatogonial proliferation and differentiation

After detecting the presence of RyRs in testicular germ cells and having obtained proof that these channels were functionally active, we addressed the issue whether germ cell proliferation and differentiation could be influenced by interfering with RyR activity. For these experiments we used cultures of immature testicular fragments in the presence of high concentrations of ryanodine, which are known to block the RyR channel activity (Meissner, 1994). Ryanodine can act as both an agonist or an antagonist of RyRs, depending on its concentration. It is accepted that below 10 μ M, ryanodine activates RyR channels whereas at concentrations higher than 100 μ M it alters channel's conductance by locking it in a closed state (Buck et al., 1992; Meissner, 1994). Initially, we

investigated in our system whether the treatment with high doses of ryanodine resulted in the block of RyR channels. As shown in Fig. 6, germ cells that were preincubated with 300 μM ryanodine for 1 hour were unable to respond to caffeine, whereas non-pretreated germ cells retained the ability to release Ca^{2+} . This type of pharmacological approach has been used in several studies to prove the involvement of RyR channels in different cellular functions (Ferrari et al., 1998; Ferrari and Spitzer, 1999; Pisaniello et al., 2003). Therefore, we asked whether administration of high doses of ryanodine could affect two major events that are peculiar of the early phases of spermatogenesis, i.e. spermatogonial proliferation and progression towards meiosis. Testicular fragments from 7-day-old mice were treated with 100 μM and 300 μM ryanodine in the presence of 20 ng/ml FSH, which is necessary to

maintain the progression of germ cell differentiation. After 72 hours of treatment, testicular fragments were fixed, sectioned and tubules containing BrdU-labelled spermatogonia were counted. Spermatogonial proliferation was assessed by evaluating the percentage of tubules containing more than five BrdU-labelled spermatogonia (Fig. 7A). Spermatogonial progression through meiosis was evaluated by counting percentage of tubules with more than five meiotic cells (mostly leptotene-pachytene primary spermatocytes) recognized by morphological analysis (Fig. 7B). The results showed a significant gradual reduction in the number of proliferating spermatogonia and a significant increase in the number of meiotic cells when testicular cultures were treated with FSH and ryanodine compared with those treated with FSH alone. No effect was seen when low (4 μM) concentration of ryanodine was used (not shown).

Morphological analysis and germ cell apoptosis assessed by TUNEL assay allowed us to exclude a nonspecific cytotoxic effect of ryanodine on germ cells. The specificity of the ryanodine effect was further tested by culturing testicular fragments in the presence of FSH and the membrane permeant 8-Br-cADPR, which acts as antagonist of cADPR, the endogenous ligand of RyR channels. A similar effect to ryanodine on spermatogonial proliferation and differentiation was observed with 100 μM (not shown) and 300 μM 8-Br-cADPR (Fig. 7A,B). In the absence of FSH, ryanodine did not affect either spermatogonial proliferation or meiosis compared with control cultures (MEM alone), suggesting that the observed effects were strictly dependent on the presence of FSH. The possibility that high doses of ryanodine per se could activate FSH signalling was ruled out by the evidence that extracellular cAMP levels produced by isolated Sertoli cells under FSH stimulation were similar with or without ryanodine (data not shown). To analyse the kinetic of RyR block-dependent events, testicular fragments were cultured for 24, 48 and 72 hours after addition of 300 μM ryanodine (Fig. 8A,B). The results demonstrated that the increase in meiotic cell number already occurred after 48-hour treatment while the reduction in spermatogonia proliferation was observed only after 72-hour treatment.

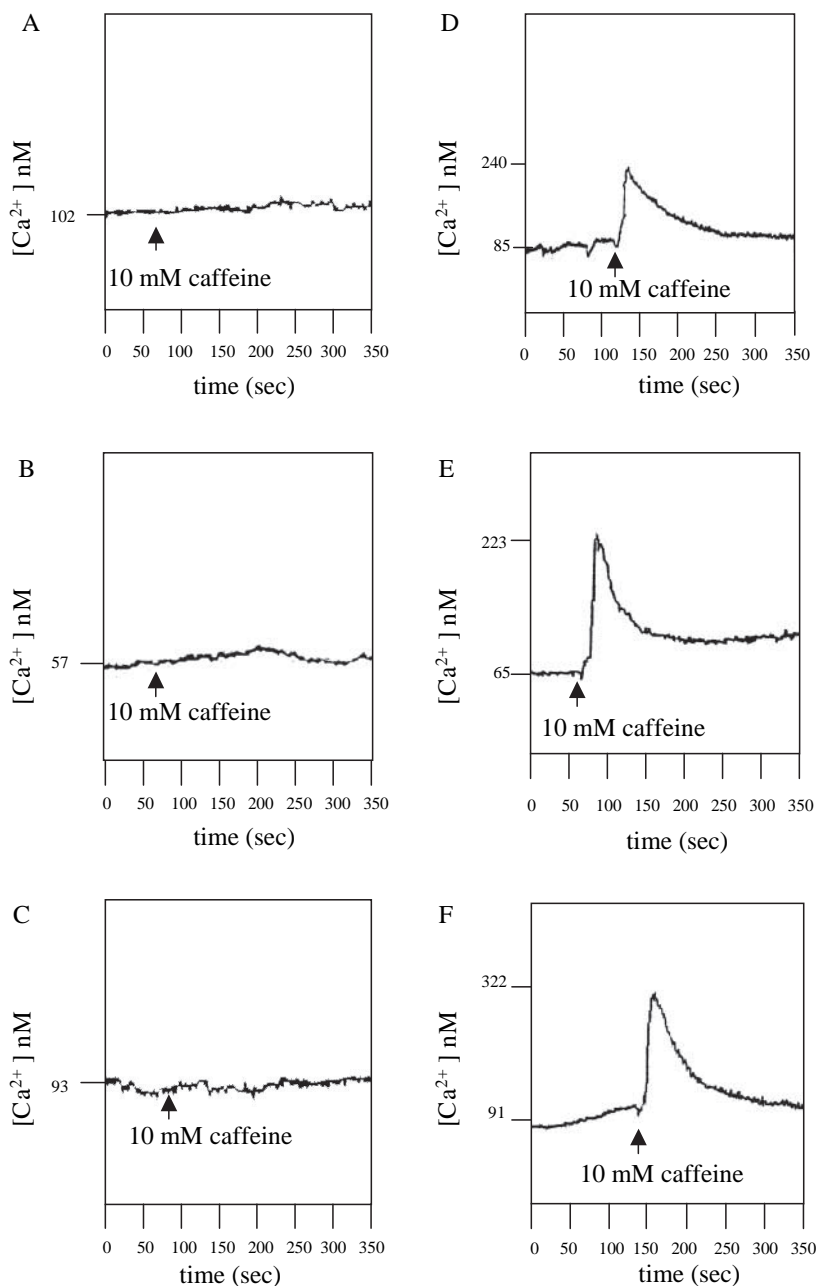


Fig. 4. Caffeine-induced Ca^{2+} release in spermatogonia, spermatocytes and spermatids. Effects of 10 mM caffeine on $[\text{Ca}^{2+}]_i$ release in Fura-2-loaded spermatogonia (A,D), pachytene spermatocytes (B,E), round spermatids (C,F). A single cell was equilibrated in KHH, then caffeine was added as pointed by the arrow. Panels (A,B,C) represent response to caffeine following cell preincubation in plain MEM. Panels (D,E,F) represent caffeine-induced Ca^{2+} release following cell preincubation in MEM added with 10 mM CaCl_2 . The signal was calibrated with 5 μM ionomycin. $[\text{Ca}^{2+}]_i$ values are representative of six different experiments for each cell type.

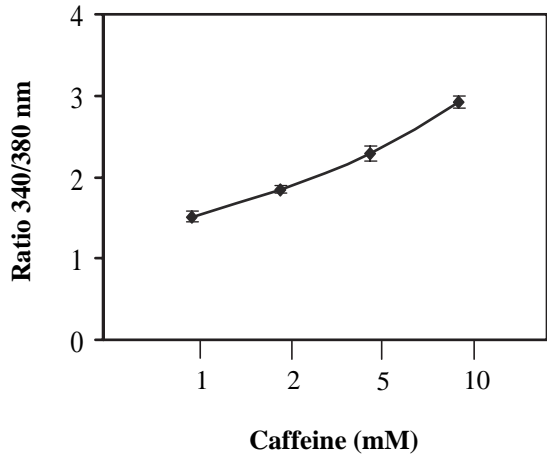


Fig. 5. Dose response curve of germ cells to caffeine under conditions of increased extracellular calcium. Stimulation with caffeine (1–10 mM) induced a concentration dependent increase in Ca^{2+} release. Values are expressed in terms of fluorescence ratio at the two excitation wavelengths and are the mean \pm s.d. of three independent experiments.

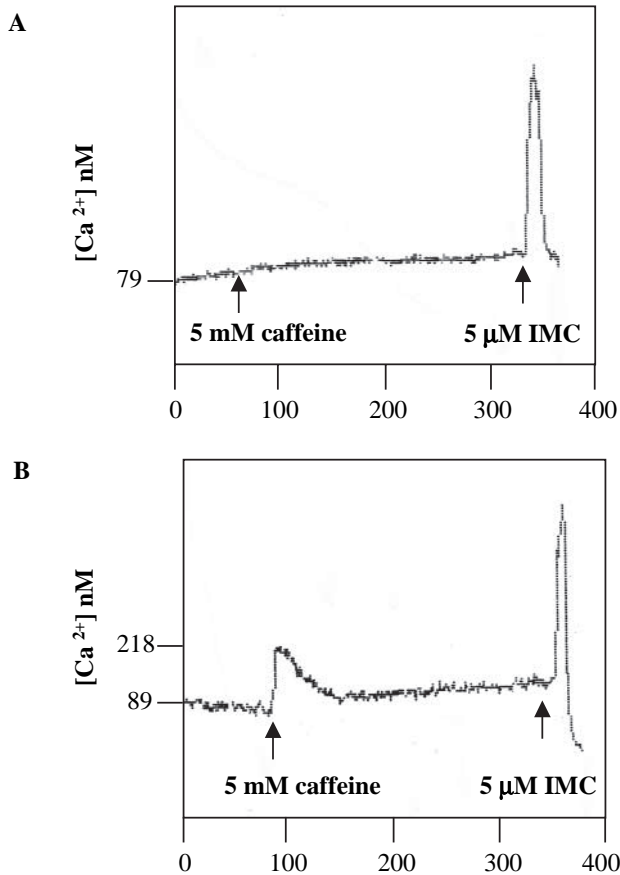


Fig. 6. Treatment with 300 μM ryanodine abolishes caffeine induced Ca^{2+} release in germ cells. Cells were preloaded with 10 mM CaCl_2 , washed and either incubated (A) with or (B) without 300 μM ryanodine for 1 hour with before measuring caffeine-dependent Ca^{2+} -release. Ionomycin (IMC) was added to elicit further release of intracellular Ca^{2+} . The traces are typical of three independent measurements.

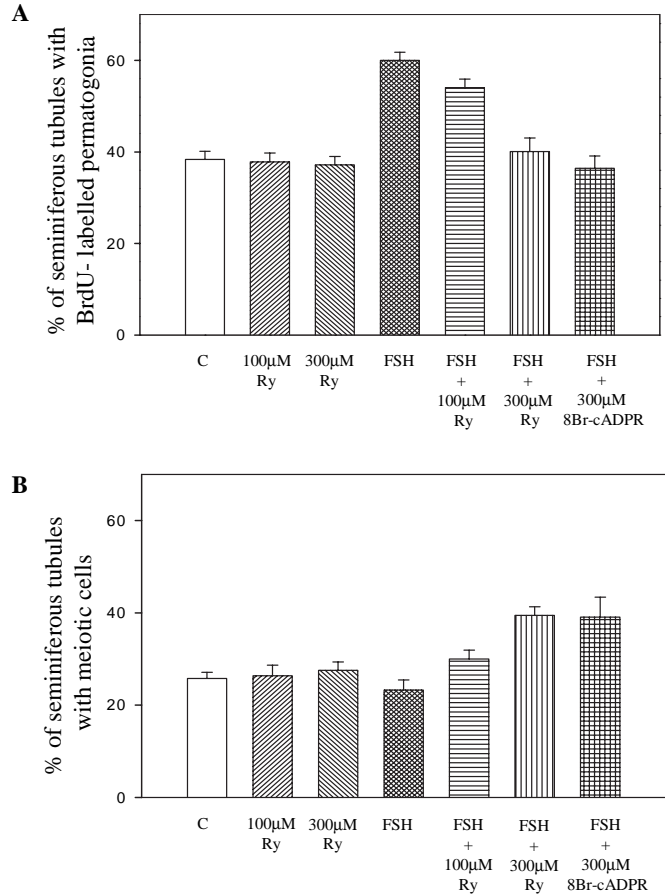


Fig. 7. Effects of blocking RyR channels on spermatogonia proliferation and differentiation. Testicular fragments were cultured for 3 days with 100 μM or 300 μM ryanodine (Ry) or 20 ng/ml FSH (Ry) alone, and combinations of 20 ng/ml FSH with 100 μM Ry, 300 μM ryanodine or 300 μM 8-Br-cADPR, and eventually labelled with BrdU for 5 hours. (A) Percentages of tubules with BrdU-labelled spermatogonia; (B) percentages of tubules containing meiotic cells. Cell counting was performed as described in Materials and Methods. Values are the mean \pm s.e.m. of three independent experiments. FSH+Ry 300 μM versus FSH: $P < 0.001$; FSH+300 μM 8-Br-cADPR versus FSH: $P < 0.001$.

Discussion

The aim of this study was to investigate the expression, functional activity and the potential role of RyRs in spermatogenic cells. We provide direct evidence that spermatogonia express RyR1, but not RyR2 and RyR3, and that the receptor is indeed functional ryanodine sensitive calcium channel. Moreover, we demonstrate that RyR-dependent Ca^{2+} transients appear to interfere with spermatogonial differentiation. In fact, by blocking RyR channels with high concentrations of ryanodine in in vitro cultured fragments from immature mouse, we revealed a reduced spermatogonial proliferation and accelerated maturation of early meiotic cells. Interestingly, inhibition of Ca^{2+} mobilization achieved by treatment of testis fragments with 8-Br-cADPR, the membrane permeant antagonist of the physiological RyR ligand cADPR, resulted in significant reduction in spermatogonia proliferation and enhanced meiotic

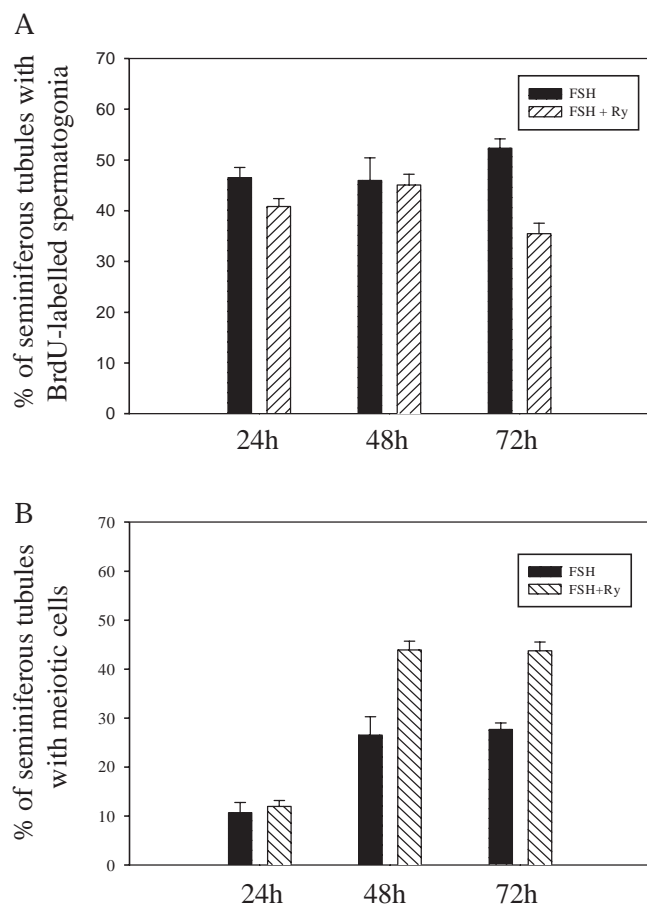


Fig. 8. Spermatogonial proliferation and differentiation are independently affected by RyR inhibition. Testicular fragments were cultured for 24, 48 and 72 hours in the presence of 20 ng/ml FSH, with or without 300 μ M ryanodine. (A) Percentages of tubules displaying BrdU-labelled spermatogonia. (B) Percentages of tubules displaying meiotic cells. Labelling and counting were performed as described in Materials and Methods. Each value represents the mean \pm s.e.m. of at least 200 tubules for each experimental condition, obtained in two independent experiments. (A) FSH+Ry versus FSH at 72 hours: $P < 0.001$; (B) FSH+Ry versus FSH at 48 and 72 hours: $P < 0.001$.

progression similar to those observed after treatment with high concentrations of ryanodine. The organ culture of immature testis used in this study represents a very powerful, and at the moment, the only experimental model to investigate the mechanisms involved in full premeiotic germ cell development until meiotic onset (Boitani et al., 1993). In fact, despite the very poor survival of spermatogonia after isolation, the organ culture system allows the maintenance of the architecture of the seminiferous epithelium, particularly preserving the interactions between germ cells and Sertoli cells. The possibility that germ cell loss by apoptosis was responsible for the decline in BrdU-labelled spermatogonia can be ruled out on the basis of overall good morphology of the seminiferous epithelium and because the TUNEL assay performed at the end of three days of culture in the presence of ryanodine did not detect any testicular cell degeneration. Furthermore, the finding that the ryanodine/8-Br-cADPR treatment induced a concurrent significant increase of the percentage of tubules

with meiotic cells stands in favour of the lack of any toxic effect of high doses of ryanodine/8-Br-cADPR used to block Ca^{2+} release from intracellular stores. The two events affected by RyR blockade appear not to be temporally correlated as the effect of ryanodine upon spermatogonial proliferation was observed after 72 hours of treatment, whereas the effect upon meiosis began to appear after 48 hours and was maintained afterwards. These results suggest that RyR inhibition interferes independently with both mitotic and meiotic phases of spermatogenesis. Accordingly, cell proliferation was inhibited in favour of an increased stimulation of melanocyte pigmentation in human melanocytes cultured in presence of ryanodine (Kang et al., 2000). In addition, our findings demonstrate that ryanodine effect is direct upon germ cells and is not mediated by Sertoli cells as the somatic cells of the tubules do not express any of the three RyR isoforms. However, it is interesting to note that the effect of ryanodine was observed only when testicular fragments were cultured in the presence of FSH, which controls spermatogonial proliferation and differentiation (Boitani et al., 1993) by acting on Sertoli cells that are the only cells expressing FSH receptors in the testis and responding to the gonadotropin by secreting a number of factors that regulate germ cell maturation.

Transients evoked by RyRs have been shown to be involved in cell differentiation mechanisms in excitable systems, such as skeletal muscle cells and neurons. Indeed, normal *in vitro* and *in vivo* differentiation of *Xenopus* myocytes has been shown to be strictly dependent on RyR activity and Ca^{2+} transients (Ferrari et al., 1996; Ferrari et al., 1998; Ferrari and Spitzer, 1999). More recently, it has been demonstrated that the activity of RyR channels is required for foetal murine myoblast differentiation because the selective block of RyR results in the inhibition of the differentiative program (Pisaniello et al., 2003). In the intact mammalian cerebellum as well as in neuronal cultures it was also observed that changes in RyR expression resulted in functional changes in Ca^{2+} signalling transients during normal neuronal development (Mhyre et al., 2000).

The molecular mechanisms underlying RyR activation and Ca^{2+} release in germ cells remain to be clarified. On the basis of the data on the effect of 8-Br-cADPR reported in this study, one possible candidate that might be involved in modulating RyR function is cyclic ADP-ribose (cADPR). This molecule, discovered as an endogenous activator of RyR in sea urchin eggs (Clapper et al., 1987), is produced by the ectoenzyme CD38 in several cell systems (Barone et al., 2002; Khoo and Chang, 2002). It is worth mentioning that we observed that spermatogenic cells expressed a higher molecular weight isoform of CD38 (data not shown), thus suggesting that cADPR might be synthesized by this ectoenzyme in testicular germ cells.

Another interesting finding of this paper is the expression pattern of three RyR isoforms in germ cells at more mature stages of differentiation, including pachytene spermatocytes and round spermatids. We report here that RyR1 and RyR3, but not RyR2, mRNAs are present in these cell types, in agreement with previous *in situ* hybridisation data (Giannini et al., 1995). The low abundance at which the RyRs are expressed in male germ cells with respect to other tissues has been an obstacle to their functional characterization. We did succeed, however, in demonstrating a caffeine-dependent Ca^{2+} release

in germ cells under conditions of increased extracellular Ca^{2+} , which are known to enhance the sensitivity of the channels, therefore making them susceptible to activation (Mironneau et al., 2001; Mironneau et al., 2002). In contrast, epididymal spermatozoa from mouse (present findings) as well as bull (Ho and Suarez, 2001) did not contain any RyRs. Moreover, rat spermatozoa failed to release Ca^{2+} following stimulation with caffeine and ryanodine, even though they were responsive to $\text{Ins}(1,4,5)\text{P}_3$ (Walensky and Snyder, 1995). Further support to the idea that RyRs are not crucial for reproduction is based on the finding that RyR3 knockout mice show normal spermatogenesis and fertility (Komazaki et al., 1998), while the lack of RyR1 results in early postnatal death of the animals (Takeshima et al., 1994).

In conclusion, our results demonstrate that germinal RyRs are able to function as Ca^{2+} release channels and they may play a role during the onset of spermatogenesis. RyR1 presumably contributes with its Ca^{2+} signalling in regulating the transition of spermatogonia from a proliferating phase to meiosis, and both RyR1 and RyR3 might act in advanced developmental phases.

We thank Tiziana Menna for her technical assistance. FSH was obtained through National Hormone and Pituitary Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and A. F. Parlow. This work was supported by the following grants: Telethon Italy, MIUR and PAR University of Siena to V.S., MIUR and ASI to C.B., MIUR cofin 2001 and Dept of Health Special Program 2001 to M.S.

References

- Barone, F., Genazzani, A. A., Conti, A., Churchill, G. C., Palombi, F., Ziparo, E., Sorrentino, V., Galione, A. and Filippini, A. (2002). A pivotal role for cADPR-mediated Ca^{2+} signaling, regulation of endothelin-induced contraction in peritubular smooth muscle cells. *FASEB J.* **16**, 697-705.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.
- Berridge, M. J., Bootman, M. D. and Roderick, H. L. (2003). Calcium signalling, dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* **4**, 517-529.
- Boitani, C., Geremia, R., Rossi, R. and Monesi, V. (1980). Electrophoretic pattern of polypeptide synthesis in spermatocytes and spermatids of the mouse. *Cell Differ.* **9**, 41-49.
- Boitani, C., Politi, M. G. and Menna, T. (1993). Spermatogonial cell proliferation in organ culture of immature rat testis. *Biol. Reprod.* **48**, 761-767.
- Buck, E., Zimanyi, I., Abramson, J. J. and Pessah, I. N. (1992). Ryanodine stabilizes multiple conformational states of the skeletal muscle calcium release channel. *J. Biol. Chem.* **267**, 23560-23567.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.
- Clapper, D. L., Walseth, T. F., Dargie, P. J. and Lee, H. C. (1987). Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. *J. Biol. Chem.* **262**, 9561-9568.
- Ferrari, M. B. and Spitzer, N. C. (1999). Calcium signaling in the developing *Xenopus* myotome. *Dev. Biol.* **213**, 269-282.
- Ferrari, M. B., Rohrbough, J. and Spitzer, N. C. (1996). Spontaneous calcium transients regulate myofibrillogenesis in embryonic *Xenopus* myocytes. *Dev. Biol.* **178**, 484-497.
- Ferrari, M. B., Ribbeck, K., Hagler, D. J. and Spitzer, N. C. (1998). A calcium signaling cascade essential for myosin thick filament assembly in *Xenopus* myocytes. *J. Cell Biol.* **141**, 1349-1356.
- Fitzsimmons, T. J., Gukovsky, I., McRoberts, J. A., Rodriguez, E., Lai, F. A. and Pandol, S. J. (2000). Multiple isoforms of the ryanodine receptor are expressed in rat pancreatic acinar cells. *Biochem. J.* **351**, 265-271.
- Giannini, G., Conti, A., Mammarella, S., Scrobogna, M. and Sorrentino, V. (1995). The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J. Cell Biol.* **128**, 893-904.
- Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.
- Ho, H. C. and Suarez, S. S. (2001). An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca^{2+} store is involved in regulating sperm hyperactivated motility. *Biol. Reprod.* **65**, 1606-1615.
- Kang, H. Y., Kim, N. S., Lee, C. O., Lee, J. Y. and Kang, W. H. (2000). Expression and function of ryanodine receptors in human melanocytes. *J. Cell. Physiol.* **185**, 200-206.
- Kho, K. M. and Chang, C. F. (2002). Identification and characterization of nuclear CD38 in the rat spleen. *Int. J. Biochem. Cell Biol.* **34**, 43-54.
- Komazaki, S., Ikemoto, T., Takeshima, H., Iino, M., Endo, M. and Nakamura, H. (1998). Morphological abnormalities of adrenal gland and hypertrophy of liver in mutant mice lacking ryanodine receptors. *Cell Tissue Res.* **294**, 467-473.
- Meissner, G. (1994). Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* **56**, 485-508.
- Meistrich, M. L. and van Beek, M. E. A. B. (1993). Spermatogonial stem cells, assessing their survival and ability to produce differentiated cells. *Methods Toxicol.* **3**, 106-123.
- Mhyre, T. R., Maine, D. N. and Holliday, J. (2000). Calcium-induced calcium release from intracellular stores is developmentally regulated in primary cultures of cerebellar granule neurons. *J. Neurobiol.* **42**, 134-147.
- Mironneau, J., Coussin, F., Jeyakumar, L. H., Fleischer, S., Mironneau, C. and Macrez, N. (2001). Contribution of ryanodine receptor subtype 3 to Ca^{2+} responses in Ca^{2+} -overloaded cultured rat portal vein myocytes. *J. Biol. Chem.* **276**, 11257-11264.
- Mironneau, J., Macrez, N., Morel, J. L., Sorrentino, V. and Mironneau, C. (2002). Identification and function of ryanodine receptor subtype 3 in non-pregnant mouse myometrial cells. *J. Physiol.* **538**, 707-716.
- Morena, A. R., Boitani, C., Pesce, M., de Felici, M. and Stefanini, M. (1996). Isolation of highly purified type A spermatogonia from prepubertal rat testis. *J. Androl.* **17**, 708-717.
- Ozawa, T. (2001). Ryanodine-sensitive Ca^{2+} release mechanism in non-excitable cells. *Int. J. Mol. Med.* **7**, 21-25.
- Pisaniello, A., Serra, C., Rossi, D., Vivarelli, E., Sorrentino, V., Molinaro, M. and Bouche, M. (2003). The block of ryanodine receptors selectively inhibits fetal myoblast differentiation. *J. Cell Sci.* **116**, 1589-1597.
- Rossi, D. and Sorrentino, V. (2002). Molecular genetics of ryanodine receptors Ca^{2+} -release channels. *Cell Calcium* **32**, 307-319.
- Santi, C. M., Darszon, A. and Hernandez-Cruz, A. (1996). A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. *Am. J. Physiol.* **271**, C1583-C1593.
- Scarpino, S., Morena, A. R., Petersen, C., Froysa, B., Soder, O. and Boitani, C. (1998). A rapid method of Sertoli cell isolation by DSA lectin, allowing mitotic analyses. *Mol. Cell. Endocrinol.* **146**, 121-127.
- Serrano, C. J., Trevino, C. L., Felix, R. and Darszon, A. (1999). Voltage-dependent Ca^{2+} channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. *FEBS Lett.* **462**, 171-176.
- Suzuki, M., Kurihara, S., Kawaguchi, Y. and Sakai, O. (1991). Vitamin D3 metabolites increase $[\text{Ca}^{2+}]_i$ in rabbit renal proximal straight tubule cells. *Am. J. Physiol.* **260**, F757-F763.
- Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H. and Noda, T. (1994). Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature* **369**, 556-559.
- Tarroni, P., Rossi, D., Conti, A. and Sorrentino, V. (1997). Expression of the ryanodine receptor type 3 calcium release channel during development and differentiation of mammalian skeletal muscle cells. *J. Biol. Chem.* **272**, 19808-19813.
- Trevino, C. L., Santi, C. M., Beltran, C., Hernandez-Cruz, A., Darszon, A. and Lomeli, H. (1998). Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis, possible functional implications. *Zygote* **6**, 159-172.
- Tunwell, R. E. and Lai, F. A. (1996). Ryanodine receptor expression in the kidney and a non-excitable kidney epithelial cell. *J. Biol. Chem.* **271**, 29583-29588.
- Walensky, L. D. and Snyder, S. H. (1995). Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J. Cell Biol.* **130**, 857-869.