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T-type Ca^{2+} channels and α_{1E} expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction

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Abstract There is pharmacological evidence that Ca^{2+} channels play an essential role in triggering the mammalian sperm acrosome reaction, an exocytotic process required for sperm to fertilize the egg. Spermatozoa are small terminally differentiated cells that are difficult to study by conventional electrophysiological techniques. To identify the members of the voltage-dependent Ca^{2+} channel family possibly present in sperm, we have looked for the expression of the α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} genes in mouse testis and in purified spermatogenic cell populations with RT-PCR. Our results indicate that all 5 genes are expressed in mouse testis, and in contrast only α_{1E}, and to a minor extent α_{1A}, are expressed in spermatogenic cells. In agreement with these findings, only T-type Ca^{2+} channels sensitive to the dihydropyridine nifedipine were observed in patch-clamp recordings of pachytene spermatocytes. These results suggest that low-threshold Ca^{2+} channels are the dihydropyridine-sensitive channels involved in the sperm acrosome reaction.

Key words: T-type Ca^{2+} channel; Sperm acrosome reaction; α_{1E} subunit; Dihydropyridine; Spermatogenesis; RT-PCR

1. Introduction

Ca^{2+} channels play a key role in cell signalling [1,2]. Mammalian spermatozoa must undergo Ca^{2+}-dependent exocytosis of the acrosomal granule to fertilize the egg [3]. ZP3, a sulfated glycoprotein from the zona pellucida, a thick extracellular glycoprotein coat surrounding the egg, is the main mediator of sperm binding and the acrosome reaction (AR) in mammals [3]. ZP3 activates a transduction mechanism dependent on extracellular Ca^{2+} that increases intracellular Ca^{2+} ($[Ca^{2+}]_i$) and pH (pH_i), and leads to acrosomal exocytosis. These changes are inhibited by pertussis toxin (PTX) indicating their regulation by G_i proteins [4,5].

Pharmacological evidence indicates that voltage-dependent Ca^{2+} channels are fundamental in this exocytotic process. High affinity binding sites for the L-type voltage-dependent Ca^{2+} channel antagonist PN200-110, were detected in bull and ram sperm membranes [5]. Several inorganic divalent cations like Co^{2+} and Ni^{2+}, and dihydropyridine antagonists, equally block acrosomal exocytosis triggered by a combined elevation of pH_i and a K^+ induced depolarization (mouse, bull and ram spermatozoa) or by ZP3 in bull sperm [5]. Depolarizing conditions that appear to open Ca^{2+} channels, bypass the inhibition of the ZP3-induced exocytosis produced by PTX. These experiments indicate that opening of sperm voltage-dependent Ca^{2+} channels is enough to trigger the AR when pH_i is increased, and that the activation of these channels, which involves a PTX sensitive G protein, is a required step in the ZP3 signal transduction pathway [5].

The direct study of sperm Ca^{2+} channels has been difficult due to their small size and complex geometry [6]. In addition, spermatozoa are terminally differentiated cells lacking the machinery for protein synthesis. Therefore, all ion channels required for cell function must be synthesized during spermatogenesis. As an approach to study Ca^{2+} channels in mouse sperm we looked for transcripts of the genes coding for the subunit that contains the pore and the voltage sensor of several voltage-dependent Ca^{2+} channels (α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E}) in both testis and in purified spermatogenic cell populations. We found that spermatogenic cells have mainly transcripts of the α_{1E} gene. We also obtained whole-cell patch-clamp recordings in pachytene spermatocytes. These cells only functionally express T-type Ca^{2+} channels sensitive to nifedipine and Ni^{2+} in their plasma membrane. Since the sperm AR and the uptake of Ca^{2+} that triggers it are also inhibited by these blockers, it is likely that a T-type Ca^{2+} channel is involved in inducing this reaction.

2. Materials and methods

2.1. Spermatogenic cell dissociation and purification for RT-PCR

Testes from adult CD-1 mice were used as a source of germ cells. They were decapsulated, and the seminiferous tubules suspended in EKRB (in mM: 120.1 NaCl, 4.8 KCl, 25.2 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 11 glucose, 1 glutamine, 1×essential amino acids (Sigma), 1×non-essential amino acids (Sigma)), adjusted to pH 7.2 with CO_2. Spermatogenic cells were obtained as described [7], resuspended into EKRB 0.5% BSA and filtered through an 80 μm mesh Nytex nylon filter (Tetko Inc., Lancaster NY). The single-cell suspension of germ cells was sedimented at unit gravity through a 2–4% BSA linear gradient generated on a staput chamber (Johns Scientific, Ontario) at 4°C. Gradients were collected after 2.5 h in 10-ml fractions, which were assessed for cell morphology and purity by light microscopy using Nomarski optics, and similar fractions were pooled.

2.2. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs from seminiferous tubules and spermatogenic cells were extracted as described [8]. Prior to each reverse transcription (RT) reaction, 5 μg of total RNA were dissolved in 10 μl water and digested with 1 U RNase-free DNase (RQ1, Promega Co., Madison) in the transcription buffer (in mM: 50 KCl, 2.5 $MgCl_2$, 20 Tris-HCl, pH 8.4) for 10 min at 37°C, and the DNase was heat inactivated at

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65°C for 15 min. At this point, random hexamers were added to a final concentration of 2.5 ng/μl, and quickly chilled on ice. RT was achieved with the Superscript preamplification system (Gibco BRL, Gaithersburg MD), according to the manufacturer's instructions. Similar results were obtained by priming the cDNA synthesis with oligo-dT_{12-18}. For the polymerase chain reaction we designed oligonucleotides against α_{1A} (5'-CGGGATCCCGAAGCAGTCGATGGCGCAGAGA and 5'-GGAATTCCTTGTTGGGCCCTTCCCAGTAC), α_{1B} (5'-CGGGATCCCGACCGAATGGCCGCCCTTCGAA and 5'-GGAATTCCTGAGGATGGCGAAGAAGAGCA), α_{1C} (5'-CGGGATCCCGCAATGCAGCTGCAGGACTTGC and 5'-GGAATTCCTTATGCCCTCCTGGTTGTAGC), α_{1D} (5'-CGGGATCCCGGCCATGCAGCACTATGAGCAA and GGAATTCCCCATGATGACAGCCACGAAGA), and α_{1E} (5'-GGATCCAGCAGGAACCGACAAGGAACC and 5'-GAATTCGGTGGCCAGGATCATGTACTC) Ca^{2+} channel subunits. Amplifications were performed on a PTC-100 programmable thermal controller (M.J. Res. Inc., Watertown MA) with Taq DNA polymerase (Boehringer, Mannheim, Germany). PCR reactions were carried out in a final volume of 50 μl having 200 μM dNTP, 250 μM each primer, 1.5 mM $MgCl_2$. Mixtures were overlaid with mineral oil, and PCR was initiated by adding 2.5 units of Taq DNA polymerase at 85°C ('hot start') per reaction. Cycling was 94°C for 3 min during the first cycle, annealing at 56°C for 1 min, extension at 72°C for 2 min, and denaturation for 45 s during 35 cycles. Amplimer identity was checked by direct double-strand sequencing of the gel purified fragment with the dsDNA Cycle Sequencing System (Gibco BRL), according to the supplier's instructions.

2.3. Whole-cell patch-clamp experiments

Spermatogenic cells were obtained as described [7], except that Ca^{2+} free solutions were used. The dissociated cells were stored at 4–10°C and remained healthy for at least 12 h. An aliquot of the cell suspension was placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon Corp.), and was continuously superfused with recording medium (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 1 $NaHCO_3$, 0.5 NaH_2PO_4, 5 Na-HEPES, 5 glucose, 10 $CaCl_2$, pH 7.35) at 20–23°C. Most cells included in this study were at the pachytene stage of differentiation, although similar recordings were obtained from round spermatids as well. Pachytene spermatocytes are easily recognizalbe under phase contrast microscopy because of their large diameter (> 16 μm), and distinct condensed chromatin (see Fig. 3C,D). Ca^{2+} currents were recorded with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA) connected to the pipette and the bath by Ag-AgCl wires. The pipette internal solution was (in mM: 110 Cs-methanesulfonate, 10 CsF, 15 CsCl, 2 Cs-BAPTA, 4 ATP-Mg, 10 phosphocreatine, 5 Cs-HEPES, pH 7.35). Glass borosilicate pipettes were pulled to tip diameters of about 1.5 μm, having resistances ranging between 2 and 5 MΩ when filled with pipette solution. Records were low-pass filtered at 2 kHz (4-pole Bessel filter), digitized at 30 kHz and analyzed off-line. A p/4 pulse protocol was used routinely to minimize leak and capacitive currents from current records [9], and series resistance was compensated by at least 50%. Nifedipine-containing extracellular solutions were prepared fresh from concentrated stock solutions. They were pressure-applied (10 lb/inch^2) via a puffer pipette located within 200 μm from the cell under examination, which was controlled by a Picospritzer II device (General Valve, Fairfield, NJ). Application of normal external solution with or without vehicle did not induce significant changes in Ca^{2+} currents. IC_{50}s were estimated according to: $IC_{50} = ([B](1-F_B))/F_B$, where [B] corresponds to the blocker concentration, and F_B is the fraction of the peak current blocked at a −20 mV test potential in the presence of the blocker [10].

3. Results and discussion

Spermatozoa are small terminally differentiated cells with a complicated geometry. This has limited the characterization of their ion channels [11,12]. Only three patch-clamp studies have been reported on sperm, two in the sea urchin [13,14], and one in mammals describing a cGMP-regulated cation channel [15]. Incorporation of isolated sperm plasma membranes to planar bilayers has revealed the presence of Ca^{2+} channels, among other types, in sea urchin and mammalian spermatozoa [16–21].

Since mature sperm lack the protein synthesis machinery and most RNAs, the only way to study gene expression and protein assembly is in the progenitor spermatogenic cells. We used mouse testis as starting material to look for Ca^{2+} channel gene transcripts. Voltage-dependent Ca^{2+} channels are heteromultimeric membrane proteins coded by a superfamily of related genes. The α_1 subunit, which contains both the pore and the voltage-sensor, is the main subunit [22]. Six α_1 sub-

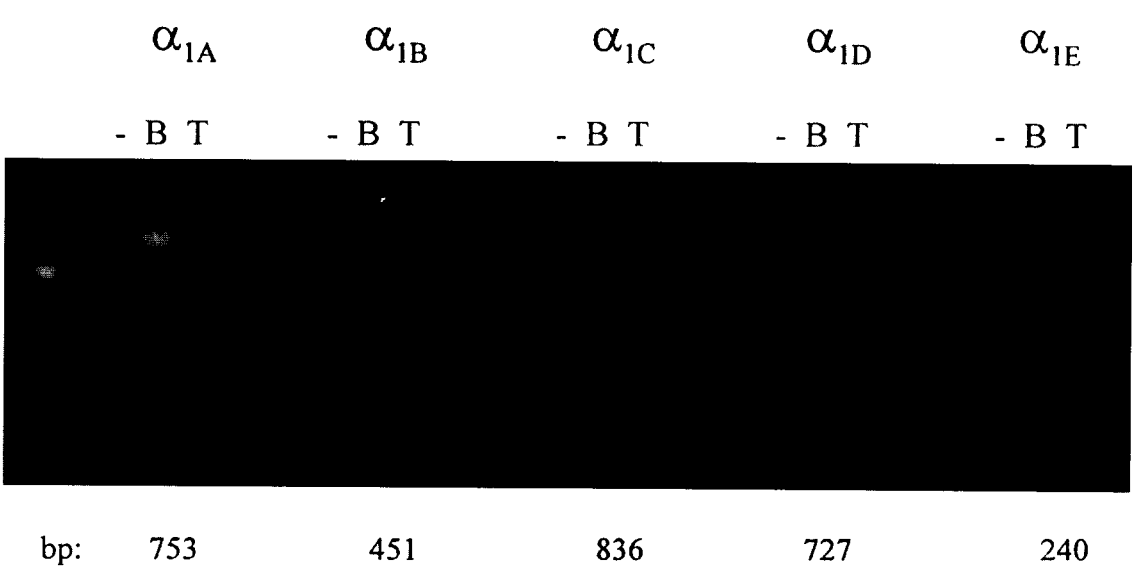

Fig. 1. RT-PCR experiments showing the expression of α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} Ca^{2+} channel subunit genes in mouse brain (B) and testis (T) RNA. Total mouse brain and testis RNAs were DNase I-treated before reverse-transcribed. The first-strand cDNAs were amplified with specific primers against α_1 subunits for 35 cycles. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The identity of the amplicons was confirmed by double-strand sequencing. The minus sign indicates reactions without cDNA, as controls. Numbers on the bottom indicate amplicon sizes in bp. The DNA molecular weight marker was a 100-bp DNA ladder having a more intense 600 bp band.

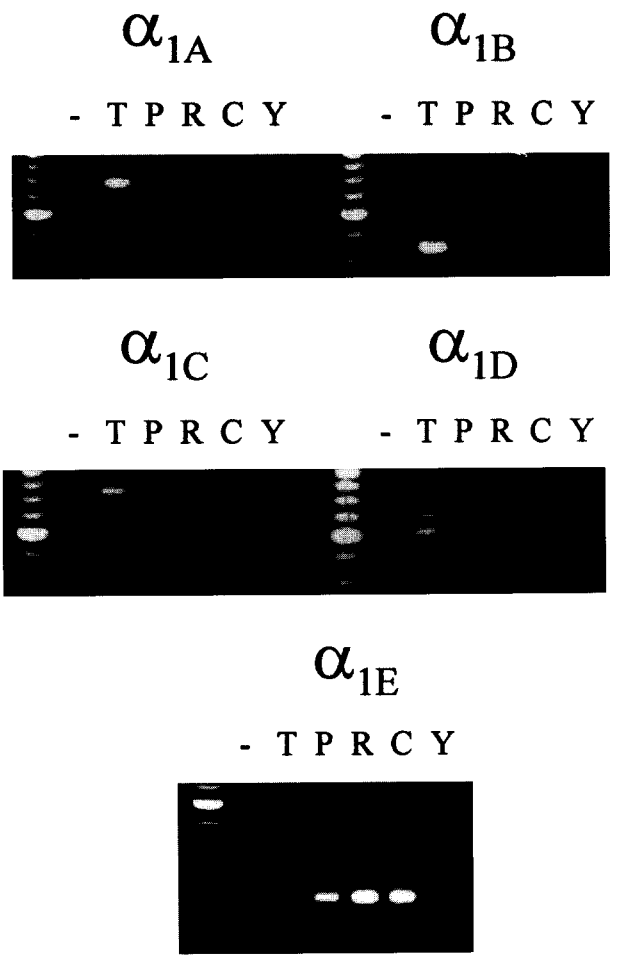

Fig. 2. RT-PCR experiments showing the expression of Ca^{2+} channel α_1 subunits in pachytene spermatocyte (P), round (R) and condensing (C) spermatid and residual body (Y) RNA. Testis (T) was included for comparison. Experimental conditions were as in Fig. 1.

unit genes have been described in mammals, now termed α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E} and α_{1S} [23].

As a first approach to the molecular identification of the Ca^{2+} channel genes expressed in sperm, we performed RT-PCR experiments with specific oligonucleotides against α_{1A}–α_{1E} in mouse testis cDNA. Fig. 1 shows the electrophoretic pattern of the amplimers obtained in mouse brain (lanes labeled B) and testis (lanes labeled T). The expected sizes (in bp) were: 753 for α_{1A}, 451 for α_{1B}, 836 for α_{1C}, 727 for α_{1D} and 240 for α_{1E}. We obtained RT-PCR amplimers with the expected sizes for all tested oligo pairs, and the amplimer identity was confirmed by double-strand sequencing (Torres and Liévano, unpublished results). Only with α_{1D} did we obtain a double band, probably indicating the existence of alternatively spliced products in the testis. These experiments show that all five α_1 subunit genes are expressed in the mouse testis, which is not surprising, since testis contains intertubular tissue (blood and lymphatic vessels, nerve fibers, and Leydig, Sertoli, myoid cells), and the different types of spermatogenic cells [24]. Multiple α_1 gene expression has also been reported in excitable cell lines [25].

In purified spermatogenic cells, however, α_1 subunit gene expression is different, as shown in Fig. 2. RT-PCR experiments in pachytene spermatocytes (P), round (R) and condensing (C) spermatids, and residual bodies (Y) showed detectable expression of the α_{1E} gene, giving the expected 240 bp amplimer in all cDNAs. Although less intense, there were also PCR positives for α_{1A} in round and condensing spermatids, and in the residual bodies. This is probably a minor transcript since the same amount of cDNA was used in all samples. Fig. 2 shows that the α_{1B}, α_{1C} and α_{1D} transcripts are absent in spermatogenic cells. Thus, these cells basically only have α_{1E} transcripts that could be physiologically relevant.

The participation of L-type Ca^{2+} channels during the sperm AR in mammals and in sea urchins has been invoked because of the inhibitory effect of dihydropyridines and diphenylalkylamines on this reaction and on the Ca^{2+} uptake that occurs along with it [5,26,27]. However, it is known that μM concentrations of dihydropyridines are able to block low-threshold T-type currents in some preparations [25,28,29]. The fact that high dihydropyridine concentrations (μM) are required to inhibit the AR makes it difficult to distinguish whether an L- or a T-type Ca^{2+} channel is involved in this process. In an attempt to answer this question we carried out whole-cell patch-clamp recordings in pachytene spermatocytes. Typical whole-cell Ca^{2+} currents recorded from these cells are shown in Fig. 3A. They were evoked from a holding potential of −80 mV to test pulses in the range of −70 to +40 mV. These Ca^{2+} currents were transient and had fast kinetics, they activated at low depolarizations, and displayed activation and inactivation kinetics typical of T-type channels. The *I-V* curve obtained from the records in Fig. 3A is shown in Fig. 3B. Analogous inactivating, low-threshold Ca^{2+} channel currents were described in rat spermatogenic cells [30]. Our whole-cell records do not show evidence for the presence of high-voltage activated, non-inactivating Ca^{2+} channels, such as L-type channels. A typical pachytene spermatocyte is shown in Fig. 3C,D. We assessed the effects of dihydropyridines on the pachytene spermatocyte Ca^{2+} currents. Fig. 3E shows superimposed traces elicited by depolarization to −20 mV, obtained before, during and after the application of 2 μM nifedipine. It is evident from the records that nifedipine is able to reversibly block the T-type Ca^{2+} channels with an estimated IC_{50} = 8.1 μM (n = 5). The channels blocked by nifedipine correspond to T-type Ca^{2+} channels, as shown by the superposition of the normalized Ca^{2+} currents in the presence and absence of the blocker (Fig. 3E). It is known that T-type channels are blocked by external Ni^{2+} [31]. Spermatocyte T-type channels are also sensitive to Ni^{2+}, with an estimated IC_{50} = 150 μM (n = 4). A preliminary account of these currents has been presented [32], and a complete electrophysiological characterization submitted [33].

T-type channel molecular identification still remains elusive. The only cloned member of the low-threshold Ca^{2+} channels is the rat brain α_{1E} subunit [34]. This channel shows transient kinetics when expressed in *Xenopus* oocytes, and is sensitive to external Ni^{2+} (IC_{50} = 28 μM). As the low-threshold Ca^{2+} channels, α_{1E} is able to transport Ca^{2+} only slightly more efficiently than Ba^{2+} [35]. This is also the case for the spermatogenic cell expressed Ca^{2+} channels, both in rat [30] and in the mouse [33].

Heterologous expression of several structurally related members of the α_{1E} has shown a large variation in the voltage-dependence of channel activation [35–38]. The biophysical properties of heterologously expressed α_1 subunits depend on the auxiliary subunits coexpressed, particularly the β sub-

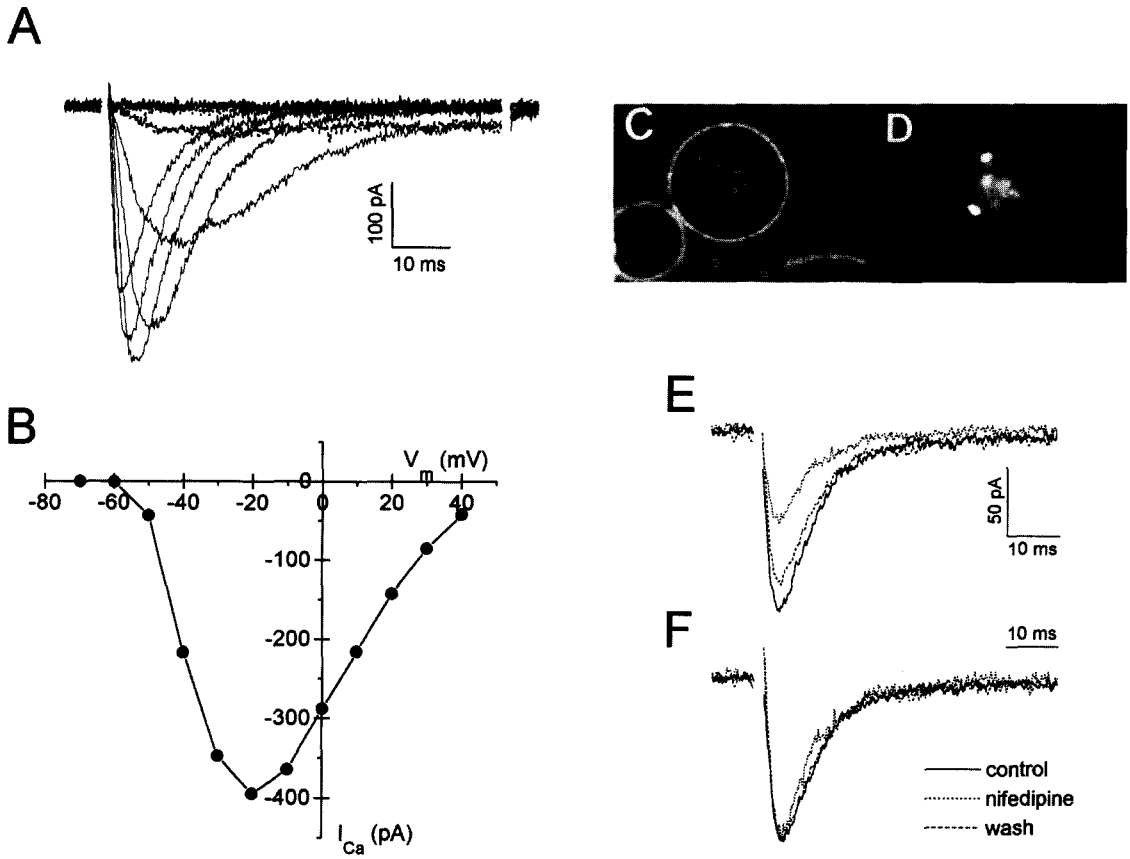

Fig. 3. (A) Family of Ca^{2+} currents recorded in primary spermatocytes from adult mice, evoked by stepping membrane potential to voltages between −70 and +10 mV, in 10 mV increments, from a holding potential of −80 mV. A portion of the non-compensated capacitive transients were blanked for clarity. (B) Current-voltage relationship obtained from a complete set of records, including those shown in A. (C,D) Micrographs of an acutely dissociated mouse spermatogenic cell in the pachytene stage of maturation obtained under phase contrast (C) and epifluorescence (D), after chromatin staining with the dye Hoechst 33342 at 1 μg/ml. (E,F) Acute effects of nifedipine application. Nifedipine (2 μM) was pressure-aplied for 20 s onto the spermatogenic cell while Ca^{2+} currents were elicited by step depolarizations to −20 mV every 10 s. Nifedipine induced a rapid and partially reversible inhibition of Ca^{2+} currents. Superimposed traces were obtained before, during and after the application of nifedipine. (F) Traces represented in E were normalized and superimposed for comparison. Note that activation and inactivation kinetics are not affected by nifedipine application.

units [39]. The phenotypic expression of Ca^{2+} channels is thus a complex function of the cell's metabolic status, molecular repertoire, and specific isoforms being transcribed and translated [40]. Although the presence of Ca^{2+} channel α_1 subunit transcripts does not guarantee the functional expression of Ca^{2+} channels [25], the coexistence of α_{1E} transcripts and T-type Ca^{2+} channels in mouse spermatogenic cells may suggest that T-type channels arise from an α_{1E} subunit isoform in conjunction with other auxiliary subunits. Our results, however, do not rule out the possibility that T-type channels arise from an α_1 subunit other than those tested. There is also evidence suggesting the participation of at least two types of Ca^{2+} channels in the AR, both in the sea urchin [41] and in the mouse [42].

Mammalian spermatozoa must undergo changes after leaving the testis to become competent for fertilization. These changes occur in the male (epididymal maturation) and female reproductive tracts (capacitation and AR), and involve important variations in the ionic composition of the external media (reviewed in [6,43,44]). Increases in extracellular $[K^+]$ known to occur during the sperm's journey [45], could depolarize them and open voltage-dependent Ca^{2+} channels triggering premature exocytosis. Having T-type Ca^{2+} channels in uncapacitated sperm, which would be largely inactivated at their estimated resting membrane potential (around −40 mV; [46,47]), instead of L-type which would not be as inactivated, could therefore be a safeguard to contend with the environmental changes that occur during its maturation.

The following findings indicate that a T-type Ca^{2+} channel may be involved in the early events leading to the mammalian sperm AR: (a) the presence of mainly α_{1E} transcripts in pachytene spermatocytes, round and condensing spermatids (Fig. 2); (b) only T-type Ca^{2+} channels are functionally present in pachytene spermatocytes and round spermatids; (c) μM nifedipine is required to block both, the ZP3 induced increase in $[Ca^{2+}]_i$ and the AR in mouse sperm, and the T-type Ca^{2+} currents of pachytene spermatocytes (Fig. 3); (d) similar Ni^{2+} concentrations block the mouse sperm AR [5], and T-type channels in pachytene spermatocytes.

T-type Ca^{2+} currents in mouse spermatogenic cells have a low threshold, rapid inactivation and show steady-state inactivation in the −90 to −40 mV range [32]. As mentioned, at the mouse sperm resting membrane potential (∼−40 mV, [47,48]), a significant fraction of the spermatogenic T-type channels might be inactivated [30,33]. Interestingly, after capacitation, a process required by mammalian sperm to under-

go the ZP3-induced AR [3], the cells hyperpolarize to ~ −55 mV probably due to an increase in plasma membrane K^+ permeability [46,47]. In spite of this, capacitated sperm are unable to undergo AR upon depolarization by external K^+ addition [5], as would be expected if voltage-dependent L-type Ca^{2+} channels were present, or if inactivation had been effectively removed from T-type channels by capacitation. Possibly, as in sea urchin sperm [48,49], a ZP3-induced hyperpolarization could be necessary in mouse sperm to further remove inactivation from T-type Ca^{2+} channels and allow them to open and induce AR. On the other hand, binding of ZP3 to its sperm receptor(s) may activate G proteins which directly, or indirectly through second messengers, could alter the voltage-dependence of T-type channels, opening them to trigger AR. Although many unknowns remain in the complex sequence of events leading to the AR, T-type Ca^{2+} channels may play a central role in the process.

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