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Identification and Localization of T-type Voltage-operated Calcium Channel Subunits in Human Male Germ Cells

EXPRESSION OF MULTIPLE ISOFORMS*

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Suchitra Jagannathan‡, Emma L. Punt‡§, Yuchun Gu‡¶, Christophe Arnoult‖, Denny Sakkas‡‡, Christopher L. R. Barratt**, and Stephen J. Publicover‡**

*From the ‡School of Biosciences, University of Birmingham, Birmingham B15 2TT, United Kingdom, ‖CNRS, Commissariat à l'Energie Atomique/Grenoble DBMS-CIS, 17 Rue des Martyrs, Grenoble, 38054 France, and the **Reproductive Biology and Genetics Research Group, Assisted Conception Unit, Birmingham Women's Hospital, Birmingham B15 2TG, United Kingdom*

Low voltage activated, voltage-operated Ca^{2+} channels are expressed in rodent male germ cells and are believed to be pivotal in induction of the acrosome reaction in mouse spermatozoa. However, in humans, very little is known about expression of voltage-operated Ca^{2+} channels in male germ cells or their function. We have used reverse transcription-polymerase chain reaction, *in situ* hybridization, and patch clamp recording to investigate the expression of low voltage activated voltage-operated Ca^{2+} channels in human male germ cells. We report that full-length transcripts for both α_{1G} and α_{1H} low voltage activated channel subunits are expressed in human testis. Multiple isoforms of α_{1G} are present in the testis and at least two isoforms of α_{1H}, including a splice variant not previously described in the human. Transcripts for all the isoforms of both α_{1G} and α_{1H} were detected by reverse transcription-polymerase chain reaction on mRNA isolated from human spermatogenic cells. *In situ* hybridization for α_{1G} and α_{1H} localized transcripts both in germ cells and in other cell types in the testis. Within the seminiferous tubules, α_{1H} was detected primarily in germ cells. Using the whole cell patch clamp technique, we detected T-type voltage-operated Ca^{2+} channel currents in isolated human male germ cells, although the current amplitude and frequency of occurrence were low in comparison to the occurrence of T-currents in murine male germ cells. We conclude that low voltage activated voltage-operated Ca^{2+} channels are expressed in cells of the human male germ line.

The AR[1] of spermatozoa is crucial for fertilization. In humans, male factor infertility is highly correlated with failure of AR and/or the events that activate AR (1–4). It is believed that the primary activator of AR in mammals is binding of the spermatozoon to the ZP (5), although AR can also be induced by progesterone (6–8). In both instances, activation of exocytosis requires a multiphasic entry of Ca^{2+} through plasma membrane ion channels (9–12).

Stimulation of mouse spermatozoa with purified ZP3 induces a brief (200 ms) $[Ca^{2+}]_i$ transient, which is blocked by antagonists of VOCCs (13). This transient activates a separate sustained $[Ca^{2+}]_i$ influx, probably through store-operated channels incorporating Trp2, which leads to AR (12–14). Application of the patch clamp technique to the study of ion channels in mature spermatozoa has proved to be very difficult and has so far been limited to cell-attached recordings and use of artificial bilayers (Ref. 15, and see "Discussion"). Unfortunately, this approach has (so far) provided little information on expression of VOCCs. However, the whole cell clamp technique has been applied successfully to mouse spermatogenic cells (16–18). These cells express a LVA, transient VOCC current with voltage dependence and kinetics very similar to those of expressed, recombinant α_{1G} and α_{1H} LVA channels (19–21). No high voltage activated (HVA)-VOCC current was observed. The pharmacological sensitivity of the spermatogenic cell LVA current resembles that of ZP-induced $[Ca^{2+}]_i$ signal in spermatozoa, suggesting that influx through this channel is the primary response to ZP binding (17, 22). Intriguingly, it has been shown recently that the LVA currents of mouse late spermatids are partially blocked by ω-conotoxin GVIA (23). The effects of ω-conotoxin GVIA on the ZP-induced $[Ca^{2+}]_i$ signal and AR have not yet been investigated.

Immunostaining and RT-PCR of rodent testicular tissue and germ cells have demonstrated the presence of both transcripts and proteins for HVA-VOCC α_1 subunits (α_{1A}, α_{1B}, α_{1C}, α_{1E}; Refs. 23–30) and β subunits (29). Expression of recombinant α_{1E} subunits can result in T-like currents (31). However, male germ cells of α_{1E} knockout mice possess normal T-currents, indicating that α_{1E} channels do not mediate the currents seen in these cells (32). Espinosa *et al.* (28) used RT-PCR to investigate expression of LVA channel transcripts in mouse germ cells. Appropriate products were generated using primers directed against the -COOH termini of α_{1H} and α_{1G}. However, Jacob *et al.* (33), using primer pairs against various regions of α_{1G}, could obtain products only with primers encoding domain IV and the -COOH terminus in rat testis mRNA. Antibodies for LVA channels are not available.

Very little is known about the expression and roles of VOCCs in human male germ cells. The effects of VOCC antagonists on progesterone-induced $[Ca^{2+}]_i$ signaling in spermatozoa have

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§ Recipient of a Biotechnology and Biological Sciences Research Council studentship.

¶ Present address: Medical Research Council Clinical Sciences Center, Division of Medicine, Hammersmith Campus, Imperial College School of Medicine, London W12 ONN, United Kingdom

‡‡ Present address: Dept. of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06520-8063.

[1] The abbreviations used are: AR, acrosome reaction; ZP, zona pellucida; ZP3, zona pellucida glycoprotein 3; VOCC, voltage-operated calcium channel; LVA, low voltage activated; HVA, high voltage activated; RT, reverse transcription; NGP, neoglycoprotein; DIG, digoxigenin; Ω, ohm(s).

been studied extensively, but findings have been both variable and contradictory (34–40). Preliminary data suggest that human ZP induces a pimozide-sensitive $[Ca^{2+}]_i$ signal in human spermatozoa (41, 42). NGPs, which have been proposed to act in a similar manner to human ZP, induce elevation of $[Ca^{2+}]_i$ and AR in human spermatozoa (37, 43, 44). NGP-induced AR is blocked by VOCC antagonists (44, 45) and mibefradil, a semi-specific blocker of T-type channels, blocks NGP-induced $[Ca^{2+}]_i$ signaling and AR with similar potency (37, 46). Using molecular and immunohistochemical techniques, Benoff and colleagues (27) have provided evidence for the presence of a number of isoforms of the HVA α_{1C} subunit in human testis and spermatozoa. However, only preliminary data are available on expression of LVA channels. Son *et al.* (46) obtained a 489-bp RT-PCR fragment of α_{1H} with human testicular mRNA. Transcripts for α_{1G} were not detected in human testicular mRNA (46) or mRNA isolated from human spermatozoa (33).

To understand the processes that underlie AR in human spermatozoa, and to assess the accuracy of the mouse model of AR, it is vital that the nature of the Ca^{2+} channels involved is elucidated. As a vital, first step, we have undertaken the detection, sequencing, and localization of T-channel subunit transcripts from the human testis and have applied the patch clamp technique to immature human germ cells.

MATERIALS AND METHODS

PCR

PCR forward and reverse primers were designed to sequences of α_{1G} from the human brain (GenBank™ accession no. AF029229) and α_{1H} from the human heart (GenBank™ accession no. AF051946). PCR products were amplified on human, adult, normal testis cDNA (Invitrogen, Groningen, Netherlands; Origene Technologies, Inc., Rockville, MD) using in-house primer pairs (Table I) and the Expand High Fidelity PCR System containing thermostable *Taq* DNA polymerase and a proofreading enzyme (Roche Diagnostics, Mannheim, Germany). A human brain cDNA library provided by the Medical Research Council, Human Genome Mapping Project Resource Center, United Kingdom, was used as positive control. Touchdown PCR was carried out on a Primus 25 legal thermocycling PCR system (MWG Biotech Ltd., Ebersberg, Germany). The cycling conditions were 94 °C for 3 min 30 s, followed by six cycles of 94 °C, 30 s; 64 °C, 30 s; 72 °C, 3 min; with a touchdown temperature change of −0.5 °C/cycle. A further 40 cycles were carried out at 94 °C, 30 s; 61 °C, 30 s; 72 °C, 3 min, followed by a final extension at 72 °C for 10 min. The annealing temperatures for different primer pairs were altered in the range of 64 to 58 °C depending upon the T_m (melting temperature) of the primer pairs in use. Corresponding changes in touchdown temperatures were made. The annealing temperature of the second set of PCR cycling conditions was kept 3 °C below the annealing temperature of the first set of cycling conditions.

The PCR products were run on agarose gels and purified using a QIAquick™ Spin gel extraction kit (Qiagen GmbH, Hilden, Germany) and either sequenced directly (MWG Biotech. Ltd.) or cloned into a pGEM®-T Easy cloning vector (Promega).

Isolation of Germ Cells

Human Cells—Cells were obtained by two methods.

For RT-PCR and for the first series of electrophysiological recordings, testicular tissue was removed from patients who were attending for treatment at the Assisted Conception Unit, Birmingham Women's Hospital, Birmingham, United Kingdom (Human Fertilization and Embryology Authority Center 0119). Mature, motile spermatozoa were found in all biopsies used for cell isolation. Sufficient testicular tissue was initially isolated for the treatment of patients by intracytoplasmic sperm injection, whereas the remainder was used to isolate cells undergoing spermatogenesis. Ethical approval was obtained from the local ethics committee (0374 and 0420). A total of six biopsies were used for isolation of cells, three of which had been frozen in sperm freezing medium (MediCult Ltd., Copenhagen, Denmark) after selection of cells for intracytoplasmic sperm injection and subsequently thawed for isolation of germ cells and patching. The extracted testicular tissue was placed in a Petri dish containing *in vitro* fertilization medium (Scandinavian IVF, Gothenburg, Sweden) and the seminiferous tubules teased out using needles. Following this, the tissue was then passed through a series of smaller gauge needles starting at 26 through to 18. Once the cells were sufficiently dissociated, a 50-μl droplet of the cell suspension was placed in a Petri dish along with two 50-μl droplets of clean medium. The droplets were covered with oil (OvOil, Scandinavian IVF). The Petri dish was then placed on a Nikon Microscope under 200× magnification, and individual cells were removed from the cell suspension using a Narashige micromanipulator. Micropipettes with a 10–15-μm inner diameter were used to individually select germ cells (spermatocytes and spermatids). The classification of germ cells was the same as that previously published by Johnson and colleagues (47). Once an individual germ cell was selected, it was placed in a clean medium droplet. This procedure was repeated until sufficient germ cells were isolated. The droplet was then aspirated using a pipette, and the contents were either placed in an Eppendorf tube for RT-PCR (see above) or incubated overnight in a Petri dish for attachment to gelatin-coated slides prior to electrophysiological recording (see below). For RT-PCR, a total of 75 cells from the six biopsies were pooled before extraction of mRNA. For patch clamping, cells from five of the biopsies were used.

For the second series of electrophysiological recordings, seminiferous tubules were isolated from the testes of a patient undergoing an orchidectomy (ethical authorization number DGS 2001/0211) and incubated at 37 °C for 30 min in 3 ml of solution containing (mM): NaCl (150), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), NaH_2PO_4 (1), $NaHCO_3$ (12), D-glucose (11), pH 7.3, and collagenase type IA (1 mg/ml; Sigma). Tubules were rinsed twice in collagenase-free medium and cut into 2-mm sections. Spermatogenic cells were obtained by manual trituration and attached to culture dishes coated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA). The cells obtained were primarily pachytene spermatocytes and round spermatids.

Mouse Cells—Testes were dissected in phosphate-buffered saline and transferred to a Petri dish containing Earle's balanced salts medium (Sigma) containing 1% (w/v) trypsin (Invitrogen) in RNase-free water. Seminiferous tubules were teased out using a sterile needle and the sample incubated at 37 °C for 2 h with intermittent shaking. The aqueous medium was then pipetted out from the Petri dish, and mineral oil (Sigma) was overlaid. Germ cell isolation, including overnight incubation for attachment, was then carried out as described above for isolation of cells from biopsies taken for intracytoplasmic sperm injection (method 1 above). For patch clamp, untrypsinized tissue was used to isolate germ cells. After isolation, cells were allowed to attach to gelatinized slides before recording (see below).

RT-PCR on Germ Cells

For both human and mouse preparations, total RNA was isolated from 75–100 germ cells using the StrataPrep® Total RNA Microprep kit (Stratagene) as per the manufacturer's protocol (48). The expected yield of RNA was 50–100 ng. RNA was eluted in a total volume of 60 μl of elution buffer. 30 μl was used for reverse transcription. The RT reaction was carried out in a total volume of 60 μl in presence of 5 mM $MgCl_2$, 0.8 mM dNTP, 1.5 units of recombinant RNasin® (1 unit/μl), 1× RT buffer (10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% (v/v) Triton® X-100), 1.5 μg of random hexamers (0.5 μg/μl), and 48 units of avian myeloblastosis virus reverse transcriptase enzyme (15 units/μl) (Promega). The reaction mix was incubated for 10 min at room temperature, followed by 60 min at 42 °C. The sample was placed in a boiling water bath for 5 min and on ice for 5 min to inactivate the enzyme. The RT mix was stored at −70 °C for further use or used immediately in PCR.

PCR reaction was carried out using 5 μl of the RT mix from RNA isolated from human or mouse germ cells, 1× RT buffer, 0.64 μM primer pairs (A1G9F-A1G10R specific to α_{1G} and HHS1-HHAS1 specific to α_{1H}, respectively), and nuclease-free water to make up the total reaction volume to 25 μl.

Touchdown PCR, similar to that described under "PCR," was carried out in the presence of *Taq* DNA polymerase enzyme (Promega). Primers internal to the region amplified were used to confirm the PCR products obtained. Control PCR reactions employed (i) human or mouse β actin primers (Origene) to amplify 614 and 575 bp, respectively, and (ii) primer pairs matching the human T200 leukocyte common antigen precursor gene sequence (GenBank™ accession no. AH007396; see Table I). PCR was carried out using RT mix from germ cell RNA and testis cDNA (Invitrogen) as template. Amplification conditions were hot start at 94 °C for 3 min 30 s, followed by 35 cycles of 94 °C, 30 s; 64 °C, 30 s; 72 °C, 1 min. The PCR product obtained using cDNA as template was sequenced to confirm its identity.

TABLE I

Sense and antisense primers used in the amplification of transcripts for α_{1G} and α_{1H} subunits and the leukocyte common antigen precursor

No.	α_{1G} primers			
	Sense primer		Antisense primer	
	Name	Sequence	Name	Sequence
1	HGS4	5′-ATGGACGAGGAGGAGGAT-3′	HGAS2	5′-TGTCTCCTCTGCTCATCC-3′
2	HHS8	5′-TGCGTGACCCTGGGCATG-3′	HGAS3	5′-GGGAGGCTGAAATTCTCAG-3′
3	HGS6	5′-CTGAGCGTGGACCTGGAG-3′	HGAS5	5′-ATGATGTCGACCCAGCCC-3′
4	HGS7	5′-GCCTGGATCGCCATCTTC-3′	HGAS6	5′-TGGTGGTGATGGTGGTGG-3′
5	HGS9	5′-AGCTGCTCTCGCTCCCAC-3′	A1G10R	5′-GGCTGGTGAAGACGATGTTG-3′
6	A1G9F	5′-TTGGCCGGGGAATCATGATC-3′	HGAS7	5′-TCGGGCTCTGATTCGGAC-3′
7	HGS12	5′-TGACCTTCGGCAACTACGTGC-3′	HGAS12	5′-TCCAGCTGCTTGCAGCGCTC-3′
8	HGS18	5′-AAGCAGCTGGACCAGCAGG-3′	HGAS18	5′-GACAAGGACCACGTGGTCG-3′
9	HGS17	5′-TCCTGTGTCACCGATCATC-3′	HGAS17	5′-ATGTTCAGGACAAAGAAGGCC-3′
10	HGS11	5′-CTGAACATGTTTGTGGGTGTGG-3′	HGAS11	5′-TGACACCTGTGATGAAGAGGTC-3′
11	HGS11	5′-CTGAACATGTTTGTGGGTGTGG-3′	HGBAS1	5′-TCAGCCATCTGCTTCTCCTTAC-3′
12	HGS16	5′-CACTTGTGCACCAGCCACTAC-3′	HGAS16	5′-CTCTCCTCCAGGTGCTTCATC-3′
13	HGS13	5′-ACTTTGTGTCCTTCGTGCTGAC-3′	HGAS13	5′-ACAAGACGGAGCCTGACTGAG-3′
14	HGS14	5′-TGTCGGCATGGGAGCACTG-3′	HGAS14	5′-CTCCTCTGCTCATCCAGCC-3′
15	HGS1	5′-GAAGTGCTACAGCGTGGAGGC-3′	HGAS1	5′-CCAGGTCTGCTGGGTCAGAGG-3′
No.	α_{1H} primers			
	Sense primer		Antisense primer	
	Name	Sequence	Name	Sequence
1	HHS7	5′-ATGACCGAGGGCGCACG-3′	HHAS9	5′-CTCGGAGCCGCACTCAAC-3′
2	HHS8	5′-TGCGTGACCCTGGGCATG-3′	HHAS10	5′-CCTGACAAAGGCACTGTCC-3′
3	HHS9	5′-GGACAGTGCCTTTGTCAG-3′	HHAS8	5′-CGGCACACGTTGTAGTAC-3′
4	HHS2	5′-CCAGTACTACAACGTGTGCC-3′	HHAS2	5′-GATCTTCTCGTAGGGATCAG-3′
5	HHS1	5′-GGCTGGGCACCATGAACTAC-3′	HHAS1	5′-GTGAATTCATAGACGCCACG-3′
6	HHS4	5′-GGTGAGCTCAGCGGCTC-3′	HHAS13	5′-CAGCAGCCTGTGCAGAAC-3′
7	HHS10	5′-TACCATGAGCAGCCCGAG-3′	HHAS14	5′-CGTGTCGGATCTGTTGGC-3′
8	HHS14	5′-CAACTATGTGCTCTTCAACCT-3′	HHAS21	5′-ACAGGGAGAACTTCGGAGGC-3′
9	HHS14	5′-CAACTATGTGCTCTTCAACCT-3′	HHAS20	5′-TGGTCTGGAGTTCTCTGAGC-3′
10	HHS17	5′-GCGATGCCAACAGATCCGAC-3′	HHAS18	5′-ATCATCTCCGCCACGAAGATG-3′
11	HHS12	5′-GTGGCGGAGATGATGGTG-3′	HHAS17	5′-TAGTGGCTGGTGCACAGC-3′
12	HHS15	5′-TTCATCTCCTTCCTGCTCATCG-3′	HHAS19	5′-CATGGTGATGACGTTGACACAG-3′
13	HHS15	5′-TTCATCTCCTTCCTGCTCATCG-3′	HHAS22	5′-GCGAGTAGTCGGCATAGTAGG-3′
14	HHS15	5′-TTCATCTCCTTCCTGCTCATCG-3′	HHAS23	5′-CGAAGGTCACGAAGTAGACGG-3′
15	HHS16	5′-TCTACTTCGTGACCTTCGTGC-3′	HHAS25	5′-GAGCTGGTGATGTGGCTGAC-3′
16	HHS19	5′-ACGAGGAGGTCAGCCACATC-3′	HHAS24	5′-AGAGGAAGCTCTGGATTCTGG-3′
17	HHS18	5′-TCTTGGACGGTAGCCACAGTG-3′	HASUTR1	5′-TCTCCTGACGGGAGGCTGAC-3′
No.	Leukocyte primers			
	Sense primer		Antisense primer	
	Name	Sequence	Name	Sequence
	SLEUKO1	5′-ACACCCATTTTGTTATACTTATGC-3′	ASLEUKO1	5′-AAGTTGTTCTAAGTCAGTAGAATG-3′

In Situ Hybridization

A digoxigenin (DIG) labeling and detection kit (Roche; Ref. 49) was used to make DNA probes with PCR-amplified products. The products used were a 395-bp product from the COOH-terminal end of α_{1G} (PCR product no. 15; Fig. 1) and a 373-bp product from the I-II linker region of α_{1H} (PCR product no. 5; Fig. 1). The PCR product was cloned into pGEM®-T Easy vector and DIG label incorporated into the cloned DNA template by PCR carried out for 25 cycles at 94 °C, 1 min 45 s; 55 °C, 1 min 30 s; 72 °C, 2 min. The annealing temperature was 59 °C for $\alpha 1_H$. 0.4 μl of Expand High Fidelity PCR enzyme (1 unit/μl; Roche) was used in a 50-μl reaction containing 5 μl each of 10× DIG DNA labeling mix, 10× HF buffer, 1.6 pmol of primer, and 0.1 μg of plasmid DNA. DIG labeled DNA was run on a 1.5% (w/v) agarose gel. Labeled probe, migrating at a higher molecular weight than unlabeled cDNA, was eluted from the gel using QIAquick spin gel extraction kit. *In situ* hybridization was carried out on adult human testis sections (Novagen; Peterborough Hospital Tissue Bank, United Kingdom). Probe mixture and hybridization conditions were as described in the Roche manual with some modifications. Sections were de-waxed (Histoclear) for 10 min, rehydrated, and digested with 0.1 μg/ml proteinase K (Sigma) for 5 min at 37 °C. Sections were hybridized overnight at 42 °C and sequentially washed twice for 15 min at 20 °C with 5× SSC and once for 10 min at 42 °C with 1× SSC. Probe hybrids were localized using 1:500 dilution of alkaline phosphatase anti-DIG antibody (Fab fragments; Roche). Alkaline phosphatase activity was finally stained with freshly prepared solutions of 45 μl of nitro blue tetrazolium chloride (3 mg/ml) and 35 μl of 5-bromo-4 chloro-3 indolyl phosphate (3 mg/ml) in 1 ml of detection solution (0.1 M Tris-HCl, pH 8.5, 0.05 M $MgCl_2$, 0.1 M NaCl; Ref. 50). The reaction was stopped with 10 mM Tris-HCl, pH 8.1, and 1 mM EDTA. Sections were mounted using DABCO (Sigma), an aqueous mounting medium and observed under bright-field illumination (Zeiss Axioskop 2). Control hybridization and localization reactions were carried out using non-DIG-labeled probe, using unconjugated DIG (blind probe), without the anti-DIG antibody and without use of nitro blue tetrazolium chloride and 5-bromo-4 chloro-3 indolyl phosphate. Positive reactions were also carried out on other tissues to confirm localization of hybridization.

The distributions of α_{1G} and α_{1H} transcripts within seminiferous tubules, were compared by counting stained cells. For each of the two probes, 20 tubule profiles derived from two different samples were examined. The numbers of stained spermatogenic cells and Sertoli cells were counted, and the distributions of staining (spermatogenic:Sertoli) for the two transcripts were compared using a chi-square contingency table.

Electrophysiology

Two series of electrophysiological recordings were made. For the first series, cells were isolated from human testicular biopsies taken for intracytoplasmic sperm injection and from mouse testes (see above). After incubation overnight for cell attachment (see above), extracellular saline was exchanged for recording one containing 108 mM $BaCl_2$ and 10 mM HEPES, pH corrected to 7.6 with NaOH (maximum Na^+ content ~3 mM; Ref. 51). Under these conditions, T current amplitudes are typically increased by ~70% and the voltage sensitivities of current activation and inactivation are shifted by ~+30 mV (52–54). Patch electrodes were pulled from filamented 1.5-mm glass capillaries (Clark

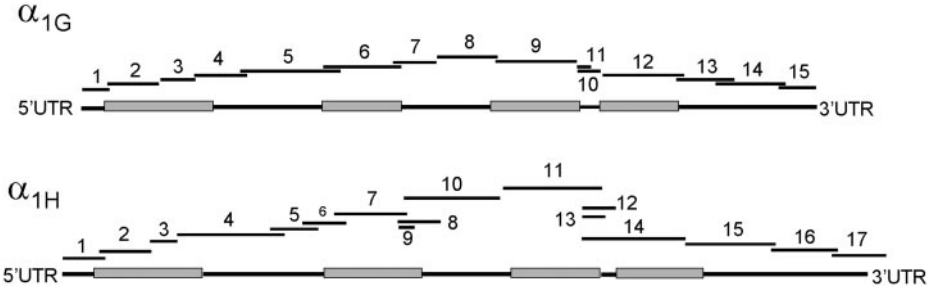

FIG. 1. **Position and overlap of the PCR products used to obtain full sequence of α_{1G} (*top panel*) and α_{1H} (*lower panel*) from human testis cDNA.** Linear representations are drawn to scale, *boxes* showing putative transmembrane α-helical regions. Product *numbering* relates to column 1 of Table I, where the primers used are tabulated.

Electromedical GC150TF) and fire-polished. Electrodes were back-filled with saline containing 150 mM CsCl, 5 mM EGTA, 10 mM D-glucose, 10 mM HEPES. pH was corrected to 7.3 with CsOH. Pipette resistance was 3–7 MΩ. All recordings were made using the whole cell variant of the patch clamp technique. Seals of up to 10 GΩ were achieved prior to breakthrough. Resulting whole cell input resistances in cells considered suitable for recording were in the order of 1–2 GΩ. Recordings were commenced within 1–2 min of breakthrough, using a Warner PC501A amplifier with filter set at 2 kHz. Signals were passed to an IBM-compatible PC, via a CED 1401 data acquisition interface. Acquisition and analysis of signals was carried out using WCP version 2.1 (Strathclyde Electrophysiology Software). Cells were held at −60 mV, and families of currents were generated by applying a series of 400-ms voltage steps, starting with a step to −40 mV and incrementing by 10 mV up to +60 mV (11 steps in all). Depolarizing steps were interspersed with hyperpolarizing steps, which were used for leak subtraction using a P/4 protocol. Recordings were carried out at room temperature (20–21 °C). Statistical comparison of the frequency of occurrence of LVA currents in human and mouse cells was carried out using a chi-square contingency table.

The second series of recordings were obtained from cells isolated from a patient undergoing orchidectomy (see above). Cells were separated by trituration and identified visually before patching. After attachment, the extracellular saline was exchanged for recording saline containing (mM): NaCl (100), KCl (5), $CaCl_2$ (10), $MgCl_2$ (1), TEA-Cl (26), sodium lactate (6), HEPES (10), 3.3 D-glucose, pH 7.4 (adjusted with 1 N NaOH). Pipettes were pulled from Corning no. 7052 glass (Gardner Glass Co., CA) and fire-polished. The pipette solution consisted of the following components (mM): cesium glutamate (130), D-glucose (5), HEPES (10), $MgCl_2$ (2.5), Mg_2ATP (4), EGTA-Cs (10), pH 7.2 (adjusted with 1 N CsOH). Pipette resistance was 5–7 MΩ. Whole cell currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Union City, CA) during depolarizing steps from a holding potential of −90 mV to test potentials between −60 mV and +30 mV (10-mV increments) and analyzed using Biopatch (BioLogic, France). All traces were corrected for leak and capacitance currents, and filtered at 2 kHz. All recordings were made at room temperature (~25 °C).

RESULTS

PCR on Testis cDNA—Initial attempts to amplify full-length α_{1G} and α_{1H} transcripts, or to obtain full-length sequence from two or three amplification products, proved unsuccessful. However, using primers designed to generate products of 250–1000 bp, we were able to generate a series of PCR products. Using this strategy we obtained the complete sequence from human testicular cDNA for both α_{1G} and α_{1H} (Fig. 1). During this procedure it became apparent that, for certain regions, more than one transcript was represented in the testicular cDNA. With α_{1G} primers, we routinely detected variation in the III-IV linker region. Using the nomenclature of Monteil *et al.* (55), the isoforms detected were α_{1G}-a, α_{1G}-b, and α_{1G}-bc, which encode three different, intracellular III-IV loops. Primers HGS11 and HGAS11 (α_{1G} PCR product 10; Fig. 1, *top panel*), which span the relevant region, generated two bands of ~220 and 240 bp (Fig. 2). Sequencing confirmed that the smaller (222 bp) product was α_{1G}-b. These primers will generate a product of 244 bp from α_{1G}-a template and a product of 240 bp from α_{1G}-bc, which would not be expected to separate clearly on the gel used. However, the sequence of the larger (240 bp) product, determined on two occasions from different PCR reactions, was unambiguously that of α_{1G}-bc. To investigate directly the presence of transcripts for α_{1G}-a, we used the specific primer HGBAS1 in combination with HGS11 (α_{1G} PCR product 11; Fig. 1, *top panel*). Reactions with these primers generated a band of 144 bp, which was confirmed, by sequencing, to be the appropriate portion of α_{1G}-a (Fig. 2). In accord with the findings of Monteil *et al.* (55), the two α_{1G}-b isoforms were always observed together. We did not detect any of the α_{1G}-e isoforms, which include an insertion in the II-III linker region of the molecule, or the d or f isoforms, which include insertions in the COOH-terminal region. With the α_{1H} primer pair HHS15-HHAS19 (α_{1H} PCR product 12; Fig. 1, *lower panel*), we obtained two products of ~300 and 280 bp using human testicular cDNA. The sequence of the larger product corresponded to the human cardiac form previously described by Perez-Reyes and colleagues (α_{1H}-a; see Ref. 56). The smaller product gave a 282 bp sequence, which was an α_{1H} isoform with a deletion in the III-IV linker region (α_{1H}-b; Fig. 3, *upper panel*). The presence of both isoforms was confirmed by use of an internal primer HHAS22 (α_{1H} PCR product 13; Fig. 1, *lower panel*), which again generated the expected product and a truncated product (Fig. 3). Comparison of the cDNA sequence with the human genomic sequence confirmed that this deletion was because of alternative splicing of the gene, omitting cassette exon 26 (GenBank™ accession no. AF051946; Fig. 3, *lower panel*). This isoform of α_{1H} was recently detected in rat brain (21), but this is the first report of such a deletion in the human (GenBank™ accession no. AJ420779). Using both primer pairs, we could detect only the longer (α_{1H}-a) isoform in positive control reactions using a human brain cDNA library (Fig. 3).

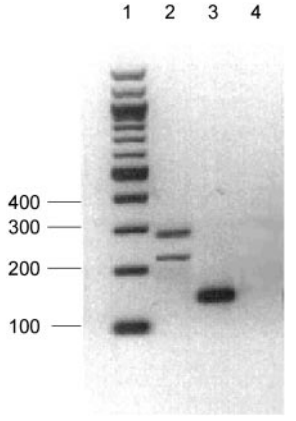

FIG. 2. **PCR of α_{1G} from human testis cDNA, using primers in the III-IV linker region.** *Lane 1*, 100-bp DNA ladder; *lane 2*, PCR products generated from human testicular cDNA using primers HGS11-HGAS11. Two products of 222 and 240 bp were shown by sequencing to correspond to the α_{1G}-b and α_{1G}-bc isoforms. *Lane 3*, PCR product generated from human testicular cDNA using primers HGS11-HGBAS1 (α_{1G}-a-specific primer pair). The product (144 bp) was shown by sequencing to be the appropriate portion of α_{1G}-a. *Lane 4*, no template control. Gel used was 3% (w/v) agarose. Gray scales in this and subsequent gel images have been inverted to improve clarity.

Negative control reactions (no template) were carried out for every primer pair, and all failed to generate a product. To

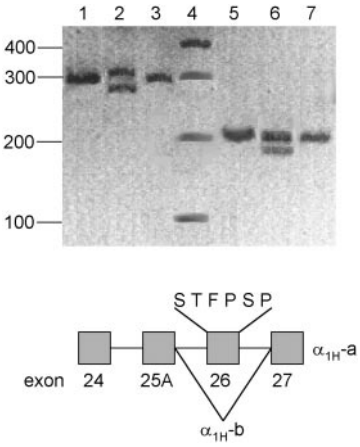

FIG. 3. **PCR of α_{1H} from human testis cDNA, using primers in the III-IV linker region.** *Upper panel*, 4% (w/v) agarose gel showing results of PCR reactions using primer pairs HHS15-HHAS19 (*lanes 1–3*) and HHS15-HHAS22 (*lanes 5–7*). *Lanes 1* and *5* show PCR products generated from human brain cDNA, *lanes 2* and *6* show PCR products generated from human testicular cDNA, *lanes 3* and *7* show PCR products generated from a human α_{1H}-a clone, and *lane 4* is a 100-bp DNA ladder. Both primer pairs generated two products from testicular cDNA. Sequence analysis showed that this was because of the presence in the testis of a deleted isoform (α_{1H}-b) in which exon 26, encoding six amino acids (STFPSP) is spliced out (*lower panel*).

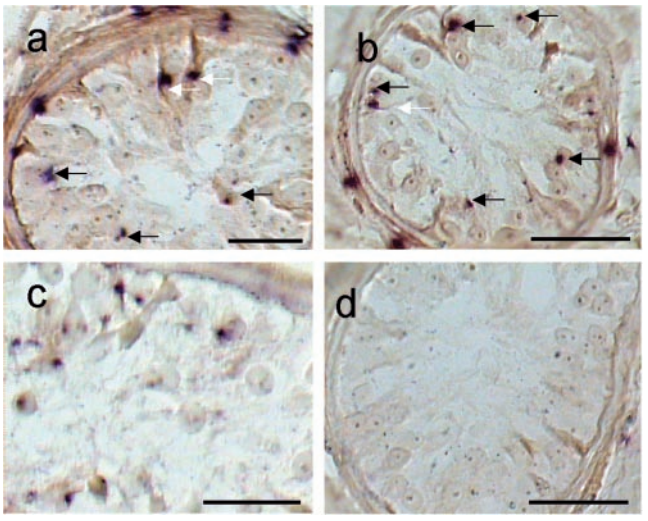

FIG. 4. **Localization of transcripts by *in situ* hybridization.** *a* shows localization of α_{1G}, *b* and *c* show localization of α_{1H}, and *d* shows a tubule profile from a control reaction in which unconjugated DIG (blind probe) was used. *Black arrows* in *a* and *b* show *purple-brown* staining of germ cells. *White arrows* in *a* and *b* show staining of larger, Sertoli-like cells. *c* shows a section of a tubule stained for α_{1H} in which a number of germ cells are stained. *Scale bar* equals 25 μm.

assess any effects of genomic DNA contamination, a series of reactions were carried out, for α_{1G} and α_{1H}, employing primers against an intronic region in the II-III linker region, paired with primers against sequences within domains II and III. These reactions generated the predicted products with genomic DNA, but failed to generate any product with testicular cDNA. PCR was also carried out with primers in the untranslated region and the COOH terminus, spanning an intronic sequence. With genomic DNA, larger products were obtained for both for α_{1G} and α_{1H} than with testicular cDNA. The size of the products from genomic DNA corresponded to amplification of a product including the intronic region.

cDNA samples from four different biopsies (each from a different donor) were used for these studies. Each sample gave positive results with all the primer pairs with which it was used (each primer pair was used on two to four different samples) except for one, which gave no product for any primer pairs directed against the region from the II-III linker to the 3′ end of α_{1H}. Positive controls, run in parallel, gave the expected product, raising the possibility that this individual (reported as fertile) expressed a truncated α_{1H} transcript.

In Situ Hybridization—To identify the cell types responsible for the LVA-VOCC subunit transcripts detected by RT-PCR, *in situ* hybridization was carried out with sections from human testicular biopsies. Although described as "normal," there were few if any late germ cells in these sections and no spermatozoa were visible (Fig. 4). However, "spherical" cells resembling spermatogonia and primary spermatocytes were clearly discernible, as were larger elongated cells, which we tentatively identified as Sertoli cells. Despite the relatively low abundance of germ cells, purple-brown staining in these cells was detected using both the α_{1G} and α_{1H} probes (Fig. 4, *a–d*). Transcripts for both LVA subunits were also detected in the Sertoli-like cells and in extratubular cells of the testis. Within the tubules, α_{1H} transcripts were present primarily in the germ cells, with few of the elongated, Sertoli-like cells stained, but α_{1G} transcripts were distributed equally between the Sertoli-like cells and germ cells (Fig. 4; $p < 0.001$). Control sections, using a "blind" probe mixture, did not show any staining (Fig. 4*d*). Human heart tissue used as a positive control showed staining of vascular tissue for both α_{1G} and α_{1H}, but no staining of myocytes (LVA-VOCCs are expressed in cardiac myocytes only during hypertrophy; Refs. 57 and 58). No staining was observed in other portions of the reproductive tract, such as ductus deferens.

RT-PCR on Germ Cells—As an alternative method for confirmation of the presence of LVA-VOCC transcripts in spermatogenic cells, RT-PCR was carried out on mRNA extracted from isolated human male germ cells. Cells were individually selected from biopsy material as described above. After preparation of germ cell cDNA, PCR was carried out using primer pairs for α_{1G} and α_{1H}, both of which had previously been shown to generate a product when used with testicular cDNA. Products of appropriate size were detected in both cases (Fig. 5) and their identity confirmed by use of internal primers. To investigate the presence of α_{1G} isoforms in cDNA isolated from germ cells, we used the primer pairs HGS11 and HGAS11 (product 10). Two bands were detected as in testicular cDNA, but there was insufficient product for sequencing. Use of HGS11 with HGBAS1 (product 11) confirmed the presence of α_{1G}-a. We conclude that germ cell cDNA contained α_{1G}-a, α_{1G}-b, and probably a_{1G}-bc (see above). For α_{1H} the primer pair HHS15-HHAS19 (product 12), as with testicular cDNA, generated two products of ~300 and 280 bp. Use of internal primers confirmed the identity of α_{1H}-a and α_{1H}-b (data not shown). Negative control reactions (no template) failed to generate products. Control reactions using intronic primers, as described above, confirmed that products were not a result of genomic DNA contamination.

To confirm that the human germ cell cDNA had not been contaminated by accidental inclusion of lymphocytes in the isolated cells, primers were designed to match the leukocyte common antigen precursor T 200, a lymphocyte marker sequence. PCR with these primers failed to generate a product with human male germ cell cDNA, but produced a product of the appropriate size with human testicular cDNA (Fig. 5). Sequencing of the product confirmed identity.

PCR was also carried out, using α_{1G}- and α_{1H}-specific primer pairs (α_{1G} product 6, α_{1H} product 5; Fig. 1), on mouse testis cDNA (Origene) and cDNA generated from mouse germ cells. As with the human, products of appropriate size were obtained

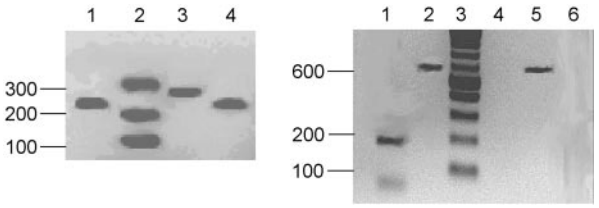

FIG. 5. **PCR of human male germ cell cDNA.** *Left panel*, T-channel primers: *Lane 1* shows results of PCR reaction using primer pairs HHS1 and HHAS1 (α_{1H}), *lane 3* shows results of PCR reaction using primers A1G9F-A1G10R (α_{1G}), *lane 2* is a 100-bp DNA ladder, and *lane 4* shows product generated from the α_{1H}-a clone using HHS1-HHAS1. *Right panel*, controls. *Lanes 1* and *4* show products generated using gene-specific primers SLEUKO1 and ASLEUKO1 for the T200 leukocyte common antigen precursor gene. Product was obtained from testis cDNA (*lane 1*) but not from cDNA derived from germ cells (*lane 4*). *Lanes 2* and *5* show that primers directed against actin generated a product both from testis and germ cell cDNA. *Lane 6* is a no template control. Sequence analysis confirmed the identity of all products. Both gels were 2% (w/v) agarose.

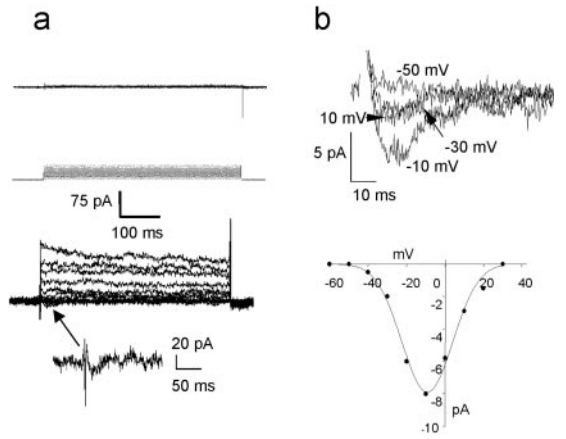

FIG. 6. **LVA currents in germ cells.** *a*, currents recorded from intracytoplasmic sperm injection biopsies (first series of recordings). *Upper panel* shows a "typical" family of traces obtained from a human germ cell isolated from a previously frozen biopsy taken for intracytoplasmic sperm injection. The cell (bathed in 108 mM $BaCl_2$) was held at −60 mV and stepped to −40, −30, −20, −10, 0, 10, 20, 30, 40, 50, and 60 mV (*center panel*). *Lower panel* shows records obtained from a freshly prepared (not previously frozen) cell, which was subjected to a similar stimulus protocol but in which an outward current was induced by depolarizing voltage steps. In this cell a very small transient inward current (*arrow*) was detectable in leak-subtracted traces obtained with steps up to −10 mV (*inset*). *b*, detection of LVA currents in human male germ cells freshly isolated from an orchidectomy. *Upper panel* shows currents, from a round spermatid, activated by stepping from −90 mV to −50, −30, −10, and 10 mV. *Lower panel* shows the current voltage relationship for this current. Similar currents were observed in a second round spermatid.

from both sources of cDNA using both sets of primers. Identity of the products was confirmed by use of internal primers (data not shown). Negative control reactions (no template) failed to generate products. Control reactions using intronic primers, as described above, confirmed that products were not a result of genomic DNA contamination.

Electrophysiology—Rodent male germ cells, held under whole cell clamp, express a LVA VOCC current with kinetics similar to those seen upon expression of recombinant α_{1G} and α_{1H} VOCC subunits. Because our PCR and *in situ* studies showed the presence of both α_{1G} and α_{1H} VOCC subunits in human male germ cells, we used the patch clamp technique to investigate VOCC currents in these cells. The first series of recordings (on cells isolated from biopsies taken for intracytoplasmic sperm injection) were carried out in high Ba^{2+} saline to maximize the amplitude of any VOCC currents. Recordings were attempted from over 70 cells, but only 50 of these maintained good seals after breakthrough (input resistance 1–2 GΩ). 35 of these cells were from previously frozen biopsies and 15 from fresh tissue. In six cells we recorded voltage-activated outward currents (Fig. 6*a*). Two of these currents were seen in cells from previously frozen biopsies, but these were much smaller than those recorded in freshly prepared cells. In one of the fresh cells, a very small "possible" LVA inward current (approximately −15 pA) was present. The current activated at approximately −30 to −20 mV (typical for a T current in this saline), but, at potentials positive to −10 mV, it was occluded by a considerably larger outward current (Fig. 6*a*). In contrast, when mouse male germ cells were isolated and prepared for recording in a similar manner, LVA currents were clearly present in at least 6 of the 20 cells examined ($p < 0.0005$). In the high Ba^{2+} saline used for these recordings, peak current occurred at ~+10 mV, as reported previously for LVA currents recorded under these conditions (51–54, 59).

In a second series of recordings on cells isolated from human testis (tissue removed during an orchidectomy), seven pachytene spermatocytes and round spermatids were tested (seal resistance >1GΩ). In two of these cells (both identified as round spermatids), small voltage-activated inward currents (maximum amplitude approximately −8 pA) were detected (Fig. 6*b*). The currents activated at −50 to −40 mV, peak amplitude occurring at −10 mV. Inactivation was rapid with a time constant of ~15 ms. These characteristics are consistent with those of currents generated by α_{1G} or α_{1H} T-type channels.

DISCUSSION

Despite the fact that T-type VOCCs are believed to play a critical role in ZP-induced AR in rodent spermatozoa (9, 17), almost nothing is known of the expression or function of these channels in $[Ca^{2+}]_i$ signaling in human male germ cells. We report here, for the first time, the presence in human testis of full-length transcripts for both α_{1H} and α_{1G} subunits. The unavailability of specific antibodies for the LVA α_1 subunit family is a significant problem in determining the tissue distribution of these channels. Therefore, to confirm the expression of LVA subunit transcripts in male germ cells, two alternative approaches were employed.

(i) RT-PCR, using RNA isolated from male germ cells, detected the presence of transcripts for both α_{1G} and α_{1H}. The cells were individually selected, by micromanipulation, by an experienced embryologist (D. S.), according to strict criteria (47). Although contamination of the germ cell sample cannot be completely discounted, we consider this to be most unlikely. PCR reactions designed to detect markers of potential contaminating cells did not generate products.

(ii) Use of *in situ* hybridization revealed the presence of transcripts for both α_{1G} and α_{1H} in germ cells, as well as in other cells of the seminiferous tubules and interstitium. The relative distribution of *in situ* staining suggests that α_{1H} transcripts are present primarily in germ cells with little staining in the elongated Sertoli-like cells, but that α_{1G} is expressed at least as strongly in other cell types as in germ cells. The sections contained few "late" germ cells and therefore may have under-represented the number of cells expressing the transcripts. However, even under these conditions, the presence of transcripts in germ cells was clear. On the basis of these two complementary findings, we conclude that both α_{1H} and α_{1G} are expressed in human male germ cells and are therefore potentially involved in Ca^{2+} signaling during spermatogenesis or in the mature gamete (see below).

LVA Channel Types and Isoforms in Human Testis—RT-PCR of human testicular and germ cell cDNA and male germ cell cDNA revealed the presence not only of transcripts for α_{1G} and α_{1H} subunits, but also of multiple isoforms of both sub-

units. Because expressed recombinant LVA channels have shown considerable differences in properties, both between different subunits and between subunit isoforms (19, 21, 60), this diversity potentially provides considerable variation in T-channel properties between testicular cell types or stages of differentiation. The shorter variant of the two α_{1H} isoforms that we detected in the testis (α_{1H}-b; which has a deletion in the III-IV linker) has not been reported previously in human tissues but has recently been detected in rat brain (21), where it is widely distributed. In contrast, reactions with human brain cDNA generated a robust product for α_{1H}-a, but α_{1H}-b could not be detected (Fig. 3). The rat α_{1H}-b isoform appears to function over more positive voltage ranges, for both activation and inactivation, than human α_{1H}-a (19, 21), although it remains to be seen whether this reflects splice variation or other (species) differences in the molecules. The range of α_{1G} splice variants detected in the testis includes α_{1G}-b, which, of the five variants expressed in HEK-293 cells by Chemin *et al.* (60), had the most positive voltage range for steady-state inactivation. α_{1G}-b and α_{1G}-a (primarily neuronal (55) but also present in human testis and germ cells) have steady-state inactivation ranges differing by >10 mV. Clearly, it is important that the distribution of these diverse T-channel subunits between cell types and stages of differentiation is elucidated, because a change from α_{1G} to α_{1H} or even from α_{1G}-a to α_{1G}-b during germ cell differentiation could have profound effects on cell function.

Functional Expression of LVA Subunits in Human Male Germ Cells—To examine the functional characteristics of the LVA VOCC subunits that we detected by RT-PCR, we applied the whole cell patch clamp to human male germ cells. In our first series of recordings, we used cells obtained from biopsies taken for intracytoplasmic sperm injection. Of the 50 records considered to be reliable (see results), only one "possible" LVA current was observed (Fig. 6*a*). In contrast, when mouse germ cells were prepared similarly, we observed T-type currents in 6 of 20 cells. Peak current occurred at ~10 mV (Fig. 6). LVA currents in rat osteoblasts (primarily α_{1G}; Ref. 54)[2] and rat marrow stromal cells (primarily α_{1H}),[2] recorded under identical conditions, gave maximal currents at 0–10 and 10–20 mV, respectively (52, 54). In a second series of experiments, using freshly prepared cells obtained from an orchidectomy, we observed T-currents in two of seven cells (both identified as round spermatids; Fig. 6*b*). Apart from the use of different biopsies, the difference in success rate between the two series of experiments may reflect other factors. First, we consider that use of previously frozen material may be inappropriate because, as well as our failure to find any T currents in such cells, the robust outward currents seen in the fresh cells used in the first series of experiments (Fig. 6*a*) were small or absent in frozen-thawed cells. Second, it may be important to record currents immediately after isolation rather than after overnight incubation. Although T-currents were observed in mouse cells prepared in this way, their frequency of occurrence (~1 in 6) was low compared with that seen in cells used on the day of isolation.[3]

All of the currents that we did observe were very small (10–15 pA) compared with the currents of rodent cells reported here and in previous studies (16–18). We conclude that human male germ cells express T currents but that, in immature cells, their amplitude and possibly their frequency of occurrence is low. Furthermore, the currents were observed in late germ cells (spermatids). We consider it likely that, in the human, functional expression of these channels occurs at a late stage of germ cell differentiation and/or during epididymal maturation, the channels being functionally significant primarily in the mature cell (see below). Preliminary patch clamp recordings from pachytene spermatocytes of cat, sheep, rabbit, and guinea pig failed to reveal the presence of any VOCC currents,[3] suggesting that rodent cells may be unusual in their expression of significant T currents at an immature stage. Functional expression of HVA VOCCs in human and possibly rodent male germ cells may be similarly restricted to mature spermatozoa. Transcripts for various HVA channels have been detected in rodent and human male germ cells (24–28), the encoded proteins can be detected in spermatogenic cells and mature spermatozoa (23, 27, 29, 30), and there is evidence for function of such channels in mature cells (23, 61). However, only LVA currents are detected in patch clamped spermatogenic cells.

The relationship of the channels described here to those detected using other techniques is difficult to assess. Although the use of whole cell patch clamp to characterize expression of ion channels in mature spermatozoa has so far proved impossible, Ca^{2+} channels have been detected by cell-attached recording and by inclusion of sperm membrane proteins in artificial bilayers. Darszon and colleagues (62), using patch clamp, detected a Ca^{2+}-permeable but poorly selective cation channel in mouse spermatozoa. Using insertion of sperm proteins into artificial lipid membranes, the same group have observed a high conductance Ca^{2+} channel in sea urchin and mouse spermatozoa that resembles the ryanodine receptor (63). Tiwari-Woodruff and Cox (64), also using artificial bilayers, observed a Ca^{2+} channel from porcine spermatozoa, which displayed sensitivity to dihydropyridine drugs, but showed no voltage dependence. Similar studies with human sperm proteins have resulted in detection of various channels, including a Ca^{2+}-selective channel that showed voltage sensitivity (65, 66), but the conductance of this channel was considerably higher than that of native or recombinant T channels. There has been only one report of patching of human spermatozoa, in which Weyand *et al.* (67) described a cyclic nucleotide gated Ca^{2+} channel. It appears that currents corresponding to the transcripts described here are yet to be detected in mature cells.

LVA VOCCs and AR—It has been known for some time that the influx of Ca^{2+} (and consequent AR) that occurs upon binding of mammalian spermatozoa to the ZP requires opening of VOCCs (9, 68). More recently it has been proposed that ZP-induced Ca^{2+}-influx in mouse spermatozoa is mediated by LVA channels (9, 17). Not only are LVA currents the only detectable VOCCs in mouse spermatogenic cells, these currents display sensitivities to organic and inorganic antagonists that closely resemble those of the ZP-induced $[Ca^{2+}]_i$ signal in mature spermatozoa (17, 18).

Because of the difficulty of working on human tissues, almost nothing is known about the participation of VOCCs in ZP-induced $[Ca^{2+}]_i$ signaling in human spermatozoa. Furthermore, there are known to be significant differences between the mouse and human in sperm-ZP interaction (69). The data reported here confirm that both α_{1H} and α_{1G} transcripts are present in human male germ cells and that functional channels are formed, consistent with participation of either or both of these channels in ZP-induced Ca^{2+} influx. Interestingly, the pharmacology of NGP-induced AR in human spermatozoa resembles that of α_{1H} (46), a finding that complements the apparent preferential expression of α_{1H} in germ cells (see above). However, in comparison to the mouse, the involvement of LVA currents in the AR of human spermatozoa is far from established. Comparison of the pharmacology of the α_{1H} isoforms reported here with the pharmacology of ZP-activated $[Ca^{2+}]_i$ signaling in human spermatozoa should allow significant pro-

[2] Y. Gu, T. Snow, S. Jagannathan, and S. J. Publicover, unpublished data.

[3] C. Arnoult, unpublished data.

gress in determining whether these subunits contribute significantly to ZP-induced Ca^{2+}-influx and AR in the human. A similar study on mouse male germ cell LVA VOCC subunits may be necessary to establish firmly the identity of the currents detected in immature germ cells.

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