Molecular identification of Ca^{2+} channels in human sperm

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Abbreviations: AR, acrosome reaction; HVA, high voltage-activated; LVA, low voltage-activated; VACCs, voltage-activated Ca^{2+} channels

Abstract

The acrosome reaction is a Ca^{2+} -dependent exocytotic process that is a prerequisite step for fertilization. External calcium entry through voltage-activated Ca^{2+} channels is known to be essential in inducing the acrosome reaction of mammalian spermatozoa. Due to their complex geometry, however, electrophysiological identification of sperm Ca^{2+} channels has been limited. Here we identified Ca^{2+} channel mRNAs expressed in motile human sperm using RT-PCR and their levels were compared using RNase protection assays. L-type, non-L-type, and T-type Ca^{2+} channel mRNAs were detected by RT-PCR using degenerate primers. Cloning and sequencing of the PCR products revealed $\alpha 1\text{B}$, $\alpha 1\text{C}$, $\alpha 1\text{E}$, $\alpha 1\text{G}$, and $\alpha 1\text{H}$ sequences. RT-PCR using specific primers repeatedly detected $\alpha 1\text{B}$, $\alpha 1\text{C}$, $\alpha 1\text{E}$, $\alpha 1\text{G}$, and $\alpha 1\text{H}$ mRNAs, and additionally $\alpha 1\text{I}$ mRNA. But $\alpha 1\text{A}$ and $\alpha 1\text{D}$ messages were not detected. Relative expression levels of the detected Ca^{2+} channel subtypes were compared by RNase protection assays. The abundance of detected mRNA messages was in the following order: $\alpha 1\text{H} \geq \alpha 1\text{G} \geq \alpha 1\text{E} \geq \alpha 1\text{B} > \alpha 1\text{C} > \alpha 1\text{I}$. These findings indicated that human motile sperm express multiple voltage-activated Ca^{2+} channel RNAs among which T-type and non-L-type channel messages are likely to be predominantly expressed. Based on their rel-

ative expression levels, we propose that not only T-type but also non-L-type calcium channels may be major gates for the external calcium influx, required for the acrosome reaction.

Keywords: Ca^{2+} channels; human spermatozoa; RT-PCR; RNase protection assay

Introduction

The acrosome reaction (AR) is an obligatory event happening prior to sperm penetration into eggs. The essential step, a Ca^{2+} -required exocytosis, has been suggested to be coupled with external calcium entry through voltage-activated Ca^{2+} channels (VACCs) (Florman, 1994; Arnoult *et al.*, 1999; Darszon *et al.*, 1999). In the process of sperm contacting eggs, chemical signals such as ZP3 from zona pellucida of eggs were identified to induce increases of pH and membrane depolarization, subsequently coupled with calcium influx through activated VACCs in the sperm membrane (Babcock and Pfeiffer, 1987; Foresta *et al.*, 1993).

Due to their pivotal involvement in the AR, VACCs in mammalian sperm have been extensively explored using pharmacological, electrophysiological, or molecular-biological tools. Some pharmacological studies have suggested that L-type channels were involved in the AR, which was supported by direct detections of $\alpha 1\text{C}$ mRNA messages from human and rodent sperm by RT-PCR (Florman *et al.*, 1992; Goodwin *et al.*, 1997; Goodwin *et al.*, 2000). Non-L-type $\alpha 1\text{A}$ and $\alpha 1\text{E}$ mRNA messages were also amplified from mouse spermatogenic cells by RT-PCR (Liévano *et al.*, 1996). Their expression was confirmed in the protein level using specific antibodies for VACCs (Serrano *et al.*, 1999). More recently, a pharmacological study reported that $\alpha 1\text{B}$ and $\alpha 1\text{E}$ might be main channels for external calcium influx in rodent sperm (Wennemuth *et al.*, 2000).

In contrast to the published reports addressing possible roles of high voltage-activated (HVA) Ca^{2+} channels, direct electrophysiological recordings of Ca^{2+} currents in mouse spermatogenic cells displayed typical low voltage-activated (LVA) T-type Ca^{2+} currents, characterized by low voltage activation, slow deactivation, and fast activation and inactivation kinetics, suggesting that T-type channels might be major Ca^{2+} entry pathways in the spermatogenic cells (Livano *et al.*, 1996; Santi *et al.*, 1996; Arnoult *et al.*, 1998; 1999). Due to their complex geometry, however, electrophysiological characterization of VACCs in mammalian mo-

tile spermatozoa has not been feasible.

Recently Roche developed mibefradil, a novel specific blocker for T-type channels, which has been broadly used for pharmacological identification of T-type channels (McDonough and Bean, 1998; Martin *et al.*, 2000). Several studies have shown that pre-incubation of mibefradil sensitively inhibited the mannose-BSA induced AR of capacitated mammalian spermatozoa in a dose-dependent manner, implying that T-type channels might be mainly involved in AR as gates for Ca^{2+} entry (Brandelli *et al.*, 1996; Blackmore and Eisoldt, 1999; Son *et al.*, 2000).

Recent molecular cloning and expression studies identified at least three genes assigned to T-type Ca^{2+} channels with $\alpha 1\text{G}$, $\alpha 1\text{H}$, and $\alpha 1\text{I}$ isoforms (Perez-Reyes, 2003). PCR primers designed on the basis of the reported T-type channel sequences successfully detected $\alpha 1\text{G}$ and $\alpha 1\text{H}$ mRNAs from human and mouse spermatocytes, suggesting heterogeneity of T-type Ca^{2+} currents in mammalian sperm (Espinosa *et al.*, 1999; Jagannathan *et al.*, 2001).

Compared to the extensive efforts for the identification of VACCs in mouse spermatocytes, VACCs in human sperm have been poorly identified. Up to now, existence of only $\alpha 1\text{C}$, $\alpha 1\text{G}$, and $\alpha 1\text{H}$ mRNA messages in human spermatocytes was reported (Goodwin *et al.*, 2000; Jagannathan *et al.*, 2001). However, their relative abundance has not been compared. In this present investigation, therefore, we made an attempt to identify VACC mRNA subtypes in human spermatozoa and compare their relative expression levels using the combined tools of RT-PCR and RNase protection assays.

Materials and Methods

Isolation of sperm and total RNA

Sperm were repeatedly donated from 3 healthy men among the authors, of which ages are in twenties or thirties. Semen samples observed under a compound microscope displayed normal sperm in density, motility, and morphology, based on the WHO criteria (WHO, 1992). Samples were allowed to liquefy for 30 min at room temperature. The spermatozoa mixture was washed twice by centrifugation (300 g, 5 min) with Ham's F-10 medium. Motile spermatozoa were isolated by a swim-up method (Son *et al.*, 2000). This isolation process was repeated 3 times.

Total RNA was isolated from the human spermatozoa using the guanidinium thiocyanate-phenol extraction (Chomczynski and Sacchi, 1987; Noh *et al.*, 2001), which was repeated at least 3 times to improve the quality of sperm RNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

First strand cDNA was synthesized from 0.5 μg of human sperm RNA or human brain RNA (Clontech, Palo Alto, CA) using AMV reverse transcriptase (Takara, Kyoto, Japan) (Hwang *et al.*, 2002). Degenerative and specific PCR primers used to amplify L-type, non-L-type, and T-type Ca^{2+} channel $\alpha 1$ subunits are listed in Table 1 (Vajna *et al.*, 1998). Each PCR reaction was carried out in a final volume of 50 μl having 200 μM dNTP, 50 pM forward and reverse primers, 1.5 mM MgCl_2 , and 2.5 U of Taq polymerase (Takara, Kyoto, Japan).

The PCR reaction was first denatured at 94°C for 1 min, and then performed 35 cycles consisting of denaturation at 94°C for 30 s, annealing for 30 s at different temperatures depending on primer pairs (refer to Table 1), and extension at 72°C . RT-PCR products amplified by degenerative primers were cloned in pGEM-T vector (Promega, Madison, WI) and transformed into competent cells. Ten PCR products cloned were sequenced and compared to the reported Ca^{2+} channel sequences.

Ribonuclease protection assays

A cRNA synthesis kit and a ribonuclease protection assay kit were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The anti-sense RNA synthesis and RNase protection assays were performed according to the protocols supplied by the manufacturer. Briefly, anti-sense RNA probes of human Ca^{2+} channel subtypes were synthesized based on 1 μg of the vectors containing Ca^{2+} channel PCR products using T3 or SP6 RNA polymerase. The information including vectors, directions of cDNA inserts, restriction enzyme sites used for linearization, and sizes of riboprobes were summarized in Figure 3. Some of Ca^{2+} channel cDNA were subcloned into pEZ-T vector (RNA, Seoul, Korea) to utilize proper restriction enzyme sites. The synthesized RNA probes were separated through a 10% polyacrylamide gel and estimated probe sizes were purified. Specific activities of the probes were greater than 1.0×10^9 dpm/g and varied less than 10% between subtype-specific probes. Three μg of total human sperm RNA was hybridized to the ^{32}P -labeled anti-sense riboprobe of 2×10^5 cpm for 10 h at 50°C in the hybridization buffer. The hybridization was digested by RNase A and RNase T1 at 30°C for 30 min. The protected products from the digestion reactions were resolved on 10% polyacrylamide gels, which were exposed to X-ray films. The portions corresponding to bands detected on X-ray films were cut out and their radioactivities were counted as cpm using a scintillation counter.

Table 1. Primer sets used for RT-PCR. The primers to detect L-, non-L-, and T-type channels were degenerately designed on the basis of the amino acid sequences written in the parentheses. The conserved sequences for L-, non-L-, and T-type channel are from Domain III S6 and IV S6, Domain I S3 and I pore, and Domain III S6 and IV S3 regions, respectively. Some of the specific primer sets were adapted or modified from a previous report (Vajna *et al.*, 1998). Elongation times for L-type and α 1G sequences were 60 s, while elongation times for the other channel sequences were 30 s. The abbreviations used represent as follows: AT, annealing temperature; F, forward primer; R, reverse primer; S, C/G; R, A/G; Y, C/T; D, A/G/T; H, A/T/C; N, A/C/G/T.

Subunit	Primer location (nt)	Nucleotide sequence	AT (°C)	Size (bp)	GenBank accession number
L-type (CaV1)	F (FFMMNIFV) R (FISFYMLC)	TTYTTYATGATGAAYATHTTYGT ANAGCATRTARAARCTDATRAA	55	945 for α 1S 927 for α 1C 933 for α 1D	
Non-L-type (CaV2)	F (GWNVMDF) R (TMEGWTD)	GGNTGGAAYGTNATGGAYTT TCNGTCCANCCYTCCATNGT	52	461 for α 1B 464 for α 1A 464 for α 1E	
T-type (CaV3)	F (GVVENF) R (INPTII)	GGCGTSGTSGTSGAGAACTT GATGATGGTGGGRTTGAT	57	488 for α 1H 524 for α 1G 470 for α 1I	
α 1S (CaV1.1)	F 3,385-3,408 R 4,327-4,306	TTCTTCATGATGAACATCTTTGT AGAGCATGTAGAAGCTGATGAA	65	1,043	NM000069
α 1C (CaV1.2)	F 2,874-2,894 R 3,385-362	GCCGCGCCGAGTCAAGTCTA GCTCCTCCGGGTCTGCATCTCATC	65	512	L04569
α 1D (CaV1.3)	F 1,137-1,158 R 1,737-1,717	CGAACGGAGGCATCACCAACTT ACGGCGGCCCTACATCTTCTG	65	601	M83566
α 1F (CaV1.4)	F 3,405-428 R 4,313-4,290	TTCTTCATGATGAACATCTTCGT AGAGCATGAAGAAGCTGATGAA	65	809	NM005183
α 1A (CaV2.1)	F 4,672-4,691 R 4,983-4,961	TCCTCAAGCATTCGGTGGAC TGCAGGCCCTCTCATTTTTT	65	461	M94172
α 1B (CaV2.2)	F 638-657 R 1,098-1,079	GGCTGGAACGTGATGGACTT TCAGTCCAGCCCTCCATGGT	65	461	M94172
α 1E (CaV2.3)	F 106-124 R 411-385	GCGGCCGCCTACAAGCAGA AAAGCAAAAGATCCCAATGAAATAAGG	60	306	L27745
α 1G (CaV3.1)	F 4,234-4,256 R 5,124-5,107	ATCGGCAACATTGTAGTCATCTG GATGATGGTGGGGTTGAT	57	891	NM018896
α 1H (CaV3.2)	F 4,733-4,752 R 5,221-5,204	GGCGTCGTGGTTCGAGAACTT GATGATGGTGGGGTTGAT	57	489	AF051946
α 1I (CaV3.3)	F 4,101-4,121 R 4,647-4,630	CCACAACCCCTGGATGCTGCT GATGATGGTGGGATTGAT	57	547	NM021096
GAPDH	F 56-79 R 339-362	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	60	307	BC026907

Results

Numerous studies have demonstrated that Ca²⁺ influx through VACCs is essential for the exocytotic events of mammalian acrosome reaction. However, it is not

still clear what VACC subtypes are expressed in mature human spermatozoa. In this report, we identified VACC mRNA messages in motile human sperm and compared their relative expression levels.

We first examined whether or not VACC channel

subfamilies (L-, non-L-, and T-type) are expressed in human spermatozoa. Three pairs of degenerate PCR primers (Table 1) were designed on the basis of consensus amino acid sequences found in the VACC subfamilies. RT-PCR using the degenerate primers amplified the PCR products from not only human sperm but also brain RNA. Their sizes were similar to the estimated ones of L-type, non-L-type, and T-type Ca^{2+} channel signals. The band intensities amplified from sperm RNA were generally weaker than those from brain RNA (Figure 1). These findings indicated that human motile sperm express multiple types of VACC mRNA messages including L-, non-L-, and T-type calcium channels. Interestingly, T-type channel products amplified from the human sperm RNA were separated into two bands around 500 base pairs on an agarose gel (Figure 1C), suggesting that at least two subtypes or splice variants of T-type channels messages were expressed in the spermatozoa. The detected patterns by RT-PCR were similar between total RNAs isolated from different semen samples.

The amplified PCR products were purified and cloned into pGEM-T vectors for further analysis. Ten PCR products per each Ca^{2+} channel subfamily were sequenced and compared to previously reported Ca^{2+} channel sequences for identification. According to nucleotide similarity, the PCR products were identified as follows: all of the 10 L-type channel products sequenced were identical to the human $\alpha 1\text{C}$ (L04569); among the 10 non-L-type channel PCR products, 7 sequences were identical to the $\alpha 1\text{B}$ (M94172) and the other 3 were the human $\alpha 1\text{E}$ sequence (L27745); among the 10 T-type channel PCR products, 6 sequences were the human $\alpha 1\text{H}$ (Genbank access no, AF051946) and the other 4 were the $\alpha 1\text{G}$ (AF190860).

The two bands representing T-type signals in Figure 1C turned out that the bigger band is $\alpha 1\text{G}$ cDNA (524 bp) and the smaller one is $\alpha 1\text{H}$ cDNA (488 bp). Among them, any splice variant was not detected, suggesting that the detected $\alpha 1\text{G}$ and $\alpha 1\text{H}$ sequences are predominant forms expressed in human spermatozoa. Together, these RT-PCR data indicate that motile human spermatozoa express at least 5 subtypes of VACC $\alpha 1$ subunits, including L-type $\alpha 1\text{C}$, non-L-type $\alpha 1\text{B}$ and $\alpha 1\text{E}$, and T-type $\alpha 1\text{G}$ and $\alpha 1\text{H}$ Ca^{2+} channels.

Expression of VACC mRNAs in human spermatozoa was re-examined by RT-PCR using the 10 pairs of specific primers (Table 1). The PCR products were separated on 1% agarose gels (Figure 2). Among L-type channel signals, only $\alpha 1\text{C}$ specific band appeared on the gel (Figure 2A), and its expression level in the spermatozoa was lower than in brain. On the contrary, $\alpha 1\text{D}$ messages were not detected in human spermatozoa, but in human brain (Figure 2B). The other 2 L-type channel messages (retinal $\alpha 1\text{F}$ and skeletal $\alpha 1\text{S}$) were detected from neither sperm nor brain RNA (data not shown).

Among the three non-L-type channel members, $\alpha 1\text{B}$ and $\alpha 1\text{E}$ signals were amplified from human sperm RNA, but the $\alpha 1\text{A}$ signals were not (Figure 2C-E). Comparatively, all the three subtype mRNAs of the non-L-type subfamily were apparently detected from human brain RNA, as previously reported (Perez-Reyes and Schneider, 1994).

Among the three T-type channel members, not only $\alpha 1\text{G}$ and $\alpha 1\text{H}$ but also $\alpha 1\text{I}$ signals were detected as bands on the gels (Figure 2F-H). Comparison of the band intensities corresponding to the three T-type channel messages implied that $\alpha 1\text{G}$ and $\alpha 1\text{H}$ RNAs

Figure 1. Ca^{2+} channel subfamilies in human spermatozoa. RT-PCR was performed using degenerate primers, which were designed to specifically amplify VACC subfamilies (Table 1). RT-PCR products for L-type, non-L-type, and T-type Ca^{2+} channel transcripts were separated on 1% agarose gels with size markers. The band sizes on the gels correspond to the calculated ones of L-type, non-L-type, and T-type Ca^{2+} channels (about 930, 460, and 500 bp, respectively; refer to Table 1). Subsequent cloning and sequencing revealed that $\alpha 1\text{C}$, $\alpha 1\text{B}$, $\alpha 1\text{E}$, $\alpha 1\text{G}$, and $\alpha 1\text{H}$ cDNAs were amplified from human sperm mRNA. The abbreviations represent as follows: M, DNA size marker; -, negative control (without reverse transcriptase); S, human sperm RNA; B, human brain RNA. The marked arrows ($\leftarrow$) and numbers indicate detected PCR products and their expected sizes.

Figure 2. Human sperm Ca²⁺ channel subtypes detected by RT-PCR. The expression of $\alpha 1C$, $\alpha 1D$, $\alpha 1A$, $\alpha 1B$, $\alpha 1E$, $\alpha 1G$, $\alpha 1H$, and $\alpha 1I$ transcripts in human brain and sperm RNA was examined by RT-PCR using the specifically designed PCR primer pairs (Table 1), some of which were modified from a previous report (Vajna *et al.*, 1998). PCR products were separated on 1% agarose gels with size markers. Except $\alpha 1A$ and $\alpha 1D$ (B, C), 6 subtype transcripts were appeared as detectable bands on the gels (A, D-H). However, $\alpha 1F$ and $\alpha 1S$ messages were detected from neither brain nor sperm RNA (data not shown). The abbreviations and symbols used are same to the above.

were predominantly expressed, while $\alpha 1I$ RNA was weakly in human mature sperm. A series of RT-PCR utilizing the specific primers repeated the same results obtained using the degenerate primers and additionally found $\alpha 1I$ signals. Also, similar expression patterns were detected by RT-PCR between sperm samples (data not shown). The repeated findings supports that LVA and HVA VACC subtypes are expressed in human spermatozoa.

Due to the limitation of RT-PCR to evaluate relative abundance of mRNA messages, expression levels of the Ca²⁺ channel mRNAs in the human spermatozoa were compared using RNase protection assays for which the PCR products cloned were used as substrates to *in vitro* transcribe anti-sense probes. The synthesized anti-sense probes and protected signals were separated on a gel and exposed to an X-ray film. Protected signals of VACCs appeared to be bands slightly smaller than synthesized antisense probes (Figure 4A). $\alpha 1H$, $\alpha 1G$, $\alpha 1E$, and $\alpha 1B$ messages were detected as predominant messages, whereas $\alpha 1C$ and $\alpha 1I$ were detected as weak messages. Their relative abundance was evaluated by comparing the radioactivities of protected messages. The normalized protected messages were abundant in the

following order: $\alpha 1H \geq \alpha 1G \geq \alpha 1E \geq \alpha 1B > \alpha 1C > \alpha 1I$ (Figure 4B). These results suggested that T-type Ca²⁺ channels and non-L-type channels ($\alpha 1G$, $\alpha 1H$, $\alpha 1B$ and $\alpha 1E$) might be major gates through which calcium influx is essential for inducing the acrosome reaction of human spermatozoa.

Discussion

In the present study, we demonstrated that at least six subtypes of VACC transcripts pertaining to LVA and HVA Ca²⁺ channels were expressed in the human spermatozoa. Their expression levels were detected as follows: $\alpha 1H \geq \alpha 1G \geq \alpha 1E \geq \alpha 1B > \alpha 1C > \alpha 1I$. These results suggested that both subfamilies of LVA and HVA Ca²⁺ channels might play pivotal roles as the gates for the external Ca²⁺ entry. However, it should be further investigated whether the detected levels of these mRNA messages are similar to those of Ca²⁺ channel proteins in sperm membrane.

Some previous papers reported that T-type channels were identified to be the major conductors inducing the AR (Brandelli *et al.*, 1996; Blackmore and Eisoldt, 1999; Son *et al.*, 2000), whereas other re-

Figure 3. Diagrams of $\alpha 1B$, $\alpha 1C$, $\alpha 1E$, $\alpha 1G$, $\alpha 1H$, and $\alpha 1I$ cDNAs in vectors. GAPDH cDNA cloned by RT-PCR was also schematically diagramed in the bottom. Inserted cDNA names and directions (5' to 3') were marked on the maps according to Ca^{2+} channel $\alpha 1$ cDNAs, which were cloned by RT-PCR (Figures 1 and 2). Each isoform specific antisense cRNA probe was synthesized using T3 or SP6 RNA polymerase according to the inserted direction of VACC isoform cDNA after linearizing by marked restriction enzyme. Synthesized probes were used for RNase protection assays (Figure 4). Synthesized and protected probe sizes were calculated and marked. To synthesize antisense probe of $\alpha 1B$ cDNA, for example, the 5' end was cut by Sac II, and then $\alpha 1B$ riboprobe was synthesized based on the linearized cDNA using SP6 RNA polymerase. Expected sizes of $\alpha 1B$ riboprobe before and after RNase digestion are 553 and 461 bases, which were marked below the cDNA map.

ports did emphasize the potent contribution of calcium entry through HVA calcium channels (Goodwin *et al.*, 2000; Wennemuth *et al.*, 2000). These contradictory findings might be compromised by our detection of

Figure 4. Relative expression levels of Ca^{2+} channel $\alpha 1$ subtype messages examined by RNase protection assays. Each riboprobe was synthesized based on the Ca^{2+} channel cDNA cloned by RT-PCR. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen and its cDNA was also cloned by RT-PCR (Wong *et al.*, 1994). The ^{32}P labeled probes were hybridized with 3 μ g of human sperm RNA and then the reactions were digested with RNase T1 and RNase A for 30 min. The detected level of GAPDH was used for normalization of Ca^{2+} channel mRNA signals protected from RNase protection assays. The synthesized and protected probes were separated on 10% polyacrylamide gels, which were exposed to X-ray films at $-80^{\circ}C$ for 2 days in an intensifying cassette. (A) A representative autoradiogram obtained from by an RNase protection assay. From the left, synthesized and protected probes of $\alpha 1B$, $\alpha 1C$, $\alpha 1E$, $\alpha 1G$, $\alpha 1H$, and $\alpha 1I$ Ca^{2+} channels were displayed with an internal control (GAPDH). For each Ca^{2+} channel subtype marked on the top, synthesized Ca^{2+} channel and GAPDH riboprobes were showed in the left lane and their protected probes were in the right lane. The sizes of protected signals were slightly smaller than those of synthesized probes due to digestion of non-specific sequences such as polylinker portions. The probe sizes before and after RNase digestion correspond to the expected values in Figure 3. (B) Relative expression levels of Ca^{2+} channel $\alpha 1$ mRNAs. The gel portions corresponding to the protected signals on an X-ray film were cut and their radioactivities (cpm) were counted using a scintillation counter. The cpm values were normalized to percentages and their relative values were plotted as bar graphs. The marked asterisks (*) indicates Ca^{2+} channel $\alpha 1$ probes protected from RNase digestion.

LVA ($\alpha 1G$, $\alpha 1H$, and $\alpha 1I$) and HVA ($\alpha 1B$, $\alpha 1C$, and $\alpha 1E$) VACC subtypes in human spermatozoa. Thus, calcium influx through the multiple VACCs might cooperatively participate in inducing the AR.

Among the multiple Ca^{2+} conducts associated with

the AR, T-type Ca²⁺ channels seem to play an essential role in the early phase of the sequential events triggering the AR (Florman *et al.*, 1992; Darzon *et al.*, 1999). Due to their low-voltage thresholds for activation, T-type channels may be activated by the initial depolarization occurred by the ZP3-mediated cation influx. The Ca⁺ influx via the T-type channels leads to a further depolarization that subsequently activates HVA Ca²⁺ channels in the mammalian sperm. The initial Ca²⁺ entry through LVA Ca²⁺ channels might diminish in a short time due to the fast inactivating properties of the LVA T-type channels responding to the continual depolarization, whereas the subsequently activated HVA channels might contribute to increasing and maintaining a critical Ca²⁺ concentration in the sperm cytoplasm. Thus, the crucial roles of T-type channels in the AR might originate from playing as not only the calcium entry gates in the early phase, but also the mediators stimulating the HVA Ca²⁺ channels ($\alpha 1B$, $\alpha 1E$, and $\alpha 1C$), which were detected in this study.

Wennemuth and his colleagues did not see the potent contribution of T-type channels in Ca²⁺ increment pathways elicited by application of high K⁺ solutions in mouse spermatozoa (Wennemuth *et al.*, 2000). One of the possible reasons that they did not detect the involvement of T-type channels was that their depolarization method was not by ZP3 treatment, but by switching the external solution with the high K⁺ one. The latter method is easy to depolarize the mouse sperm, but the fast depolarization to around 0 mV by the high K⁺ solution would inactivate T-type channels very quickly, thereby T-type channels absorbed into an inactivated state being not available at the condition (Arnoult *et al.*, 1999).

Taken together, we report here that human motile sperm expressed multiple Ca²⁺ channel subtypes that might play pivotal roles as the gates for the external Ca²⁺ entry. Due to their distinctive activation ranges, the possible contributions of the LVA and HVA Ca²⁺ channels to the AR might be temporarily distinguished, but sequentially connected. Combined with the slow processes including IP₃-mediated Ca²⁺ release and store-operated Ca²⁺ influx (Walensky and Snyder, 1995; O'Toole *et al.*, 2000; Ren *et al.*, 2001; Rossato *et al.*, 2001), the Ca²⁺ entry through the LVA and HVA Ca²⁺ channels might be genuine processes to guarantee the AR.

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