Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception*

Leslie O. Goodwin1,5, Nina B. Leeds1, Ian Hurley2, Francine S. Mandel1,3, Robert G. Pergolizzi1 and Susan Benoff4

1Department of Research, North Shore University Hospital, New York University School of Medicine, Manhasset, New York, 2Department of Obstetrics and Gynecology, North Shore University Hospital, New York University School of Medicine, 3Department of Public Health, Cornell University Medical College, and 4Departments of Obstetrics and Gynecology and Cell Biology, New York University School of Medicine, Manahasset, New York, USA

5To whom correspondence should be addressed

Therapeutic administration of calcium channel-blocking medications has been correlated with reduced mannose receptor expression and iatrogenic human male infertility. In this report, we investigate whether the pharmacological activity of dihydropyridines, which block calcium influx through voltage-dependent calcium channels, contributes to the production of an infertile state. An influx of extracellular calcium is an absolute requirement for the initiation of a progesterone-stimulated acrosome reaction by human spermatozoa. To determine whether dihydropyridines could inhibit progesterone-induced acrosome loss, we have studied a protein expressed in rat and human spermatozoa which is related both antigenically and by cDNA sequence to the α1 subunit of the rat cardiac muscle voltage-dependent calcium channel, which forms the pore of the channel. Using reverse transcription–polymerase chain reaction, we have isolated a 2169 base clone from rat testis mRNA whose sequence was largely identical to that of the α1 subunit of the rat cardiac muscle calcium channel, but had an 84 base change, attributable to splicing and alternate exon usage. This change inserts a peptide cassette encoding an amphipathic membrane-spanning helix that constitutes part of the ionic pore of the skeletal muscle calcium channel regulating the kinetics of activation of the calcium channel and may serve as an intramembrane dihydropyridine binding site. In parallel, human spermatozoa from fertile donors were exposed to nifedipine in vitro. Nifedipine inhibited progesterone-stimulated calcium influx and subsequent acrosome reactions in human spermatozoa at concentrations effective in excitable cells, but required a prolonged time to do so. In contrast, progesterone ligand binding was unaffected by nifedipine treatment. These data demonstrate that human spermatozoa express an L-type calcium channel which is responsive to nifedipine. Assuming sperm calcium transport pathways are highly conserved, the slow kinetics by which the blockade of the human sperm channel was obtained can be correlated with alterations in channel activation and conductance associated with isoform diversity generated by alternate splicing as observed in the rat. These data provide unequivocal evidence for the presence of functional L-type voltage-dependent calcium channels in rat and human spermatozoa. The data also define an altered binding site for calcium entry antagonists in this channel and offer a unique target for the design of new male contraceptive agents.

Key words: acrosome reaction/calcium channel/calcium influx/nifedipine/progesterone receptor

Introduction

Agonist-stimulated calcium (Ca2+) influx initiates the physiological acrosome reaction (Thomas and Meizel, 1988), a secretory event required in order for zona-bound spermatozoa to penetrate through the zona pellucida (e.g. Liu and Baker, 1994a,b; Benoff et al., 1996). Progesterone secreted by the cumulus matrix is one agonist commonly employed to study such Ca2+ influx (Oehninger et al., 1994; Krausz et al., 1995). At least two transporters are postulated to sequentially regulate progesterone-dependent elevations in sperm intracellular Ca2+ concentrations (Florman, 1994; Roldan et al., 1994; Benoff et al., 1995b). The first has the properties of a poorly selective cation channel which is not voltage regulated. Activation of this channel mediates small, transient elevations in intracellular Ca2+ content which are limited to the sperm head. The second transporter mediates sustained elevations in intracellular Ca2+ throughout the spermatozoon. This pathway is activated by depolarization and inhibited by dihydropyridines (DHPs; Florman et al., 1992). DHPs inhibit Ca2+ entry via the L-subtype (long-lasting, large current) high voltage-dependent Ca2+ channel (VDCC; Vaghya et al., 1987). These observations suggest that the second transporter is an L-like Ca2+ channel. This suggestion receives further support from studies on the effects of nifedipine, the prototype DHP (Vaghya et al., 1987),
on progesterone-stimulated diacylglycerol (DAG) production (O’Toole et al., 1995). DAG formation is a late event in the pathway leading to acrosome exocytosis, occurring after entry of Ca\(^{2+}\) (Roldan et al., 1994). Nifedipine significantly inhibits both DAG accumulation and agonist-stimulated acrosome loss. Thus, it is concluded that L-type VDCCs are active in mammalian spermatozoa. It is possible that these L-type VDCCs in the sperm membrane could serve as targets for the development of new, non-steroidal male contraceptive protocols (Benoff et al., 1994, 1995a; Hershlag et al., 1994, 1995).

VDCCs are present in a wide variety of tissues (review, Tsien et al., 1991). Biochemical and molecular biological studies have demonstrated that the L-type VDCC in cardiac muscle is comprised of at least three subunits: \(\alpha_1\), \(\alpha_2\), and \(\beta\) (Wei et al., 1991). In skeletal muscle, the L-type VDCC is formed by a complex of five proteins: \(\alpha_1\), \(\alpha_2\), \(\beta\), \(\delta\), and \(\gamma\) (Catterall, 1988; Tsien et al., 1991; Wei et al., 1991). These polypeptides are encoded by four separate genes; \(\alpha_2\) and \(\delta\) are produced from alternative transcripts of the same gene (Catterall, 1988; Wei et al., 1991; Tsien et al., 1991). The \(\alpha_1\) subunit is the best characterized component of all L-type VDCCs. Analysis of the \(\alpha_1\) protein sequence indicates that the molecule contains four repetitive regions, each comprised of six putative transmembrane segments responsible for forming the gating channel (Tanabe et al., 1987). Transfection studies have shown that the non-glycosylated \(\alpha_1\) subunit is necessary and sufficient for VDCC function (Catterall, 1988; Mikami et al., 1989), as well as for Ca\(^{2+}\) influx inhibition by DHPs (Kim et al., 1990). A considerable body of evidence developed from kinetic and X-ray diffraction analyses now indicates that VDCC antagonists are highly lipophilic, that these drugs must partition into the membrane prior to receptor binding, and that cholesterol modulates their interaction with cellular membranes (Rhodes et al., 1985; Herbette et al., 1989; Langs et al., 1990; Mason et al., 1991, 1992; Mason, 1993). These data are taken to indicate that a DHP receptor (DHPR) site of the \(\alpha_1\) subunit is in a hydrophobic environment, e.g. within the plane of the membrane lipid bilayer (Catterall and Striessnig, 1992). Receptor site mapping with site-directed anti-peptide antibodies supports this conclusion (Nakayama et al., 1991; Striessnig et al., 1991).

The primary structure of the Ca\(^{2+}\) channel expressed in mammalian spermatozoa remains to be defined. Physiological studies indicate that this channel differs from the classic L-type VDCC of somatic tissues in three important aspects. First, the relative strength of inhibition of Ca\(^{2+}\) influx by transition metal ions, e.g. nickel, cadmium, cobalt and manganese, is the reverse of that observed with typical L-type VDCCs (Florman et al., 1992; Florman, 1994). Second, the threshold sensitivity of this channel to the inhibitory effects of DHPs is lower than that of other L-type VDCCs (Florman, 1994). Third, while cholesterol enrichment activates otherwise silent DHP-sensitive VDCCs in muscle, this treatment has no effect on the activity of Ca\(^{2+}\) channels in spermatozoa (Gleason et al., 1991; Benoff et al., 1994). These data indicate that the Ca\(^{2+}\) channel specific to mammalian spermatozoa also shares characteristics compatible with those of T-type (low voltage-activated) Ca\(^{2+}\) channels (Akaike et al., 1989; Hille, 1992). Thus, the testis-specific Ca\(^{2+}\) channel has been indirectly defined as ‘L-like’, not L-type (Florman, 1994).

Lack of knowledge of the primary structure of the VDCC expressed in mammalian spermatozoa has clearly hampered the dissection of the mechanism underlying the effects of DHPs and other Ca\(^{2+}\) entry antagonists on male fecundity. Although in-vivo drug administration results in a dose-dependent, partial arrest of spermatogenesis in guinea pigs (Juneja et al., 1990), human semen parameters are unaffected by such pharmacological therapy (Benoff et al., 1994). In addition, based on studies wherein treatment of animal spermatozoa or capacitated human spermatozoa with Ca\(^{2+}\) channel blockers failed to inhibit agonist-stimulated Ca\(^{2+}\) influx and actually stimulated acrosome loss, other investigators have argued that entry of extracellular Ca\(^{2+}\) into spermatozoa is not meditated by VDCCs (Roldan et al., 1986, 1987; Fraser and McIntyre, 1989; Juneja et al., 1990). In contrast, in a clinical setting, we have reported that therapeutic administration of Ca\(^{2+}\) channel blockers for control of hypertension and migraine headache produces an infertile state in men with normozoospermic semen parameters, which is reversible by drug withdrawal as the only intervention (Benoff et al., 1994, 1995a; Hershlag et al., 1995, 1996). Spermatozoa from these infertile men are refractory to the effects of model zona ligands to induce Ca\(^{2+}\) influx and acrosome exocytosis (Benoff et al., 1994; Hershlag et al., 1995). Such data suggest that a pharmacological blockade of Ca\(^{2+}\) channels in the sperm membrane contributes to the production of iatrogenic infertility and is in significant contrast to the findings from animal studies.

Progesterone serves as a physiological agonist of extracellular Ca\(^{2+}\) influx and DAG accumulation in both human and animal spermatozoa (Thomas and Meizel, 1988; Blackmore et al., 1991; Melendrez et al., 1994; Roldan et al., 1994; O’Toole et al., 1995), suggesting that sperm Ca\(^{2+}\) transport pathways are conserved among different species. Therefore, in the current report, to resolve the controversy concerning whether blockade of agonist-stimulated Ca\(^{2+}\) entry by DHP analogues is required in order to achieve the infertile state, we have: (i) defined the primary structure of the Ca\(^{2+}\) channel expressed in rat testis; and (ii) correlated the effects of changes in this sequence specific to the rat male germ line with the effects of nifedipine on progesterone-stimulated Ca\(^{2+}\) influx and acrosome loss by motile human spermatozoa from fertile donors.

**Materials and methods**

**Products and reagents**

All polymerase chain reaction (PCR) reagents were purchased from Perkin-Elmer (Foster City, CA, USA). Dulbecco’s phosphate-buffered saline (DPBS) was purchased from Gibco Laboratories (Grand Island, NY, USA), and modified Ham’s F-10 medium was purchased from Irvine Scientific (Santa Ana, CA, USA). Unless otherwise noted, all other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) and all other enzymes were obtained from New England Biolabs (Beverly, MA, USA).
Human semen specimens

All protocols employing human subjects were reviewed and approved by the Institutional Review Board of North Shore University Hospital, New York, USA. Semen specimens from unidentified in-vitro fertilization (IVF) fertile donors, collected by masturbation after 2–3 days of abstinence for routine semen analysis, were obtained at the point of discard, for which informed consent was not required. Only fresh specimens with the following parameters were used in these studies: >20×10⁶ spermatozoa/ml, >40% motility, and at least 30% normal oval head forms with 50% or more of the head covered by the acrosome.

Isolation of rat tissue RNA

Total RNA was isolated from various rat tissues (Sprague–Dawley males aged 6 months, weighing 300–400 g) using a modified protocol of Chomczynski and Sacchi (1987). Briefly, 2 µg of total RNA was homogenized to completion in a Tissumiser (Greiner Scientific, New York, NY, USA) in 20 ml of buffer D (4.5 M guanidinium isothiocyanate, 50 mM sodium phosphate pH 7.0, 0.5% w/v lauryl sarcosine, 10 mM EDTA and 1% v/v 2-mercaptoethanol). Sodium acetate (2 M pH 4.0; 2 ml), phenol (20 ml), and chloroform (4 ml) were sequentially added and the mixture inverted after each addition. After sitting on ice for 10 min, the homogenate was centrifuged at 1500 g for 20 min at 4°C. The aqueous phase was then extracted once with an equal volume of a mix of phenol and chloroform (1:1) and centrifuged at 1500 g for 20 min at 4°C. The aqueous phase was extracted again with an equal volume of chloroform and centrifuged at 1500 g for 20 min at 4°C. The aqueous phase was separated and precipitated with an equal volume of isopropanol. The precipitate was stored at −20°C for at least 1 h and then collected by centrifugation at 12 000 g for 30 min at 4°C. The resulting pellet was washed with 70% EtOH, centrifuged at 12 000 g for 30 min at 4°C, briefly air dried and resuspended in RNase-free water (500 µl). The purity and percentage recovery of the RNA was determined spectrophotometrically.

First strand synthesis

First strand cDNA was synthesized according to the manufacturer’s instructions using a reverse transcription system kit (Promega, Madison, WI, USA). Briefly, 2 µg of total RNA was used per reaction in a total volume of 40 µl containing 5 mM MgCl₂, 1× reverse transcription buffer (10 mM Tris–HCl, 50 mM KCl and 0.1% Triton X-100), 1 mM each dNTP, 40 IU rNasIn ribonuclease inhibitor, 30 IU of avian myeloblastosis virus (AMV) reverse transcriptase and 1 µg of oligo (dT)₁₅ primer. The reaction was incubated at 42°C for 60 min and a control vector containing an insert coding for kanomycin resistance gene was transcribed in parallel as a positive control.

PCR primers

A diagram depicting the proposed structure of the prototype α1 subunit of the L-type VDCC is shown in Figure 1 (Tsiens et al., 1991; Soldatov, 1994). To amplify and clone homologous sequences found in rat testis total RNA, six sets of paired (F = forward and R = reverse) PCR primers were generated from the published sequence of the cDNA encoding the α1 subunit from rat cardiac muscle (Koch et al., 1990; Accession Nos. M59786, M34364). Oligonucleotide primers were synthesized on an Applied Biosystems Model 393 DNA Synthesizer (Foster City, CA, USA). Primers used in PCR reactions are as follows:

- RACH 2081F 5’GGAGGACACCTCGTGAGGAGCGAGAT
- RACH 3048F 5’CAGGCACACCCTCTTCAAGAACCACATTC
- RACH 3908F 5’GTGGTACCTGGTCACCTACCACTACT
- RACH 3936R 5’AGTAGGTTGAGTTGACGCCACGTACCAC

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RACH 4777R 5’CAGATTCTCTTGAAATCCATCAGGTTGA
RACH 5801F 5’GAAAGATTTAGATCTGACATTGAGGCC
RACH 6077R 5’GGCTTGCACTGGCAATGCTGGTATG
RACH 6695R 5’CAGGGTGCTGACATTAGGACCTGGCTGTC

Generation of double-stranded cDNA

The above primers were used in PCR reactions with 50–100 ng testsis (or other tissue where applicable) first strand cDNA in a 100 µl reaction containing 1× buffer (10 mM Tris–HCl pH 8.3 and 50 mM KCl), optimized for MgCl₂ (2–6 mM), 200 µM dNTPs, 5 IU Taq polymerase and 50 pM each of the forward and reverse primers. The PCR programme reaction conditions were 94°C for 30 s, 68°C for 4 min for 40 cycles in a Thermocycler model 9600 (Perkin-Elmer, Foster City, CA, USA).

Generation of PCR product and direct sequencing

An 869 bp PCR product was generated from rat testis and heart using primers RACH 3908F and RACH 4777R. This product was subjected to direct sequencing to determine the primary structure. PCR conditions were 94°C for 30 s, 68°C for 2 min for 40 cycles in a Thermocycler model 9600 (Perkin-Elmer).

Cloning of PCR product

The PCR product was visualized by ethidium bromide staining of a 1% agarose gel and gel purified using Wizard PCR Preps (Promega, Madison, WI, USA) following the protocols for fluorescence-based DNA sequencing with Taq polymerase provided by the manufacturer. All sequencing primers used to sequence pRACH62 initiated at the nucleotide base indicated in Figure 4 and were 18 nucleotides in length. Partial sequences were compiled and aligned with cardiac muscle L-type VDCC sequences (Koch et al., 1990) as well as other Ca²⁺ channel sequences (Tsiens et al., 1991; Tanabe et al., 1987; Koch et al., 1989; Mikami et al., 1989; Soong et al., 1993) with MacVector 5.0 Program (Kodak, New Haven, CT, USA). Secondary structure predictions were determined using General Protein Mass Analysis for Windows, Version 2.13a (Lighthouse data, Odense SV, Denmark).

DNA sequencing

Selected clones were sequenced using automated DNA Sequencing System Model 373A (Applied Biosystems DNA Sequencer, Foster City, CA, USA) following the protocols for fluorescence-based DNA sequencing with Taq polymerase provided by the manufacturer. All sequencing primers used to sequence pRACH62 initiated at the nucleotide base indicated in Figure 4 and were 18 nucleotides in length. Partial sequences were compiled and aligned with cardiac muscle L-type VDCC sequence (Koch et al., 1990) as well as other Ca²⁺ channel sequences (Tsiens et al., 1991; Tanabe et al., 1987; Koch et al., 1989; Mikami et al., 1989; Soong et al., 1993) with MacVector 5.0 Program (Kodak, New Haven, CT, USA). Secondary structure predictions were determined using General Protein Mass Analysis for Windows, Version 2.13a (Lighthouse data, Odense SV, Denmark).

Preparation of human semen for experimental studies

Spermatozoa were selected for motility by a ‘swim-up’ method as previously described (Bronson et al., 1987). Untreated (‘fresh’ or uncapacitated) specimens were prepared for analysis by centrifugation (500 g for 8 min) to concentrate spermatozoa. To induce capacitation, the spermatozoa were pelleted and resuspended at a density of 12×10⁶ cells/ml in Ham’s F-10 containing 30 mg/ml charcoal-delipidated (Chen, 1967) human serum albumin (HSA). The cells were subsequently incubated for 1 or 2 days at room temperature. Under these
The surfaces of motile spermatozoa were labelled with 100 µg/ml tetraconjugated BSA and differentiated by reaction with 100 mg/ml tetraconjugated BSA. In-vitro exposure of human spermatozoa to nifedipine was performed as described for Man-FITC-BSA.

Preparation of rat spermatozoa for experimental studies
Spermatozoa were collected by aspiration from the vas cauda junction of 6 month old Sprague–Dawley male rats. The spermatozoa were washed three times in DPBS, centrifuged at 500 g and immediately prepared for immunocytochemical staining.

In-vitro exposure of human spermatozoa to nifedipine
The examination of the effects of nifedipine on human sperm membrane function followed previously established protocols (Benoff et al., 1994, 1995a). Motile human sperm populations were diluted and incubated overnight in capacitation media supplemented with stimulus on acrosome status, spermatozoa were incubated for 20 min at 37°C in calcium-supplemented buffer containing 1 µg/ml progesterone (Sigma No. P0130); control reactions were performed in the absence of progesterone as well as in the presence of 0.1% ethanol (the solvent used to prepare the initial concentrated progesterone stock solution) (Benoff et al., 1996). To examine the effect of a non-physiological stimulus on acrosome status, spermatozoa were exposed to 3 µM ionomycin following the protocol of Thomas and Meizel (1988). Control aliquots were exposed to 0.5% ethanol, the solvent used to prepare the ionomycin stock solution.

Induction of acrosome reaction
To examine the effect of the cumulus-secreted progesterone on acrosome status, spermatozoa were incubated for 20 min at 37°C in calcium-supplemented buffer containing 1 µg/ml progesterone (Sigma No. P0130); control reactions were performed in the absence of progesterone as well as in the presence of 0.1% ethanol (the solvent used to prepare the initial concentrated progesterone stock solution) (Benoff et al., 1996). To examine the effect of a non-physiological stimulus on acrosome status, spermatozoa were exposed to 3 µM ionomycin following the protocol of Thomas and Meizel (1988). Control aliquots were exposed to 0.5% ethanol, the solvent used to prepare the ionomycin stock solution.

Evaluation of acrosome status
Acrosome-intact and acrosome-reacted spermatozoa were ethanol permeabilized and differentiated by reaction with 100 mg/ml tetramethylrhodamine isothiocyanate-labelled *Pisum sativum* agglutinin (RITC-PSA; Vector Laboratories, Inc., Burlingame, CA, USA), which binds to intra-acrosomal contents. Spermatozoa were scored microscopically as: (i) acrosome-intact if the labelling by RITC-PSA was uniform throughout the anterior and equatorial regions of the head; or as (ii) acrosome-reacted if the sperm heads were RITC-PSA negative or if only the equatorial segment was labelled. To discriminate between viable spermatozoa undergoing an agonist-stimulated acrosome reaction and those undergoing degenerative/spontaneous acrosome reactions, the percentages of spermatozoa exhibiting a bright band of fluorescence in the equatorial region were quantified (‘fast acrosome measure’; Tesarik et al., 1993). Acrosome status was scored by counting 300 spermatozoa in at least 20 microscopic fields as previously described (Benoff et al., 1995b).

Visualization of mammalian binding sites with fluorescein-conjugated BSA
Motile sperm populations were surface labelled with 100 µg/ml fluorescein isothiocyanate conjugate (FITC)-conjugated mannosylated BSA (Man-FITC-BSA; Sigma No. A7790) in a Ca²⁺-supplemented buffer as previously described by Benoff et al. (1993b,d). Control reactions contained 100 µg/ml FITC-conjugated BSA. Motility and viability (by eosin Y dye exclusion) were assessed at the beginning and end of the labelling protocol.

Man-FITC-BSA binding by viable spermatozoa was enumerated as whole head plus midpiece (pattern II) or equatorial/post-equatorial regions plus midpiece (pattern III) using phase contrast and fluorescence microscopy (Benoff et al., 1993b,d). Coded slides from each specimen were examined at ×400 by two observers, each scoring at least 300 spermatozoa in 10–20 microscopic fields, with 5–7% variation in scoring between observers.

Visualization of non-nuclear progesterone binding sites
The surfaces of motile spermatozoa were labelled with 100 µg/ml progesterone 3-(O-carboxymethyl) oxime:BSA fluorescein isothiocyanate conjugate (P-CMO:BSA; Sigma No. P8779) for 15 min utilizing the same buffer system employed in the Man-FITC-BSA labelling reaction (Benoff et al., 1995b). Control reactions and measurements of sperm viability and quantification of the percentages of spermatozoa exhibiting head-directed P-CMO:BSA surface label were performed as described for Man-FITC-BSA.

Evaluation of acrosome status
In all experiments, mouse monoclonal antibodies IIF7 (Leung et al., 1987) and IIID5E1 (Leung et al., 1987; Ohlendieck et al., 1991) and

![Figure 1. Schematic representation of the α1 subunit of the L-type high voltage-dependent Ca²⁺ channel (VDCC).](image-url)
Figure 2. Immunocytochemical analysis of unfixed Triton-permeabilized preparations of motile capacitated human spermatozoa from fertile donors. Comparison of the distribution of monoclonal antibody IIF7 binding (A, B) with that of anti-actin antibody binding (C, D). Labelled spermatozoa were viewed at ×600 and photographed at ×1500 on 400 ASA film with exposure times for fluorescein and rhodamine images respectively, of 50 s and 4 s. (A, C) Phase-contrast images; (B) IIF7 binding is restricted to the post-acrosomal region of the sperm head; (D) anti-actin antibody binding is observed in the human sperm head in two patterns (Benoff et al., 1997) similar to those observed for head-directed Man-FITC-BSA and anti-myosin antibody labelling (Benoff et al., 1993b,d, 1994, 1996). Pattern II (small arrow), over the region of the acrosome cap and pattern III (large arrow), labelling in the equatorial segment only.

Specimens were viewed at ×600 magnification. The proportion of spermatozoa showing different topographical patterns of labelling were assessed by inspection of mounted slides stored at 4°C for <2 weeks before analysis. Where required for documentation, identical fields were photographed on 35 mm/400 ASA TMAX film (Eastman Kodak, Rochester, NY, USA) using both phase-contrast and epifluorescence illumination. All photographs were developed for the same length of time at 68°C and printed with identical exposure times.

Measurements of intracellular free Ca2+

Aliquots of control and nifedipine-treated spermatozoa were loaded with the intracellular Ca2+ indicator dye Fura-2/AM (1 µM; Calbiochem-Novabiochem Corp., La Jolla, CA, USA) for 45 min at 37°C/5% CO2 as described by Thomas and Meizel (1988). Dye-loaded spermatozoa were diluted to a final concentration of 1.25×106/ml in FM media containing 2.5 mM CaCl2. Fluorescence was monitored against muscle actin (Sigma No. A-2668). Human and rat spermatozoa were reacted sequentially with primary antibody and tetramethylrhodamine B (TRITC) or FITC-conjugated goat anti-mouse immunoglobulin (IgG) (Sigma Nos. T-6528 and F-7634 respectively) to detect bound mouse IgG, FITC-conjugated donkey anti-sheep IgG (Sigma F-7634) to detect bound sheep immunoglobulins, or with human serum protein-preabsorbed FITC-conjugated sheep anti-rabbit IgG to detect bound anti-actin antibodies.

A total of 10 µl of human spermatozoa (50×106/ml) or rat spermatozoa (2×106/ml) were smeared onto the well of a pre-cleaned heavy Teflon coated (HTC) microscope slide (No. 10–1179; Cel-Line Associates Inc., Newfield, NJ, USA), and air dried. For detection of surface-bound epitopes, sperm samples were three times washed in phosphate-buffered saline (PBS) with 0.5 mM EDTA and 0.3% porcine gelatin. Slides were blocked with the above solution for 30 min at room temperature in a humidified chamber. The primary antibody was added to slides in the same buffer, and incubated for 30 min at room temperature. At the end of the incubation period, slides were washed three times in PBS/0.5 mM EDTA and blocked again for 30 min at room temperature in PBS/0.3% porcine gelatin. Slides were then incubated with secondary antibody to the dilution recommended by the supplier for 30 min at room temperature in the dark. Finally, slides were washed three times in PBS/0.5 mM EDTA, fixed in 3% formalin for 15 min and air dried.

To detect internal epitopes, unfixed spermatozoa were Triton-permeabilized and stained as previously described (Benoff et al., 1996).
acrosome loss after progesterone exposure (Bonaccorsi et al., 1995; Tesarik et al., 1996). Therefore, values for progesterone-stimulated Ca\(^{2+}\) influx are derived from the average relative fluorescence values obtained between 2.5 and 3.5 min after addition of P, during the plateau phase.

**Statistical analysis**

All data were analysed by paired \(t\)-tests, performed with the SAS/PC software package (SAS-Institute Inc., Cary, NC, USA). Statistical significance was set at \(P < 0.05\). Where significant differences were noted between control and nifedipine-treated aliquots of motile human spermatozoa, summary statistics are presented in the figure legends as means ± SD.

**Results**

**Detection of L-like VDCCs in human and rat sperm**

To determine whether the human sperm plasma membrane contains Ca\(^{2+}\) channels which share antigenic epitopes with L-type VDCCs expressed in somatic tissues, motile spermatozoa from fertile donors were exposed to two monoclonal and one polyclonal antibody which recognize the α1 subunit of VDCCs expressed in rabbit skeletal muscle transverse tubules (Leung et al., 1987; Ohlendieck et al., 1991; Pragnell et al., 1991). Although the sperm surface was labelled by all three antibodies, the intensity of antibody binding was markedly increased when spermatozoa were Triton-permeabilized to allow antibodies access to antigens potentially buried in the lipid bilayer. Typical results with permeabilized cells are presented in Figure 2.

All spermatozoa in the population display labelling with monoclonal antibody IIF7 directed against DHPR (Figure 2B). This finding is consistent with a prior report indicating that Ca\(^{2+}\) influx is observed in >90% of agonist-exposed fertile donor spermatozoa (Plant et al., 1995). No labelling is observed when spermatozoa are exposed only to secondary antibody, demonstrating the specificity of the reaction (not shown). All spermatozoa bind IIF7 in the same manner. The epitopes recognized by IIF7 are concentrated in the post-acrosome region. The distribution of Ca\(^{2+}\) channel antigenic epitopes in human spermatozoa clearly differs from that observed in the F-actin control (Figure 2D). Anti-actin antibody labelling is observed in two patterns, either over the entire human sperm head (pattern II) or concentrated in the equatorial region (pattern III). Separate studies have indicated that these two anti-actin staining patterns correlate, respectively, with the presence of an intact acrosome or one which has undergone exocytosis (Benoff et al., 1997).

The reaction of rat spermatozoa with antibodies directed against the α1 subunit of VDCCs was similarly examined (Figure 3). Both permeabilized and unpermeabilized preparations readily bound these antibodies, significant binding occurring to the head region. In contrast to observations of human spermatozoa, the intensity of antibody labelling was greater in the unpermeabilized preparations. This finding is consistent with the suggestion that sperm shape and lipid composition regulates susceptibility to detergent-induced plasma membrane rupture (Schweisguth and Hammerstedt, 1992; Watson et al., 1992). Nevertheless, the region of the sperm head in which antibody binding is concentrated is identical to that of human spermatozoa, specifically the post-acrosomal region (Figure 3D).

**Identification and sequence analysis of mRNA sequences encoding L-type VDCCs in rat testis**

Approximately 900 clones generated by PCR amplification of rat testis mRNA were screened, first by insert size and then by DNA sequence analysis. Four clones were isolated which exhibited >98% sequence identity with authentic rat cardiac L-type VDCC sequences. In contrast, when the sequence of these four clones was compared with the sequence of a T-type Ca\(^{2+}\) channel (Accession No. L15453; Soong et al., 1993) <25% overall sequence identity was noted. These data unequivocally demonstrate that an L-type VDCC is expressed in rat testis.

The four clones span nucleotides 2801–6695 of the rat α1 subunit. The largest clone obtained was subjected to detailed
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Rat Testis Calcium Channel Clone pRACH 62

Figure 4. Rat testis high voltage-dependent Ca\(^{2+}\) channel (VDCC) clone pRach 62. (A) Clone pRACH 62 was sequenced in both directions with the primers indicated above and below the solid line. The arrows indicate the direction of sequencing. The sequencing primers were all 18 nucleotides in length and began at the base indicated; (B) alignment of the alternatively expressed sequences in rat testis (IVS3a) and cardiac muscle (IVS3b). Sequence identity is indicated by a vertical line between the sequences of the two tissues; (C) the deduced amino acid sequence for the alternatively expressed exons is shown. Sequence identity is indicated by a line between the respective residues and an asterisk above the residue indicates a non-conservative change.

sequence analysis (Figure 4). The clone, pRach 62, starts at base 3908 and spans 2169 nucleotides. The strategy employed for sequencing pRach 62 is outlined in Figure 4A. The nucleotide sequence of this clone was determined to be identical to the corresponding region of the rat cardiac muscle \(\alpha_1\) subunit except for an 84 base change beginning at nucleotide 4104. Comparison with the gene structure and identification of consensus sequence for splice junctions (Soldatov, 1994) in mature mRNAs suggest that this differing sequence encodes an alternative exon in the IVS3 membrane spanning region (see solid bar in region IV; Figure 1). The alternate sequence expressed in testis (IVS3a) is only 58% identical to the sequence utilized in the cardiac muscle channel transcript (IVS3b) (Figure 4B).

The 35 base changes identified in the nucleotide sequence result in 13 amino acid changes in the deduced sequence of the protein (Figure 4C). The major fraction of these changes (9/13) are conservative in nature. However, four non-conservative amino acid changes were detected. Therefore, we have compared the secondary structure as predicted by Garnier et al. (1978) of the exon expressed in rat cardiac muscle with that of the alternate sequence expressed in testis (Figure 5). The

Figure 5. Analysis of predicted protein secondary structure. A comparison of the likelihood that the transmembrane regions encoded by IVS3a and IVS3b will form an \(\alpha\) helical structure. The solid line represents the analysis of the deduced amino acids encoded by the testis cDNA expressing IVS3a and the dotted line that of IVS3b. The lines above the baseline indicate a tendency or propensity for the encoded peptides to form an \(\alpha\) helix, while the lines below the baseline indicate a negative tendency or a disruption of \(\alpha\) helix formation of the encoded peptide. These data suggest that there is a significant difference in the ability of the two exons to assume an \(\alpha\) helical conformation.
conserved sequences flanking these exons were included in
the analysis to determine the effect of alternate sequence usage
on overall channel structure. These data indicate that a small
number of amino acid substitutions (4/13) can dramatically
alter the α helical structure of the channel in and around the
membrane spanning region. Although the negative charges are
conserved within the exon expressed in testis, there are
disruptions in the hydrophobic residues flanking the negative
amino acid residues on either side of the exon. In part, this is
probably due to the presence of a proline residue (a helix
disrupting residue) in place of an alanine. In addition, the
substitution of a serine for a cysteine residue suggests a
possible loss of inter- or intra-chain disulphide bond formation.

**Tissue expression and direct sequencing of the α1 subunit of the rat testis VDCC**

RACH 3908F and RACH 4777R were used in an attempt to
amplify an 869 base pair product in and around IVS3 which
potentially contains an alternatively spliced region. The expres-
sion of this portion of the α1 subunit sequence was examined
in a variety of rat tissues, including lung, liver, thymus, spleen,
skeletal muscle, heart, and testis. MgCl₂ titrations (2–6 mM)
as well as primer concentrations (10–50 pM per reaction) were
performed to optimize the reactions. Products were only
obtained when heart and testis cDNAs were used as template
(Figure 6). This finding is consistent with both the specificity
of gene expression and Ca²⁺ regulation of tissue-specific
functions, e.g. contractility or exocytosis.

We have directly sequenced the PCR products from heart
and testis cDNAs. In the heart template, only the IVS3b
sequence is detected while only the IVS3a sequence occurs in
the sequences amplified from testis. These data provide further
support for the hypothesis that the α1 subunit transcripts in
heart and testis are the products of alternate exon usage.

**Effects of nifedipine on Ca²⁺ influx in agonist-stimu-
lated capacitated human spermatozoa**

Nifedipine blood plasma concentrations of 58–580 nM are
associated with anti-hypertensive efficacy (McAllister et al.,
1986). To investigate the mechanism underlying the production
of iatrogenic infertility by therapeutic administration of Ca²⁺
entry antagonists (Benoff et al., 1994, 1995a; Hershlag et al.,
1995, 1996), these average concentrations in the circulation in
clinically effective regimens were chosen as central values in
the search for potential dose-response alterations in: (i) basal
and agonist-regulated intracellular Ca²⁺ concentrations; (ii)
agonist-stimulated acrosome loss; and (iii) activation of sperm
surface non-nuclear progesterone receptors (PRs) associated
with drug exposure.

Basal concentrations of Ca²⁺ in untreated and incubated
control aliquots from motile sperm populations from fertile
donors (n = 5) were determined and compared with intra-
cellular Ca²⁺ values in duplicate aliquots incubated for 24 and
48 h in media supplemented with nifedipine (Figure 7A). In
control aliquots, a significant increase in intracellular ionized
calcium was detected in association with capacitation. Basal
concentrations of intracellular calcium in spermatozoa exposed
to nifedipine for 24 h were, however, considerably reduced as
compared to incubated controls. After two days of nifedipine
exposure, a dose-dependent effect on incubation-associated
increases in free cytosolic Ca²⁺ was observed. In aliquots
incubated in the presence of 500 pM or 50 nM nifedipine, basal
Ca²⁺ concentrations were increased and were indistinguishable
from those of one day incubated control aliquots. In contrast,
no change in basal Ca²⁺ concentrations was observed in
aliquots supplemented with 5 or 500 µM nifedipine.

The effect of nifedipine on agonist-induced Ca²⁺ influx was
also examined (n = 4; Figure 7B). The ability of progesterone
exposure to stimulate Ca²⁺ influx develops during capacitation.
Fresh isolates are essentially refractory to the effects of
progesterone. In contrast, a significant rise in intracellular
Ca²⁺ is observed following brief progesterone treatment in
control aliquots incubated for 1 day under capacitating condi-
tions. Equivalent increases of progesterone-stimulated Ca²⁺
influx are observed in aliquots incubated in the presence of
nifedipine irrespective of dose. After 2 days incubation in
the presence of nifedipine, however, Ca²⁺ influx in response to
progesterone was completely abrogated.

To confirm these findings, we examined the effect of
progesterone exposure on acrosome status (n = 4; Figure 8A).
In control aliquots not exposed to nifedipine, an increase in acrosome loss was observed following progesterone treatment. The percentages of progesterone-stimulated acrosome loss obtained were equivalent to the percentages of spermatozoa exhibiting the secondary Ca\(^{2+}\) plateau phase after hormone treatment reported after single cell analysis by confocal laser scanning microscopy (Tesarik et al., 1996). A trend toward correlation between acrosome loss and time of incubation was also noted ($P = 0.077$). In aliquots incubated for 1 day in the presence of 500 pM or 500 nM nifedipine, the percentages of progesterone-stimulated acrosome reactions were at least as large as those of control aliquots. However, after 2 days of nifedipine exposure, the ability of incubated spermatozoa to undergo a progesterone-stimulated acrosome reaction was reduced in a dose-dependent manner. Thus, the incubation time-dependent inhibition of progesterone-stimulated acrosome
loss by nifedipine parallels that of the effect of nifedipine on agonist-regulated Ca\(^{2+}\) influx.

To demonstrate that the observed reductions in progesterone-stimulated Ca\(^{2+}\) influx and acrosome loss resulting from drug exposure were not artefacts due to alterations in the order of membrane packing associated with nifedipine-induced inhibition of cholesterol efflux (Benoff et al., 1994, 1995a; Hershlag et al., 1995), the effect of nifedipine on ionomycin-stimulated acrosome loss was examined \((n = 4; \text{Figure 8B})\). After both 1 or 2 days of nifedipine exposure, the percentages of spermatozoa exhibiting ionomycin-stimulated acrosome reactions in drug-treated aliquots were indistinguishable from those in control aliquots. These data indicate that the effects of nifedipine on human sperm function are specifically directed towards the VDCC in the sperm plasma membrane.

We have previously reported that mannose receptors and PRs are co-expressed on the acrosome reaction-inducible subpopulation of motile fertile donor spermatozoa (Benoff et al., 1995b) and that nifedipine inhibits surface expression of sperm mannose receptors (Benoff et al., 1994, 1995a; Hershlag et al., 1995, 1996). We now compare the effects of nifedipine on receptor co-expression \((n = 6; \text{Figure 9})\). Similar percentages of motile spermatozoa in fresh isolates and in duplicate aliquots incubated overnight in Ham’s F-10 containing 30 mg/ml HSA bound fluorescein-labelled mannose and progesterone ligands \((P = 0.811\) and 0.832, not significant\), confirming prior observations. Nifedipine produced a dose-dependent decrease in the percentage of capacitated spermatozoa capable of binding mannose ligands (Figure 9A), again as previously noted. In contrast, at 1 day of incubation, the percentages of sperm binding progesterone ligands in control and nifedipine-treated aliquots were increased to a similar extent (Figure 9B). These data are consistent with above findings that one day of exposure to nifedipine is without effect upon progesterone-stimulated Ca\(^{2+}\) influx and acrosome loss. More importantly, as an increase in surface binding of progesterone ligands is observed while basal values of intracellular Ca\(^{2+}\) remain low (Figure 7A), our data contradict a prior report which suggests that the ligand binding activity of the PR on human spermatozoa is activated as a consequence of mobilization of intracellular stores of Ca\(^{2+}\) (Mendoza and Tesarik, 1993).

The most significant finding of these in-vitro studies is illustrated by comparison of Figures 7B and 9B. Comparison of the percentages of sperm binding progesterone ligands in control aliquots after 1 and 2 days of incubation indicates a trend towards a time-dependent activation of latent PRs. The same trend was observed in nifedipine-treated aliquots. Even at 2 days of incubation, similar percentages of sperm binding progesterone ligands were detected in control and drug-treated aliquots. These data demonstrate that progesterone ligand binding is unaffected by nifedipine treatment. Nevertheless, progesterone does not elicit the rise in intracellular Ca\(^{2+}\) levels normally observed following ligand binding. We conclude that a pharmacological blockade of L-type VDCCs in spermatozoa is effected by nifedipine exposure and that this blockade specifically limits the ability of drug-treated spermatozoa to undergo agonist-stimulated acrosome loss.

![Figure 9](image)

**Figure 9.** Examination of the effects of nifedipine on surface expression of mannose receptors and progesterone receptors by incubated human fertile donor spermatozoa. (A) The percentages of spermatozoa in control aliquots exhibiting head-directed Man-FITC-BSA binding is increased on incubation (fresh isolate versus incubated 1 day, 6.26 ± 1.38 versus 20.84 ± 4.18; \(P < 0.002\)). In contrast, incubation in the presence of nifedipine results in a dose-dependent inhibition of mannose receptor surface expression \((500 \mu\text{M} \text{ and } 500 \mu\text{M nifedipine, } 13.21 \pm 3.57 \text{ and } 7.88 \pm 2.35 \text{ respectively, } P < 0.029 \text{ and } P < 0.003\). (B) The percentages of motile spermatozoa in control aliquots exhibiting binding of progesterone ligands is increased in a time dependent manner \((\text{fresh isolate versus incubated 1 day versus incubated 2 days, } 6.47 \pm 1.74 \text{ versus } 18.70 \pm 7.17 \text{ versus } 34.03 \pm 11.84 \text{ respectively, } P < 0.009 \text{ and } P = 0.067\). The percentages of sperm binding progesterone-CMO-BSA in nifedipine-exposed aliquots is similarly increased \((\text{control versus } 500 \mu\text{M} \text{ and } 500 \mu\text{M nifedipine, respectively, incubated 1 day, } P = 0.719 \text{ and } P = 0.864 \text{, and incubated 2 days, } P = 0.924 \text{ and } P = 0.590, \text{ not significant})

**Discussion**

A crucial aspect to responsible family planning is the involvement of both partners as well as the method of contraception. This participation would be enhanced by the development of safe, effective, and reversible male contraceptives. To date, contraception methods for men have been limited, involving barrier protection such as condoms or interruption of sperm transport by vasectomy, which may create some long-term risks (Comhaire, 1994). An alternative approach to male-oriented contraception that has received much support recently (Wu et al., 1996; World Health Organization, 1996) is to manipulate the production of hormones which lead to spermato genesis, for example, by the injection of androgens (testosterone or related male hormones) into the circulation. There are several problems with this approach, including, but not limited
to, the attainment of azoospermia and the non-reversible inhibition of spermatogenesis. The infusion of circulating androgens leads to increased irritability, diminished libido, lowered concentrations of high density lipoproteins and an increase in acne. These side-effects can be reduced with the administration of progestin but require a combination of hormones which have their own potential problems. These products are in the development stage and are expected to available in ~10 years time, and would be effective for 3 months at a time. Other possibilities of developing systemic contraceptives for men include drugs or reagents that act as antagonists to gonadotrophin-releasing hormone. The problem with this approach is that often these drugs are toxic to other cells in the testis, with the possible outcome of induced, permanent sterility.

As an alternative, Bedford (1994) has suggested that control or inhibition of sperm capacitation offers an attractive mode for male contraception. One possible way to develop such contraceptives is to co-opt mechanisms that normally regulate fertilization. It has been shown that regulation of the sterol content of the sperm plasma membrane plays an important role in capacitation (Benoff, 1993), and men with high sperm membrane cholesterol content have been found to be infertile in an IVF setting (Benoff et al., 1993c, 1995a, 1996). In this regard, it is important to note that gossypol, a binaphthalene-dialdehyde extracted from cotton seeds, appears to exert its anti-fertility effect through a similar mechanism (de Peyster et al., 1986; Huang and Urrhaler, 1986; Reyes et al., 1986). Gossypol has an efficacy rate of 98.9% and does not affect sperm cytoplasm as well. It is highly lipophilic, gossypol can interact directly with membrane L-type VDCC by antibody staining and have shown anti-fertility effect through a similar mechanism (de Peyster et al., 1986).

 Moreover, gossypol is due to the interaction of the drug with membrane lipids, which influences the membrane fluidity of the lipid microenvironment in which integral membrane proteins operate. This places a restriction on the translational and rotational ability of enzymes in the plane of the lipid bilayer, and thus effects its activity by limiting binding of substrate (Yuli et al., 1981).

In many respects, the effects of Ca\(^{2+}\) channel blockers on sperm plasma membrane structure and function mimic those of gossypol (Benoff et al., 1994, 1995a; Hershlag et al., 1995). In our laboratory, the anti-fertility activity of Ca\(^{2+}\) entry antagonists was initially identified because of the biophysical effects of these drugs on membrane cholesterol content. The partitioning of lipophilic Ca\(^{2+}\) channel blockers into the sperm plasma membrane stabilizes the membrane and inhibits the efflux of cholesterol, which is a normal part of the capacitation process (Benoff et al., 1993a,c,d, 1995a). The end result of this change in membrane organization is that mannose receptors, markers for zona binding potential and the ability to undergo zona-stimulated acrosome loss (Benoff et al., 1993a,b,c, 1995a,c, 1996) cannot be translocated to the sperm surface from sub-plasmalemmal stores. Thus, spermatozoa from men taking calcium channel blockers exhibit a markedly reduced ability to bind to the human zona pellucida. Nevertheless, Ca\(^{2+}\) channel blockers would be more attractive as potential contraceptive agents if it could be directly demonstrated that these drugs, rather than simply exerting physical effects on membrane structure, affect successive steps in the fertilization sequence, e.g. Ca\(^{2+}\) influx through L-type VDCCs and acrosome exocytosis in addition to the effects on zona binding potential. Thus, there are three major findings reported in this paper.

Firstly, we have independently demonstrated that exposure of fertile donor spermatozoa to Ca\(^{2+}\) channel blockers results in a delay in the time course of sperm capacitation. Prior studies indicate that a progressive increase in basal concentrations of ionized intracellular calcium is observed in association with capacitation (Baldí et al., 1991; Mendoza and Tesarik, 1993). Herein, we demonstrate that exposure of fertile donor spermatozoa to 500 pM or 50 nM nifedipine delays by 1 day the rise in basal cytosolic Ca\(^{2+}\) concentrations. Exposure to higher concentrations of nifedipine results in a further delay. These data are consistent with our earlier data indicating that nifedipine limits capacitation by reducing the rate of cholesterol efflux from the sperm plasma membrane (Benoff et al., 1994, 1995a; Hershlag et al., 1995). Further, these data suggest that changes in plasma membrane width and packing resulting from nifedipine-directed cholesterol retention influence not only mannose receptor expression but the ion content of the sperm cytoplasm as well.

Secondly, we have identified the human sperm plasma membrane L-type VDCC by antibody staining and have shown it to be functionally active. We have examined the effect of nifedipine exposure on the PR of human spermatozoa, which is co-expressed and forms a complex with mannose receptors (Benoff et al., 1995b). The surface appearance of these different functional binding sites is regulated by independent mechanisms. The PR is an integral plasma membrane protein in both freshly isolated and capacitated spermatozoa (Benoff et al., 1995b). In contrast to our earlier findings concerning surface expression of functional mannose receptors, we demonstrate that the PR is impervious to the alterations in sperm membrane cholesterol content effected by nifedipine exposure. A time-dependent increase in the percentages of spermatozoa expressing functional progesterone binding sites is observed under conditions where nifedipine inhibits incubation-associated increases in membrane fluidity. Nifedipine does not affect ligand binding by the PR. Rather, nifedipine appears to block the consequences of progesterone exposure, Ca\(^{2+}\) influx through L-type VDCCs and acrosome exocytosis. These data indicate that the mechanisms by which nifedipine inhibits the function of mannose receptors and PRs clearly differ.

Thirdly, our characterization of the cDNA of the L-type VDCC and its derived amino acid sequence present intriguing insight into the physiological regulation of Ca\(^{2+}\) entry in mammalian spermatozoa. The conductance and ion selectivity of L-type VDCCs in a variety of tissues suggests that they can be partitioned into several categories, as characterized by pharmacology, electrophysiology and tissue localization (Tsien
et al., 1991). This functional diversity is due, in part, to the expression of at least five genes. These genes show a large degree of sequence identity, suggesting that they are derived from a common ancestor. Additional functional diversity is generated by the use of alternatively spliced products. ‘Cassette’-like, highly conserved peptide sequences are, however, common to the spliced products of all L-type VDCC genes (Perez-Reyes et al., 1990; Snutch et al., 1991; Tsien et al., 1991; Diebold et al., 1992; Soldatov, 1994). The conservation of these peptide sequences among the five genes suggests they play an important structural/functional role in the expression of the channel. Both splicing and the common peptide cassettes are of particular relevance to our current findings.

The gene encoding the α1 subunit from rat cardiac muscle has been shown to use alternative splicing to generate isoform diversity (Koch et al., 1990; Perez-Reyes et al., 1990; Tsien et al., 1991; Diebold et al., 1992; Soldatov, 1994). The PCR primers used to amplify and clone the L-type VDCC expressed in testis were generated from this gene sequence. Herein, we present evidence that the sequence of the α1 subunit of the L-type VDCC found in rat spermatozoa is identical to that of the cardiac sequence except for one small region of 35 amino acids which resides in the membrane-spanning IVS3 region. The sequence of these amino acids is identical to that found in the α1 subunit of skeletal muscle. That we were, however, unable to amplify the region containing this sequence from skeletal muscle cDNA using primers derived from the cardiac sequence is consistent with the fact the L-type VDCC expressed in skeletal muscle is the product of a different gene (Tsien et al., 1991) and provides additional evidence for a shared peptide cassette. Given the prior demonstrations of alternative splicing, common cassettes and the fact that consensus donor/acceptor splice junction sequences (Mount, 1982) are found at the termini of this insert, we conclude that the testis-specific isoform represents a splice variant of the cardiac-type α1 gene.

Given that Ca\(^{2+}\) influx is a prerequisite to acrosome exocytosis in all mammalian spermatozoa so far studied, that antibodies to the α1 subunit of the rabbit skeletal muscle VDCC specifically react with both rat and human spermatozoa, and that the topographical distribution of antibody binding on the sperm head is similar in the two species, we think it is fair to suggest that human spermatozoa express a highly conserved homologue of the L-type VDCC sequence identified in rat testis. Therefore, we now extrapolate from the sequence data from rat to the physiological findings with human spermatozoa. Although the potential mechanism by which the IVS3 sequence expressed in the male germ line and skeletal muscle could affect the voltage gating parameters of the L-type VDCC will be discussed in detail in a separate publication, we must note here that the kinetics of activation of the skeletal muscle VDCC which occur more slowly and with different conductance than that noted in heart and smooth muscle are due in part to the alternatively spliced exon encoding IVS3a (for review see Tsien et al., 1991). These differences may help explain why 2 days of incubation in the presence of nifedipine are required to effect blockage of agonist-stimulated Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) VDCCs in fertile donor human spermatozoa. The hydrophobic side of IVS3 serves as one potential DHPR site. Changes in the hydrophobic residues and predicted secondary structure of IVS3a, as demonstrated herein, would result in a change of affinity of this subunit for nifedipine, the prototype DHP.

The identification of the primary structure of the L-type VDCC expressed in mammalian spermatozoa provides us with a valuable tool for the evaluation and diagnosis of male infertility. The presence of occult genetic mutations in the sequence of the gene encoding the channel may cause a large effect on the function of the voltage-gated Ca\(^{2+}\) channel and thus inhibit or abrogate the calcium influx necessary for the initiation of acrosome reaction leading to fertilization. This is a reasonable proposal, as recent reports have shown that microdeletions of genetic material of the Y chromosome have wide ramifications with respect to fertility, and may result in azoospermia (Najmabadi et al., 1996). The elucidation of the normospermic cDNA sequence of the VDCC allows us to compare and contrast the primary structure of those channels in patients with male infertility associated with acrosome insufficiency (Falsetti et al., 1993; Calvo et al., 1994; Lui and Baker, 1994b; Oehninger et al., 1994; Krausz et al., 1995, 1996) following the use of a blood test to obtain genomic DNA. Moreover, the specificity of the alternatively expressed exon encoding the specialized transmembrane stretch of IVS3 found in spermatozoa suggests a tempting target for pharmaceuticals that can partition into the lipid bilayer and bind to and disrupt the functioning of the channel in a very site- and tissue-directed manner. By clarifying the integral portion of the membrane involved in the gated kinetics of the sperm VDCC, we can exploit this knowledge through testing and designing analogues of dihydropyridine-binding compounds with exquisite specificity and limited pharmacological effects.

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**References**


