# VOLTAGE DEPENDENT CALCIUM CHANNELS IN MAMMALIAN SPERMATOZOA

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## 1. ABSTRACT

Calcium influx is an absolute requirement for the physiological acrosome reaction in sperm from all sources examined, both invertebrate and mammalian. Pharmacological studies suggest that the major channel in the sperm head plasma membrane responsible for modulating calcium entry and intracellular ionized calcium levels could be either an L-type (a class of high voltage-activated) or a T-type (low voltage-activated) voltage-dependent calcium channel. Patch clamp analysis of calcium currents in immature spermatogenic cells demonstrates the presence of T-type currents. Therefore, an argument has been put forth that the acrosome reaction of ejaculated sperm is regulated by a T-type calcium channel. However, indirect analysis of calcium currents in mature sperm after transfer of ion channels to planar lipid bilayers detects three current types, including that similar, but not identical, to an L-type channel, but no T-type currents. Molecular cloning of the alpha-1 pore forming subunit of calcium channels expressed in the male reproductive tract and in ejaculated sperm has resolved this controversy, demonstrating the existence of only high voltage-activated channels. Further analysis of the alpha-1 subunit isoform from rat and human testis and sperm suggests that, as a result of alternate splicing, this L-type alpha-1 subunit could produce calcium currents that were T-like, e.g., transient, rapidly inactivating with slow deactivation. Multiple splice variants of this isoform were detected in human testis, suggesting a correlation with intra-individual variation in the ability of sperm to undergo an induced acrosome reaction and with male infertility.

These variants could be developed as useful biomarkers for susceptibility to environmental and occupational toxicants. Knowledge of calcium channels structure will also contribute to design of new male contraceptives based on existing calcium channel antagonists.

## 2. INTRODUCTION

The acrosome reaction was first identified in sea urchin sperm by Dan (1), who also provided evidence for a requirement for extracellular calcium in the initiation of this specialized form of exocytosis. We now know that the mammalian sperm acrosome reaction is also a calcium-dependent process (2-4) which is an absolute requirement for zona pellucida-bound sperm to penetrate through the zona and fuse with the oolemma. Results from biophysical studies, analysis of pharmacological sensitivities and binding of specific antibodies all indicate that sperm express calcium channels which function in the acrosome reaction. These channels are the subject of the current review.

Two general classes of calcium channels have been identified: (I) calcium entry channels, and (II) calcium release channels (review, 5). The first class can be subdivided into three categories: (1) voltage-dependent calcium channels (VDCC), which are opened by depolarization, (2) receptor-operated calcium channels, which are activated by chemical messengers (e.g., glutamate), and (3) capacitative calcium entry channels,
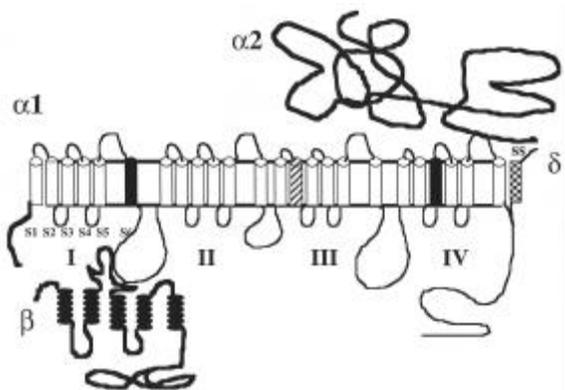
**Figure 1.** A schematic representation of a typical voltage-dependent calcium channel. The figure shown is modified from those of Tsien *et al*. (13), Heinemann *et al*. (244), Catterall (7) and Perez-Reyes and Schneider (8). The prototype VDCC is a heteromultimer composed of alpha-1, alpha-2, beta and delta subunits. The alpha-1 and beta subunits are products of different genes while the alpha-2 and delta subunits result from alternate splicing of the same primary transcript (11,12). The alpha-1 subunit forms the ionic pore and contains cytoplasmic amino and carboxy termini and four repetitive domains (I-IV), each containing six putative transmembrane segments (S1-S6). The amino terminus (thick line) and segments IS6 (filled bar), IIIS2 (diagonally hatched bar) and IVS3 (filled bar) are alternatively spliced in rat and human cardiac muscle and testis (31,126,164,165). The beta subunit binds to the alpha-1 subunit in the linker region between domain I and domain II (245,246). Although the alpha-2 subunit is externally located and linked to the membrane via its association with the delta subunit (54), the region in which these subunits interact with the alpha-1 subunit is unknown (8).

which are opened by depletion of intracellular calcium stores. The second category includes: (1) ryanodine receptors, and (2) inositol 1,4,5-triphosphate receptors. VDCCs are the primary ion channels modulating the acrosome reaction. This review will focus on expression of VDCCs in mammalian testis and sperm in relation to: (1) sensitivity of sperm to pharmacological and inorganic calcium channel antagonists, and (2) calcium currents detected in immature spermatogenic cells and after transfer of mature sperm ion channels to planar lipid bilayers.

The prototype VDCC is composed of four subunits (alpha-1, alpha-2, beta and delta) (6; see figure 1). Different alpha-1 subunits are produced by transcription from at least seven different genes and by alternate splicing of the different primary transcripts (7-9). Although these genes are located on different chromosomes (e.g., 10), sequence homologies indicate that these genes are derived from a common ancestor (figure 2A). Similarly, the beta subunit is the product both of four different genes as well alternate splicing (7,8). In contrast, the alpha-2 and delta subunits result from alternate splicing of the same primary transcript (11,12).

The alpha-1 subunit is composed of twenty-four transmembrane segments which are divided into four repetitive domains (I-IV), each containing six transmembrane segments (S1-S6) (figure 1). Potential regions of alternate splicing include the 5' end, transmembrane segments IS6, IIS2 and IVS3, and the external linker segment between IVS3 and IVS4 (8,13). The alpha-1 subunit is able to form the pore of the channel in the absence of the other subunits. Cloning of this alpha-1$_S$ subunit from skeletal muscle and its *in vitro* expression indicate that it induces calcium currents. These currents are both voltage-sensitive and inhibited by dihydropyridines (14-16). Similar findings have been obtained following transfection of the cloned alpha-1 subunits from the cardiac L-type calcium channel (alpha-1$_C$; 17) and the other alpha-1 subunits (review, 8). The structural basis for ion channel function has also been identified (7,18-25). The pore is opened by salt bridge formation between the S4 voltage sensor and segments S2 and S3. Inactivation is accomplished via the amino and carboxy termini and IS6. Secondary messenger systems involved in regulation of channel activity include G-proteins (18) and serine/threonine phosphorylation (26). Further, constitutive phosphorylation on tyrosine residues may be required to maintain VDCC in a state which is available for activation by membrane depolarization (27,28). Tyrosine phosphorylation may also serve as a link between G-protein signalling pathways and VDCC (29).

Electrophysiological studies provide evidence for the existence of both high voltage-activated (HVA; e.g., L-type) and low voltage-activated (LVA; e.g., T-type) VDCC (see figure 2A). For example, T-type currents exhibit a low voltage threshold for activation, rapid inactivation and a negative inactivation range whereas L-type currents are high voltage activated and slowly inactivating. Although Perez-Reyes and co-workers (9) have recently cloned the a neuronal alpha-1 subunit named alpha-1$_G$ which yields T-type currents when expressed in Xenopus oocytes, it is still a matter of considerable debate as to whether or not this subunit is the only alpha-1 subunit involved in the formation of native T-type channels. Divergent genotypic and phenotypic expression of native (alpha-1$_A$, alpha-1$_B$, alpha-1$_C$, alpha-1$_D$; 30; also see 31) and cloned/transiently expressed (alpha-1$_B$, alpha-1$_C$, alpha-1$_E$; 32) alpha-1 subunits has been observed, i.e., alpha-1 subunits characterized as "HVA" can produce T-type currents. In addition, antisense oligonucleotides against rat brain alpha-1$_E$ decrease T-type currents in atrial myocytes (33) and R-type currents in cerebellar granule cells (34). It has been speculated that "T-type" channels are formed by homo-oligomerization of HVA alpha-1 subunits (32,35). If this is true, it could help explain why T-type currents display a wide range of activation voltages and inactivation kinetics (32).

The above findings can also help explain why the ion sensitivity (i.e., calcium, barium) of T-type currents differs among cell types (32,36), why highly specific
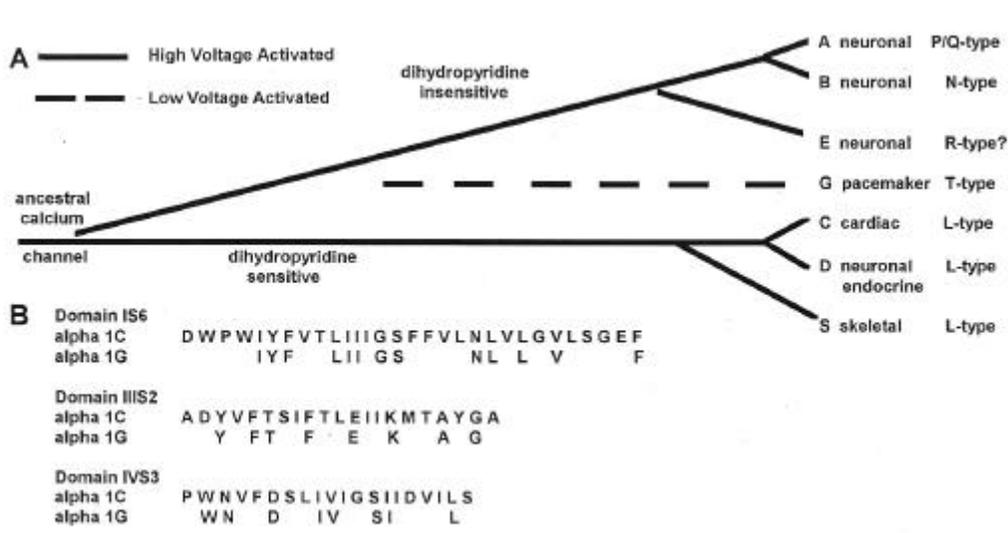
**Figure 2.** The alpha-1 subunits of VDCC were derived from a common ancestral gene. (A) Proposed familial relationships between alpha-1 subunits from different calcium channel classes were identified based on sequence homology reported by Perez-Reyes *et al*. (247) and Snutch *et al*. (248). The figure shown has been modified from that of Tsien *et al*. (13) and of Stea *et al*. (249) based on recent studies by Perez-Reyes *et al*. (9). (B) The sequences of the alpha-1$_C$ (31) and alpha-1$_G$ (9) subunits have diverged significantly. For example, the amino amino sequences of the alpha-1$_C$ subunit in regions encoding putative dihydropyridine binding sites (transmembrane segments IS6, IIIS2, IVS3) have been aligned with those of the alpha-1$_G$ subunit. Alpha-1$_G$ sequences are shown only for those amino acid residues that are identical to those in the alpha-1$_C$ sequence.

antagonists of T-type currents have yet to be identified (37-40), as well as why L-type VDCC blockers can inhibit T-type currents (41,42). With regard to L-type VDCC, the binding sites for the dihydropyridine class of calcium channel blocking drugs are located in a hydrophobic environment, e.g., within the plane of the lipid bilayer (43-46). Consistent with this, transmembrane regions IS6, IIIS2 and IVS3 have been identified as dihydropyridine binding sites (47-50). Nevertheless, the affinity for dihydropyridine binding differs among cell and tissue-subtypes based on the structure of the alpha-1 subunit expressed (47-49,51).

While the alpha-2, beta and delta subunits can modulate the kinetics, voltage dependence of activation and inactivation, and effects of dihydropyridine antagonists (e.g., 52-55; also see Section 7), the alpha-1 subunit remains the best studied. This review will concentrate on the role of alpha-1 gene expression in regulation of the biological and biophysical characteristics of mammalian sperm.

## 3. CALCIUM CHANNELS IN SPERMATOZOA

Yanagimachi and Usui (2) were the first to demonstrate that the guinea pig sperm acrosome reaction is dependent on the presence of millimolar amounts of extracellular calcium. Their studies also indicated that guinea pig sperm must undergo capacitation before they can respond to calcium. Acrosome loss by uncapacitated bovine (56) and human (57) sperm is similarly repressed.

More recent studies help explain the need for sperm capacitation preceding induction of the acrosome reaction by calcium. The physiological acrosome reaction is initiated by ligand- stimulated aggregation of receptors on the sperm head plasma membrane (58-62). Although the second messenger systems activated by ligand binding (review, 4) are functional in freshly ejaculated sperm (57), the receptor sites for physiological agonists, e.g., zona ligands and progesterone, are "unmasked" during capacitation (e.g., 60,62,63).

### 3.1. Biological findings

The acrosome reaction is mediated by efflux of potassium (64-67) and hydrogen ions and by influx of calcium (65,68-71). Membrane hyperpolarization and depolarization is induced by changes in membrane ion permeability (71-73). Studies using sperm loaded with calcium-specific indicator dyes demonstrate that extracellular calcium enters the sperm head prior to loss of acrosome content (69,74-79). L-type pharmacological calcium channel blockers and the inorganic cations, nickel(II), cobalt(II) and lanthanum(III), inhibit both calcium influx and acrosome exocytosis (66,69,70,80-83). The pharmacological antagonists also bind with high affinity to sperm membrane preparations (69,84,85). Although these findings could be interpreted to suggest that sperm could express L-type VDCC, an additional body of data brings such a conclusion into question.

If indeed sperm express L-type VDCC, they differ significantly from those in somatic tissues (41,69,79,83,84,86-89). For example, the sensitivity of sperm VDCC to nickel is greater than for cadmium, a

characteristic of T-type currents. Bay K8644 does not bind to sperm membranes or stimulate sperm VDCC function. Bay K8644 is an agonist of L-type VDCC. Significantly higher concentrations of L-type calcium channel blockers are required to inhibit sperm calcium influx and acrosome loss, a characteristic also attributed to T-type currents. Cholesterol has no effect on calcium channel activity in sperm, while cholesterol modulates L-type VDCC activity in somatic cells. As a result, the same group of investigators first characterized mammalian sperm VDCC as "L-like" (69) and later as T-type (90). In somatic cells, both L-type and T-type VDCC participate in exocytosis and similar processes (e.g., 91,92).

Examination of the secondary messenger systems regulating acrosome exocytosis has not clarified the nature of the sperm VDCC. G-proteins and tyrosine phosphorylation form part of the signal transduction pathways regulating ion channel function and calcium influx (4,73,77,82,93-100). Inhibitors of G-protein and tyrosine kinase action inhibit both calcium influx and the acrosome reaction. Sertoli cells also express L-type VDCC (101). Both G-proteins and protein phosphorylation have been implicated as being integral to the signal transduction pathway activating these channels in Sertoli cells. This pathway is regulated by follicle stimulating hormone (FSH). L-type VDCC in somatic cells are activated by G-proteins (18,102) and tyrosine phosphorylation (103). Unfortunately, T-type currents are also regulated by G-proteins and tyrosine phosphorylation (104). Further, however, in sperm the possibility exists the G-protein pathway may not be directly linked to calcium influx, as one of the actions of G-proteins is to regulate zona pellucida-induced changes in intracellular pH (73).

The patch-clamp technique has been used to demonstrate ion channel activity in whole rat and sea urchin sperm and sperm heads (105,106). Direct electrophysiological characterization of the calcium ion channels in the sperm head plasma membrane is not, however, feasible because the small size, possibly resulting in an inability to form high-resistance seals, as well as the low success rate in displaying single-channel activity (96,105,107,108). Therefore, to further characterize the ion channels expressed in the male reproductive system, calcium currents have been assessed in immature germ cells (90,109-112) and in Sertoli cells in primary culture (113). Only a single type of VDCC (T-type) was identified.

A major point in the identification of spermatogenic cell currents as T-type currents was the time course of deactivation ("tail current") after removal of membrane depolarization. A relatively slow rate of closing was observed. In somatic cells, T-type VDCC are distinguished from L-type VDCC in that the tail currents of the former are at least ten times slower than the latter (114-116). A second point in the identification of VDCC expression revolved around the sensitivity of calcium currents to nickel. T-type VDCC are strongly blocked by nickel (117). The third point of identification was the sensitivity of the calcium currents to inhibition by semi-

specific antagonists of T-type channels. (Note that T-type calcium channel blockers are also antagonists of L-type channels; e.g., 38.) No function has been ascribed the T-type VDCC in Sertoli cells as they are not directly sensitive to FSH. In contrast, the T-type VDCC has been postulated to regulate calcium ion influx during acrosome exocytosis as it was assumed that the ion channels synthesized during spermatogenesis or spermiogenesis would persist in mature sperm (107,108). Even allowing for this, the proposed regulatory mechanism for control of T-type channel activity is inconsistent with other findings on the regulation of acrosome exocytosis described above. The VDCC encoding the putative male germ line T-type current has been reported to be activated by tyrosine dephosphorylation and inactivated by tyrosine phosphorylation (111).

Calcium currents have also been indirectly studied after transfer of ion channels from whole mature sperm or from isolated head plasma membranes to planar lipid bilayers. A cation-selective channel (118,119), a high conductance, calcium-selective channel with features comparable with the ryanodine receptor (120-122) and an L-like VDCC (121) have all been identified as the major ion channel mediating gamete interaction leading to the acrosome reaction. Potential reasons for these conflicting results include: (1) the fact that mammalian sperm express multiple ion channels in their head plasma membrane (4,119,123), (2) as many as ten ion channels have been identified in a single bilayer preparation (118), and (3) in the absence of reconstituting ion channels in the presence of other components necessary for exocytosis (e.g., 124), there is no way to connect a given isolated calcium current with induction of the acrosome reaction. The most interesting of the sperm channels that was identified was sensitive to dihydropyridines but: (1) showed no inactivation, and (2) did not require strong depolarization for activation (125). If this channel is an L-type VDCC, these two characteristics could result in defining it as "T-like". Further, it must be emphasized that none of these studies provide evidence for expression of a T-type calcium currents and, thus, T-type VDCC in mature sperm. This is in direct contrast to results from studies employing immature spermatogernic cells described above.

To complicate this problem, although calcium transport pathways appear to be conserved across species (e.g., 122,126), it may not always be possible to translate results concerning the electrophysiology or the sensitivity to pharmacological or inorganic agents of calcium ion channels obtained in one species to another. Three examples are provided to support this argument. First, a tendency has been observed in mammalian testis to form cell associations of constant composition (127). However, these cell associations within the seminiferous epithelium differ among species, e.g., rat versus man (127-129). Second, on average less than 40% of human sperm are able to undergo a spontaneous or induced acrosome reaction (60,62,130-132). In contrast, more than 80% of sperm from animal systems will exhibit acrosome loss (e.g., 2). Third, the effects of calcium channel blockers on spermatogenesis and on

acrosome exocytosis differ among man and animal systems. *In vivo* administration of members of the dihydropyridine class or benzothiazepine class of these agents results in a dose-dependent arrest of spermatogenesis in guinea pigs (133,134) but spermatogenesis in the human testis is unaffected (88,135). While these drugs as well the phenylalkylamine class of calcium antagonists markedly inhibit the human sperm acrosome reaction both *in vivo* and *in vitro* (83,88,89,126,136,137), they have been reported to stimulate calcium uptake by guinea pig sperm (138) and to be without effect on acrosome loss by golden hamster sperm (139). Importantly, even findings on the effects of calcium channel blockers on the human sperm acrosome and sperm fertility potential differ among laboratories (83,88,89,126,136,140-145). Taken together, these findings suggest that more than one VDCC subtype may be capable of triggering the acrosome reaction and emphasize the need for molecular cloning in order to directly identify the exact nature of the VDCC expressed in testis and sperm.

### 3.2. Data gained from molecular cloning

There is only one report in which the expression of the different VDCC alpha-1 subunits was examined in mammalian testis (110). In this study, reverse transcription-polymerase chain reaction (RT-PCR) was performed using forward and reverse primer sets specific for alpha-$1_A$, alpha-$1_B$, alpha-$1_C$, alpha-$1_D$ or alpha-$1_E$ and whole testis RNA or RNA from purified pachytene spermatocytes, round spermatids, condensing spermatids or residual bodies as template. Expression of all five alpha-1 subunits were detected using testis RNA as template and evidence for alternative splicing of the alpha-$1_D$ was also obtained. In contrast, the alpha-$1_E$ subunit was the major gene product in RNA from spermatogenic cells, with a low level of alpha-$1_A$ expression additionally observed. In the same study, patch-clamp recordings of pachytene spermatocytes indicated the presence of calcium currents that were transient with fast inactivation but were blocked by nifedipine. Based on: (1) the fact that the alpha-$1_E$ was previously identified as a low voltage activated T-type channel based on nickel sensitivity and transient activation (146,147), (2) their electrophysiological characterization of calcium currents in immature spermatogenic cells, and (3) the concept that the genome of the haploid mature spermatozoon is quiescent (148), Lievano *et al.* (110) conclude that spermatozoa, at least in the mouse, express T-type calcium channels.

Unfortunately, Lievano and co-workers (110) were mistaken on all three counts. First, there is now a body of evidence which suggests that the haploid genome is not transcriptionally dormant (review, 149,150), although evidence that translation occurs has not yet been obtained. Second, the effects of calcium channel antagonists on alpha-$1_E$ expression and on T-type channels differ (37,151). Third, and more importantly, they directly state that the only cloned member of the T-type channels is the rat brain alpha-$1_E$. Although they admit that an unknown alpha-1 subunit code could encode T-type currents, Lievano *et al.* (110) were apparently unaware of a series of papers demonstrating that the alpha-$1_E$ subunit is in fact a high

voltage-activated VDCC (39,152-155). The actual sequence of the only putative low voltage-activated T-type alpha-$1_G$ subunit differs significantly from that of any of the high voltage-activated alpha-1 subunits including alpha-$1_E$ (9; also see figure 2B).

Taken together, the molecular data of Lievano *et al.* (110) suggest that immature mammalian germ cells contain mRNAs encoding only high voltage-activated calcium channels.

## 4. ROLE OF L-TYPE VDCC IN THE ACROSOME REACTION AND HUMAN MALE INFERTILITY

My impetus to characterize the calcium channels expressed in mammalian sperm was derived from an unexpected IVF fertilization failure with normospermic semen (88). The patient's sperm exhibited a reduced ability to undergo agonist-stimulated acrosome exocytosis. The patient was medicated with nifedipine, a calcium ion channel blocker (156,157), for hypertension control. As (1) agonist-stimulated calcium influx initiates the physiological acrosome reaction (e.g., 4,69,79,158) and (2) fertility was restored following discontinuation of nifedipine (136), these observations suggested that the observed inhibition of sperm fertilizing potential was causally related to administration of nifedipine. Subsequently, 19 additional cases of human male infertility directly attributable to calcium entry antagonists were identified (88,135,136; A. Hershlag and S. Benoff, unpublished observations).

The alpha-1 subunit of the L-type VDCC contains binding sites for nifedipine and other calcium channel blockers (43,47-49,159,160). Rat and human sperm express antigenic epitopes on their equatorial/post-acrosomal head plasma membranes which are shared with those of the alpha-1 subunit and dihydropyridine receptor of the rabbit skeletal muscle VDCC (126,161-163; see figures 3A-D). Taken together, these observations suggested that: (1) the pharmacological blockade of L-type VDCC contributed to the production of male infertility in patients taking calcium entry antagonists, and (2) PCR primers derived from somatic L-type VDCC alpha-1 sequences could be used to clone the L-type VDCC expressed in sperm.

Using such methodology, we have cloned and characterized the entire mRNA encoding the L-type VDCC alpha-$1_C$ subunit that is expressed in rat and human testis (31,126,164,165). The complete cDNA sequence and the deduced amino acid sequence of the rat testes alpha-$1_C$ subunit appears in Goodwin *et al.* (31). We have observed four main regions in which the rat testis-specific VDCC alpha-$1_C$ subunit differs from that of the L-type VDCC alpha-$1_C$ of cardiac muscle. The observed diversity is due, in part, to alternative exon usage from a single primary transcript, which we have directly demonstrated by genomic PCR analysis encompassing multiple exons and the intervening intronic sequences (164): (1) domain I segment 6 (IS6) (preferential usage of exon 8A vs. exon 8 in cardiac muscle), (2) domain III segment 2 (IIIS2) (preferential use of exon 21 vs. exon 22 in cardiac muscle), and (3) domain IV segment 3 (IVS3) (preferential use of exon 31 vs. exon
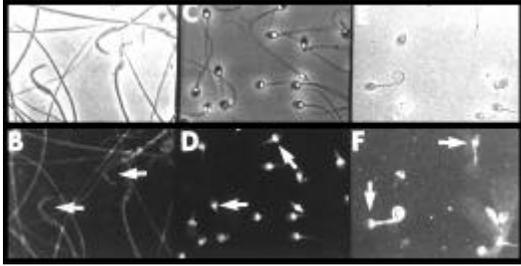
**Figure 3.** Rat and human sperm bind antibodies directed against L-type VDCC and phosphotyrosine residues. Identification of antigenic epitopes expressed by mammalian sperm. Indirect immunocytochemical labeling was performed following previously published protocols (4,126,178,250). Labeled sperm were viewed at X600 magnification with UV-epifluorescence illumination and photographed at X1500 magnification on 400 ASA film with an exposure time of 50 seconds. Paired phase (A,C,E) and epifluoresence (B,D,F) are shown. (A,B) Unfixed non-permeabilized rat sperm display unique staining with polyclonal sheep antibody 006 limited (anti-skeletal muscle dihydropyridine receptor; 251) to the post-acrosomal region of the head (126). No staining is observed with control aliquots not exposed to primary antibody (not shown). (C,D) Unfixed Triton-permeabilized preparations of motile human sperm from fertile donors bind monoclonal antibody IIF7 (anti-skeletal muscle dihydropyridine receptor; 161) over the equatorial/post-acrosomal regions of their heads (126,178). This binding pattern differs signficantly from that observed with anti-actin or anti-myosin antibodies (176,178,219,250) and is not observed in the absence of primary antibody (not shown). (E,F) Anti-phosphotyrosine monoclonal antibody clone 1G2 (Boehringer Mannheim Corp., Indianapolis, IN) binds to the tails of capacitated motile human sperm, confirming results from another laboratory (252). In addition, this antibody binds to the human sperm head in two distributions: (i) over the acrosome cap, or (ii) limited to the equatorial/post-acrosome regions (4,178). The percentages of sperm displaying clone 1G2 binding in the equatorial/post-acrosome regions is significantly increased after induction of the acrosome reaction with model zona ligand. containing mannose while the percentages of sperm displaying clone 1G2 binding over the acrosome cap was unaffected by this treatment (4,178). Typical results are shown with the arrows pointing to sperm binding clone 1G2 in the equatorial/post-acrosome regions. This distribution coincides with that observed for antibodies to L-type VDCC alpha-1 subunit (see Part D).

32 in cardiac muscle) (e.g., see figures 1 and 4) (31,126,164). Exon 8A has not previously been described in the gene encoding the cardiac alpha-1 subunit (166). Additional diversity is derived from the use of alternative promotor sequences, resulting in use of alternate exon 1A and a truncated 5' end in the testis-specific sequence (31). All the differences we have identified occur in regions of the VDCC alpha-1 subunit that regulate the gating kinetics and dihydropyridine sensitivity of the VDCC (19,22,47-49).

The regions of alternative splicing observed in the testis-specific alpha-$1_C$ subunit conform to those observed in somatic tissues (7,8,13). The amino terminus is the only region which is spliced in a tissue-restricted manner (8). The amino terminus of the testis-specific alpha-$1_C$ subunit is also expressed in brain, smooth muscle from lung and fibroblasts (167-169). The IS6 sequence observed in the testis-specific alpha-$1_C$ subunit is found in the alpha-$1_C$ subunit expressed in smooth muscle from lung (167). The IIIS2 segment of the testis-specific alpha-$1_C$ subunit is detected in brain (48,168,170). The IVS3 segment of the testis-specific alpha-$1_C$ subunit is expressed in skeletal muscle where, however, the alpha-1 subunit is the product of a separate gene (alpha-$1_S$; 13).

It is likely that two of the alternate splicing events detected, i.e., transmembrane segment IS6 and the amino terminus, could directly affect the electrophysiological characteristics of the calcium current in sperm. With regard to the former event, electrophysiological characterization of the L-type VDCC alpha-$1_C$ expressed in smooth muscle indicates that this subunit deactivates slowly, in a manner ascribed to T-type channels (171,172). This is probably the result of expression of an alternately spliced alpha-$1_C$ transcript in the region encoding exon 8 (IS5-IS6 linker/IS6) in smooth muscle and fibroblasts as compared to cardiac muscle (167), which modulates voltage-dependent inactivation (22). As the IS5-IS6 linker contributes to ion selectivity (7,173), this alternate splicing event could provide an explanation as to why the testis-specific VDCC is more sensitive to nickel and less sensitive to cadmium than classical L-type VDCC (69,79; also see Section 6). Additional changes in channel inactivation kinetics could result from the alternatively expressed amino terminus (47). The amino terminus is cytoplasmic (see figure 1). Molecular modeling using related potassium channels (174,175) indicates that the amino terminus can directly occlude the ion pore of the alpha-1 subunit.

These findings indicate that the electrophysiological properties of alpha-$1_C$ subunit expressed in rat (and human testis and sperm; see below) would not exactly fit those expected of the prototype L-type VDCC alpha-$1_C$. This conclusion is supported by studies in excitable somatic cells in culture, where divergent genotypic and phenotypic expression of alpha-1 subunits has been reported (30). Our data is also consistent with observations on calcium currents in smooth muscle *in vivo*. These cells exclusively express alpha-$1_C$ subunits (167) but display both typical L-type currents and a nifedipine-sensitive current with slow tail current decay, e.g., T-like (171,172). Nevertheless, we needed to rule out the possibility that "T-type" alpha-$1_G$ subunits were also expressed in these tissues.

Using three sets of primers derived from the rat brain alpha-$1_G$ sequence (9) and rat brain RNA as template, robust products of the expected size and sequence were obtained (176; see figure 2B). In contrast, we were unable to amplify the sequences encoding the pore of the alpha-$1_G$ subunit in rat testis RNA. Positive control reactions using actin primers and either template gave similar results. Thus, to date, there is no direct evidence for the expression of alpha-$1_G$ subunits in mammalian testis.

EXON  30  31  32  33  34
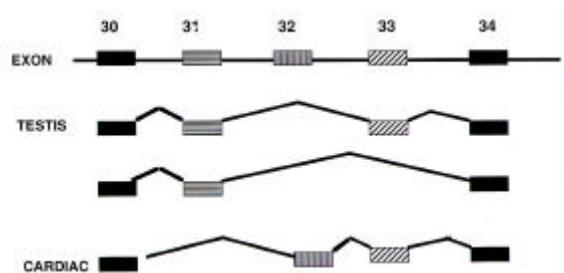
TESTIS

CARDIAC

**Figure 4.** Comparison of the splicing patterns of the alpha-$1_C$ subunit mRNA observed in human testis and cardiac muscle (165). Transmembrane region IVS3 participates both in dihydropyridine binding and in voltage gating by forming salt bridges with the S4 voltage sensor. IVS Exons 31 and 32 encode alternate IVS3 segments in the alpha-$1_C$ gene. Only exon 32 is detected in cardiac muscle. In contrast, exon 31 is expressed in testis. In 10% of the clones examined from testis RNA as template, a deletion of exon 33 was observed (see table 1). Exon 33 encodes a short linker segment between IVS3 and its voltage sensor.

**Table 1.** Catalogue of the splicing variants of the human testis alpha-$1_C$ subunit.

| Segment | Exon | Tissue-specificity | Number of clones |
|---|---|---|---|
| Amino terminus | 1 | Cardiac | 0/20 |
| | 1A | Testis | 20/20 |
| IS6 | 8 | Cardiac | 15/16 |
| | 8A | Testis | 1/16 |
| IIIS2 | 21 | Cardiac | 2/16 |
| | 22 | Testis | 13/16 |
| | 21 + 22 | Cardiac/Testis | 1/16 |
| IVS3 + linker | 31 + 33 | Testis | 36/50 |
| | 32 + 33 | Cardiac | 3/50 |
| | 31 + 32 + 33 | Testis/Cardiac | 2/50 |
| IVS3 w/o linker | 31 | Testis | 5/50 |
| | 32 | Cardiac | 3/50 |
| | 31 + 32 | Testis/Cardiac | 1/50 |

RT-PCR, cloning of the PCR products and DNA sequencing were performed using previously published protocols (31,126,164).

Importantly, analysis of the human testis-specific VDCC sequence (165) reveals additional diversity that we did not catalog in the rat (31). In contrast to the rat where essentially only two VDCC isoforms were detected in testis, 12 different alternative splicing events in alpha-$1_C$ transcripts have found in human testis (table 1), which in combinations produce at least 16 different VDCC isoforms. For example, in the IVS3 alternatively expressed region, we have observed a 72% use of exon 31 in the population (testis poly A+ RNA source pooled from 10 male subjects 10-60 yrs.). However, we have also found that 10% of the time there is a deletion following exon 31, which corresponds with exon skipping of exon 33 (figure 4). This exon acts as a linker from IVS3 to IVS4. Upon depolarization, the charged residues in S4 are thought to rotate toward the extracellular face of the bilayer. If there is a restriction on the size and/or increased rigidity of the S3 to S4 linker segments, this may have an influence on the kinetics of channel opening.

Results from RT-PCR *in situ* and from immunocytochemistry indicate that VDCC alpha-$1_C$ subunit mRNA and protein are expressed in rat testis sections throughout the seminiferous epithelium, both in all stages of the germ cell lineage and in Sertoli cells, but not in the interstitial space (164). Examination of mRNA from cultured Sertoli cells confirms that Sertoli cells express the testis-specific VDCC alpha-$1_C$ isoform (31). VDCC alpha-$1_C$ transcripts have recently been detected in RNA from ejaculated human sperm, suggesting post-meiotic VDCC gene expression (177).

In addition, we now have documented inter-individual variability in testis-specific L-type VDCC alpha-$1_C$ isoform expression. In a preliminary study of ten men, we found that each man reproducibily expressed, in ejaculated sperm, RNA populations encoding only one of the 16 isoforms (L.O. Goodwin, D.S. Karabinus, R.G. Pergolizzi and S. Benoff, submitted). In two of these cases, we detected a deletion of exons 31 through 33 (see Table 1), suggesting that the calcium ion pore would be non-functional in these men. These data suggest the existence of human sperm VDCC isoform diversity relevant to sperm fertilizing potential. The examination of this potential VDCC isoform diversity in fertile and infertile men is in progress.

Finally, we have identified two mechanisms regulating VDCC function which are unique to sperm (4).

First, channel opening is regulated by ligand-stimulated phosphorylation of unique tyrosine residues in IS6 and/or IIIS2, which is inhibited *in vivo* (178) and *in vitro* by dihydropyridines (4; e.g., see figures 3F and 5), suggesting a mechanism by which calcium transport is abrogated by these drugs. The human sperm mannose receptor plays an important role in this process: binding of mannose moieties is associated with VDCC tyrosine phosphorylation (figure 5; 4,82). The deduced amino acid sequence of the testis-specific alpha-$1_C$ subunit provides no evidence for an intrinsic tyrosine kinase activity and, thus, autophosphorylation. Rather, we suggest (4) that this phosphorylation is effected by the ZRK/hu9 tyrosine kinase activity of the sperm head which is postulated to play a regulatory role in induction of the acrosome reaction (179,180).
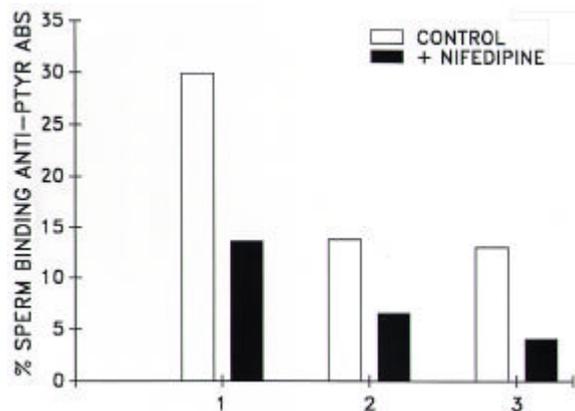
**Figure 5.** Nifedipine, an L-type dihydropyridine calcium channel anatagonist, inhibits protein tyrosine phosphorylation induced by exposure of human sperm to model zona ligands containing mannose. Typical results are shown for motile sperm populations from three fertile donors incubated overnight in capacitation media supplemented with 5 uM nifedipine (4,88,178). Control aliquots were not exposed to the drug. Acrosome loss was induced by mannose treatment (61,63,131,250) and sperm were then labeled anti-phosphotyrosine monoclonal antibody clone 1G2 (Boehringer Mannheim Corp., Indianapolis, IN) (4,178). At least 300 sperm in each aliquot were examined for antibody binding ("ANTI-PTYR ABS") in the equatorial region of the sperm head (see Figure 3F). Exposure to nifedipine resulted in a significant inhibition of the both acrosome loss (not shown) and anti-phosphotyrosine antibody binding (t-test, P <0.05). Although only one specimen from each fertile donor was analyzed, these observations confirm prior *in vitro* (4,178) and *in vivo* (178) findings.

Second, proteolysis appears to be involved in the formation of the active channel (4). Western blot of membrane proteins extracted from fertile human donor sperm and probed with antibodies specific for the L-type VDCC alpha-1 subunit indicate the presence of two antigenic protein species (figure 6, lane 2). The first species migrates between 165 to 175 kDa, consistent with the size reported for the alpha-1 subunit expressed in somatic tissues (161,163). The second species, which migrates at approximately 60 kDa, has never been reported for VDCC from somatic tissues, and was not observed in the rat germ line (164). Preliminary studies suggest that this lower molecular weight protein is not detectable in extracts from sperm from infertile men (4; figure 6, lane 1). The loss of this protein species is likely to be of clinical importance as about 50% of cases of reduced or failed fertilization in IVF exhibit normal levels of expression of receptors for human zona ligands containing mannose but a reduced ability to undergo a mannose-stimulated acrosome reaction (131,181).

These data unequivocally demonstrate that an L-type VDCC alpha-$1_C$ subunit is expressed in mammalian testis and sperm and provide evidence for a role for this subunit in the mammalian sperm acrosome reaction.

## 5. CONTRACEPTION

Men and women have similar attitudes and knowledge levels of contraception (182,183). Currently, however, primary prevention of pregnancy is largely borne by the female partner. This is due, in part, to the disadvantages of the limited number of contraceptive options available to the male, e.g., suppression of spermatogenesis (hormonal methods) or prevention of sperm transport (vasectomy; condoms) (review, 83). Lack of interest in the public sector and financial considerations have deterred the pharmaceutical industry and have limited initiatives in male reproductive research and development (184,185).

Reports on adverse effects of drugs created for other purposes have been the main source of new male contraceptive protocols (186), e.g., that therapeutic administration of calcium channel blockers for hypertension control and chronic migraine headaches results in a reversible infertile state (88,89,135-137). The latter has suggested that calcium channel blockers can be developed into non-hormonal, reversible and safe male contraceptives.

Initial studies showed that calcium channel blocker-associated sperm dysfunction was the result of inhibition of the capacitation process, limiting cholesterol efflux and surface appearance of binding sites for zona pellucida carbohydrate moieties (88,89,136). While further studies have confirmed these findings, such studies have also indicated that the pharmacological activity of calcium channel blockers which limits calcium entry into sperm is also required to inhibit the ability of human sperm to respond to agonists of the acrosome reaction (83, 126). Thus, both the biophysical effects of nifedipine on membrane structure (e.g., effects on membrane width and packing through favoring cholesterol retention) and its pharmacological calcium channel blockade limit male fertility potential. Therefore, calcium entry antagonists also effective as contraceptives must regulate both of these parameters.

With regard to inhibition of membrane cholesterol efflux, we recognize that modest chemical differences between dihydropyridine analogues substantially affect their binding to the membrane lipid bilayer, affecting drug bioavailability (44-46). For example, the charge (e.g., positive, negative or uncharged) on a particular antagonist limits that analogue to a specific region within the membrane. Limitation of drug distribution to, for example, near the hydrocarbon core/water interface or hydrophobic core affects both drug dosage requirements and duration of drug activity (45,187).

With regard to calcium channel blockade, three of the alternatively spliced regions we have identified in the testis-specific L-type VDCC, in addition to involvement in regulation of channel kinetics, are also thought to bind calcium channel blockers: IS6 (43,47,49,50), IIIS2 (48) and
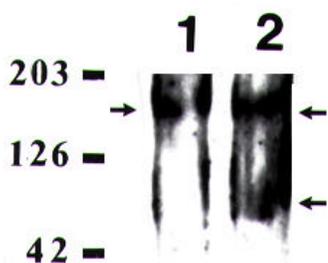
**Figure 6.** Expression of the alpha-1$_C$ subunit protein differs between fertile and some infertile men. Sperm plasma membrane extracts, SDS-polyacrylamide gel electrophoresis of sperm membrane proteins in the presence of pre-stained molecular weight size standards (Bio-Rad Laboratories, Hercules, CA), transfer of size-separated proteins to nitrocellulose membranes, reaction with monoclonal antibody IIF7 (anti-skeletal muscle dihydropyridine receptor; 161) and detection of antibody binding using Renaissance Chemiluminescence Reagent (NEN Dupont, Boston, MA) were performed as previously described (4,164). Typical results are shown. The migration of the size standards (203 kDa, 126 kDa and 42 kDa) is indicated to the left. The arrows identify the positions of protein species which bind antibody. (Lane 1) In a specimen from a patient who exhibited complete failure of fertilization *in vitro* as a result of an acrosome reaction insufficiency (131) only one protein species, $M_r$ 165-175 kDa, was reactive with monoclonal antibody IIF7. (Lane 2) In contrast, two proteins species, $M_r$ 165-175 kDa and 60 kDa, which contain antigenic epitopes which are recognized by monoclonal antibody IIF7 are observed in plasma membrane protein extracts prepared from fertile donor sperm. These data confirm prior findings (4,176).

IVS3 (47). Analysis of the predicted secondary structure and hydrophobicity of these alternatively spliced regions (31,126,164) and the marked time-dependence of *in vitro* inhibition by nifedipine of progesterone-stimulated acrosome loss by fertile donor sperm (126) indicate that the affinity of the testis-specific L-type alpha-1$_C$ isoform for calcium channel blocking drugs would differ significantly from that of alpha-1$_C$ isoforms expressed in somatic tissues.

Having identified specific changes in the structure of the alpha-1$_c$ subunit of the L-type VDCC alpha-1 of mammalian sperm, we now intend to identify or develop calcium entry antagonists that target this altered structure. (This is a well traveled route of drug development. Structure-activity requirements have been defined for drugs that block related ion channels, e.g., that for sodium) (188). Focusing on drugs which target calcium channels, nifedipine analogues have been produced which have either symmetrical ester groups or non-identical ester groups. Those with non-identical ester groups demonstrate tissue selectivity, e.g., nitrendipine lowers peripheral vascular resistance while nimodipine acts as a cerebral vasodilator (review, 160).

To describe the molecular basis for this tissue

specificity, Langs *et al*. (160,189) have used crystal structures and molecular modeling to postulate that a dihydropyridine binding cleft in the S4 transmembrane segment, Arg-X-X-Arg-P-X-X-S, is altered by changes in amino acid composition, and that these changes regulate an ester-sensing function. We suggest that this ester-sensing function of the S4 alpha helix can also be modulated by interactions with S2/3 alternate exons specifically expressed in testis. We will search for ester side chain conformations that will fit the dihydropyridine binding cleft of the testis-specific alpha-1$_C$ subunit. This seems a reasonable approach as tissue selectivity is a major criterion in the search for new calcium channel ligands (review, 51).

Naturally occurring calcium channel ligands offer an alternate source of agents to be tested for contraceptive potential. These include toxins produced by invertebrates and flora, which can bind directly to the L-type VDCC and possess calcium channel blocking properties, as well as endogenous ligands whose action may be mimicked by the existing pharmacological agents, such as small peptides identified in vascular smooth muscle, brain and erythrocytes (review, 51).

Identification of calcium entry antagonists which meet our requirements and are suitable for detailed studies of contraceptive efficacy will be facilitated by studying drug binding to expressed recombinant testis-specific alpha-1$_C$ subunit and to this recombinant protein following its insertion into membrane bilayers of differing composition. These experiments are in progress in my laboratory.

## 6.  SUSCEPTIBILITY TO ENVIRONMENTAL TOXICANTS

Although high level exposure to suspected reproductive toxicants in an occupational setting has been associated with a reduction in human male fecundity (e.g., 190), the effects of chronic low level environmental exposures to such toxicants on male reproductive health have not hitherto been systematically evaluated. A recent prospective study of consecutive couples undergoing their first cycle of fertilization *in vitro* performed by my laboratory revealed that a significant fraction of men with unexplained infertility were presenting with high blood and semen levels of exposures to suspected reproductive toxicants (e.g., lead; 191), despite the men not being occupationally exposed. More importantly, sperm dysfunction (specifically, inhibition of the induced acrosome reaction) was observed in these subjects in the absence of other outward signs such as abnormal semen parameters or altered serum hormone levels. These findings raise two issues: (1) an infertility patient population may contain a subgroup which is predisposed to strongly respond to environmental toxicant exposures, and (2) there is a pressing need to identify functional and genetic biomarkers responsive to the effects of low level exposures to environmental toxicants on human sperm fertilizing potential. VDCC isoforms may be relevant to both.
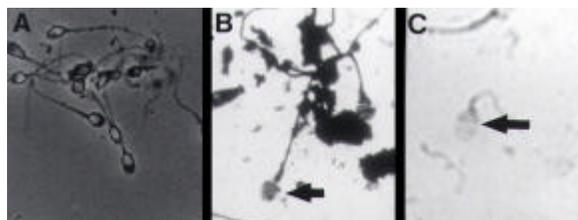
**Figure 7.** Detection of metal ion deposits in human sperm by autometallography. Motile sperm from fertile donors (n = 9) was incubated overnight in capacitation media supplemented with 22 mM zinc or 146 ug/L cadmium (176,177). Control aliquots not exposed to metal ions. Sperm were then washed free of exogenous metals and metal deposits were visualized after conversion to metal sulfides and physical development with silver lactate (253,254). Typical results from a single sperm donor are shown. Zinc and cadmium deposits co-localize with sperm head VDCCs detected by indirect immunofluorescence (see Figure 3D). (A) No silver deposits are observed on zinc-treated sperm not exposed to sodium sulfide prior to autometallography. (B) The arrow in the figure delineates the accumulation of zinc (black silver deposits) in the equatorial region of the human sperm head. (C) The arrow points to black silver deposits in the equatorial region of the human sperm head following cadmium exposure. Note that the shape of the sperm is altered by cadmium.

There is considerable evidence for differential susceptibility to a toxicant within a population. For example, in an animal system, the fertility of a strain of mice with high fecundity (Swiss) was marginally affected by exposure to ethylene glycol ethers while a strain with low fecundity (C3H) exhibited a large decline in fertility (192). Similarly, in a study of shipyard painters, ethylene glycol ether exposures produced a greater decrease in sperm counts in men who were oligospermic than in men who were normospermic (193,194). We therefore consider it significant that the calcium channel blockers nifedipine, verapamil and diltiazem protect against ethylene glycol ether testicular toxicity in rats *in vivo* (195) and in cultured rat and human testicular tissue *in vitro* (196). These observations suggest that the mechanism underlying the reproductive toxicity of ethylene glycol ethers may involve VDCC.

Calcium channel blockers also limit: (1) the neurotoxicity of organophosphorus compounds used in agriculture (197), (2) the stimulation of gastric secretion and gastric ulcer formation by ethanol and indomethacin (198), (3) liver cell death induced by the potent marine toxin maitotoxin (199), and (4) the augmentation of glucose-induced insulin secretion by ethanol in the pancreas (200). These data indicate that VDCC mediate the effects of a variety of occupational and environmental toxicants.

In many cases, cellular calcium homeostasis and calcium-mediated cell processes are thought to be critical targets for metal toxicant action (201-208). VDCC in somatic tissues have been reported to transport lead, zinc,

aluminum, mercury in addition to calcium (209-213). Similarly, permeation of zinc, cadmium, nickel, cobalt and manganese in the male reproductive tract occurs through VDCC expressed in mammalian testis and sperm (69,79; see figure 7). Nifedipine, a potent calcium channel antagonist, inhibits lead entry into bovine chromaffin cells (209) and in the frontal cortex of mouse brain (214). Nifedipine blocks cadmium-induced toxicity in mouse pre-implantation embryos by inhibiting intracellular cadmium accumulation (215). Calcium channel blockers also inhibit cadmium uptake by somatic cells (216,217). Interestingly, in a population study, calcium metabolism in men appears more sensitive to environmental metal ion exposures, e.g., cadmium, than that of women (218). These findings are important for our attempt to develop an understanding of the etiology of varicocele-associated infertility.

We (176,219) and others (220,221) have observed that sperm from infertile men with varicocele display acrosome reaction insufficiencies. Blockage in the pathway leading to acrosome exocytosis occurs at a point after initiation of signal transduction by zona ligands containing mannose. In addition, my laboratory has reported that an increase in seminal plasma cadmium levels is a defect specifically observed in specimens from infertile men with varicocele but not in specimens from men with other types of infertility (219). This increased cadmium accumulation was observed in varicocele patients who were not occupationally exposed to cadmium and who did not smoke cigarettes, another source of cadmium (222). *In vitro* exposure of fertile donor sperm to cadmium produced deficits in the acrosome reaction which mimic those observed in sperm from infertile men with varicocele (219). These observations suggest a causal relationship exists between cadmium exposures and varicocele-related acrosome reaction insufficiency.

Our *in vitro* modeling studies also indicated that cadmium must enter to sperm head in order to exert its deleterious effects (S. Benoff, unpublished observations). Sites of entry of exogenous metal ions such as zinc and cadmium were identified by autometallography. These sites co-localize with sperm head VDCC (figure 7; 176,177). The effects of metal ions on VDCC differ among cell types and among channel subtypes (69,210). We therefore suggest that the acrosome reaction insufficiency of varicocele sperm may be the result of defective calcium influx through ion channels of altered structure, possibly poisoned by cadmium.

That varicocele-associated infertility could result from the combined presence of a varicocele, a defect in cadmium metabolism and a specific L-type VDCC alpha-$1_C$ subunit isoform seems very reasonable. There is precedence for defective ion transport as the underlying cause of at least one form of human male infertility (223,224). More to the point, alterations in expression of the alpha-1 subunit of the various types of calcium channels have been documented to produce disease states in animals and in man (review, 5). For example, sequence changes, including those in transmembrane segment IIIS2, of the alpha-$1_A$ result in familial hemiplegic migraine and episodic

ataxia type-2 where patients exhibit migraine-like symptoms (225). Alternate splicing in the carboxy terminus of this subunit is linked to a severe form of human ataxia (226). The latter finding is of particular importance given that we have identified multiple splice variants of the human alpha-$1_C$ subunit that are expressed in testis and sperm.

In attempting to define the role of VDCC isoforms in the etiology of varicocele-associated infertility, we focus on the alternate splicing events regulating expression of the IS5-IS6linker/IS6 transmembrane segment (31,165,176; see Section 4 and Table 1). In related sodium channels, mutations resulting in single amino acid changes within this region regulate whether the channel will be resistant or sensitive to cadmium (227,228). We have identified a series of amino acid variants within this region in the alpha-$1_C$ sequence in rat testis (31,176). A similar survey of the human testis/sperm alpha-$1_C$ sequence is in progress.

Taken together, these observations described above indicate that (1) an important mode of action of some reproductive toxicants is disruption of normal calcium homeostasis, (2) heavy metal ion and lipophyllic toxicants exert their effects via an interaction with VDCC in the male reproductive tract, and (3) a VDCC isoform expression assay may be developed into a biomarker to permit, for the first time, generation of data concerning quantitative early warning signs of reproductive toxicity as well as earlier recognition of especially vulnerable males.

## 7. PERSPECTIVES

Changes in intracellular ion content play pivotal roles in sperm function. In particular, stepwise increases in ionized calcium are an absolute requirement for acrosome loss induced by zona pellucida glycoprotein 3, zona carbohydrate moieties and by progesterone. Molecular cloning has established that VDCC mRNAs are expressed in mammalian testis, purified immature spermatogenic cells and ejaculated sperm. Immunocytochemical and fluorescent inhibitor binding studies have localized VDCC proteins on cellular membranes. Biological studies (i.e., electrophysiology; sensitivity to pharmacological and inorganic agents) indicate that these VDCC in the sperm head plasma membrane regulate the level of intracellular calcium.

### 7.1. Conclusions

Calcium current type in sperm, as defined by patch clamp methodology and pharmacology (i.e., T-type), does not correspond with alpha-1 subunit gene expression as defined by molecular cloning (i.e., RNA sequences first attributed to high voltage-activated channels). These disparate results may not really be at odds. It appears to be a matter of semantics, i.e., whether sperm calcium channels are being defined by electrophysiology or by sequence analysis. Proteins which typically form part of high voltage-activated calcium channels can produce currents reminiscent of low voltage-activated channels following native expression in somatic cells (30) or following *in vitro* expression of cloned alpha-1 subunits (32). It is highly likely that these high voltage-activated calcium channel proteins modulate the acrosome reaction of the fertilizing

spermatozoon. Although electrophysiology is useful in defining the operational characteristics of the macromolecular assembly which forms native VDCC, so that there is no ambiguity it is suggested that in the future sperm calcium currents should be defined by the RNA sequences which encode the VDCC.

### 7.2. T-type calcium currents and VDCC subunit composition

Both HVA alpha-$1_E$ and alpha-$1_C$ mRNAs are expressed in the mammalian male germ line. However, alpha-$1_E$ expression has not been examined in ejaculated sperm. In contrast. the findings cited in Section 4 support the suggestion that the observed diversity in L-type VDCC alpha-$1_C$ subunit structure contributes to differences between men in the ability to undergo agonist stimulated acrosome loss and in human male fertility. If it can be shown that a causal relationship exists between expression of a particular alpha-$1_C$ isoform and a characteristic rate of acrosome loss, this could ultimately facilitate more rational, individualized treatment of male infertility within the setting of assisted reproduction. Identifying such a relationship would establish testis-specific alpha-$1_C$ isoforms as potential biomarkers of individual response to environmental and occupation exposures (229). In epidemiology, it could be used to elucidate the role of a single agent following exposure to complex mixtures (230). Finally, the interaction between membrane composition and membrane-imbedded channel function, isoform diversity and potential ethnic differences (e.g., 231) in isoform diversity must all be considered in the development of calcium channel antagonists as male contraceptive agents.

Differential splicing of the IS6 region (31), which participates in voltage- and calcium-dependent inactivation (8,22,23), which occurs in the testis-specific isoform of the L-type alpha-$1_C$ subunit and which results in the expression of IS6 sequence normally found in smooth muscle isoforms, may help explain physiological evidence suggesting that voltage-dependent calcium currents with properties of T-type currents are the main calcium current carrier in the male reproductive tract (90,111-113).

An alternate explanation is also possible (31). The beta subunit modulates inactivation kinetics (232,233). Four different calcium channel beta subunits have been identified which are encoded by different genes (232). Two functions have been ascribed to these beta subunits. First, the beta subunit apparently regulates folding of the alpha-1 subunit and its association with membranes (55,234,235). The beta subunits, unlike many of the alpha-1 subunits, can associate with membrane when expressed alone. Second, the beta subunits have been observed to modulate the functional properties of various alpha-1 subunits both *in vivo* (154,236,237) and when co-transfected into L cells or Xenopus oocytes (52,53,238-240). In particular, the biophysical (i.e., activation state) and pharmacological properties of the alpha-$1_C$ subunit is strongly affected by the presence of beta subunits (241). Association of beta-1, 2 or 3 subunits with a high voltage-activated alpha-1 subunit can make it express "T-like" currents (242). It is therefore possible that a similar phenomenon occurs in sperm. These

two functions may be complementary.

Beta subunit isoforms are produced by transcription of different genes and by alternative splicing of the individual transcripts (7,8,232,243). A mutation in a splice site of the beta-4 subunit gene has been associated with ataxia and seizures in the lethargic mouse (242). We therefore suggest variations in beta subunit expression may also contribute to the production of defective calcium influx, an acrosome reaction insufficiency and human male infertility. This suggestion will provide the initiative for future studies.

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