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Voltage-dependent calcium channels in mammalian spermatozoa revisited

Susan Benoff [1,2,3,11], Charles C. Chu [4,5,6,7], Joel L. Marmar [8], Rebecca Z. Sokol [9], Leslie O. Goodwin [10], and Ian R. Hurley [1]

[1] Fertility Research Laboratories, Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, New York, [2]Departments of Obstetrics and Gynecology, North Shore University Hospital, Manhasset, New York, [3]Departments of Obstetrics and Gynecology and Cell Biology, New York University School of Medicine, New York, New York [4]Laboratory of Gene Activation, Center for Immunology and Inflammation, The Feinstein Institute for Medical Research, [5]Department of Medicine, North Shore University Hospital, Manhasset, New York, [6]Department of Medicine, New York University School of Medicine, New York, NY, [7]Department of Biological Sciences, Long Island University, Brookville, NY, [8]Division of Urology, Robert Wood Johnson School of Medicine, Camden, New Jersey, [9]Departments of Obstetrics and Gynecology and Medicine, University of Southern California, Los Angeles, California, [10] Core Research Facility, The Feinstein Institute for Medical Research, [11]350 Community Drive, Room 125, Manhasset, New York 11030

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1. ABSTRACT

The last few years have seen an explosion in the number of voltage-dependent ion channel sequences detected in sperm and testes. The complex structural paradigm of these channels is now known to include a

pore-forming alpha$_1$ subunit(s) whose electrophysiological properties are modulated by an intracellular beta subunit, a disulfide-linked complex of a membrane-spanning delta subunit with an extracellular alpha$_2$ subunit, and a transmembrane gamma subunit. Many of these are alternatively spliced. Furthermore, the known number of genes coding each subtype has expanded significantly (10 alpha$_1$, 4 beta, 4 alpha$_2$delta, 8 gamma). Recently, the CatSper gene family has been characterized based on similarity to the voltage-dependent calcium channel alpha$_1$ subunit. From among this multiplicity, a wide cross-section is active in sperm, including many splice variants. For example, expression of the various alpha$_1$ subunits appears strictly localized in discrete domains of mature sperm, and seems to control distinct physiological roles such as cellular signaling pathways. These include alpha$_1$ alternative splicing variants that are regulated by ions passed by channels in developing sperm. Various combinations of ion channel sequence variants have been studies in research models and in a variety of human diseases, including male infertility. For example, rats that are genetically resistant to testes damage by lead seem to respond to lead ions by increasing alpha$_1$ alternative splicing. In contrast, in varicocele-associated male infertility, the outcome from surgical correction correlates with suppression of alpha$_1$ alternative splicing, Ion channel blockers remain attractive model contraceptive drugs because of their ability to modulate cholesterol levels. However, the large number of sperm ion channel variants shared with other cell types make ion channels less attractive targets for male contraceptive development than a few years ago. In this review, the genetics, structure and function of voltage-dependent calcium channels and related CatSper molecules will be discussed, and several practical clinical applications associated with these channels will be reported.

2. INTRODUCTION

It has long been recognized that calcium plays a crucial role in mammalian fertilization in several ways: [1] regulation of the acrosome reaction (review, 1-3), [2] regulation of capacitation (4-6), and [3] sperm recognition/binding of zona carbohydrate ligands (7). The role of calcium fluctuations in sperm physiology in invertebrates and vertebrates has recently been elegantly reviewed (8) and other recent publications re-emphasize the importance of calcium in fertilization, by identification of two novel calcium ion channels that are required for sperm motility and successful reproduction (9,10). These papers suggest that calcium entry is controlled by ion channels and, in fact, that the study of differential ion channels' expression in the male germ-line has already provided a partial understanding of the mechanisms producing some forms of human infertility (i.e., 11-13). Furthermore, it is important to also remember that the ion channels that transport calcium are also capable of transporting toxic metals, such as lead and cadmium (14,15), which have negative effects on reproductive potential (review, 16,17). For example, lead may not only antagonize the action of calcium but may also mimic the biological effects of calcium (18). Cadmium is a well-known calcium channel blocker but, upon entry into cells via the same calcium channels, cadmium effectively competes with calcium for calcium binding sites in many intracellular proteins and can also perturb calcium second messenger systems (e.g., 19,20). Thus, calcium homeostasis is a critical target for toxicant actions (21) in testis and sperm (e.g., 22). The channels that participate in these events are the subjects of the current review.

Calcium channels can be divided into two major classes, calcium entry channels (i.e., voltage-gated or voltage-dependent calcium channels [VDCCs], ligand-gated calcium channels, capacitative calcium entry channels) and calcium release channels (i.e., ryanodine receptors, inositol 1,4,5-triphosphate receptors). VDCCs trigger the release of calcium upon membrane depolarization (23). Ligand-gated calcium channels are receptors that open Ca^{2+} ion pores upon binding of chemical messengers (e.g., glutamate). Capacitative calcium entry channels are opened by depletion of intracellular calcium stores. A sequential interaction between members of both general classes of calcium channels has been proposed to control initiation of the mammalian egg-induced acrosome reaction, with activation of VDCCs being an early step in this pathway (24,25). VDCCs have also been shown to participate in the response to toxic exposures (12,16,17). To provide a detailed discussion of all the calcium channels expressed in the mammalian male germ line in terms of their structure would be a daunting task. This review will focus on expression of the VDCC and related CatSper gene families in mammalian testis and sperm based largely on our own research and clinical experience.

3. STRUCTURE OF VDCC AND CATSPER

3.1. VDCC classification

Multiple voltage-dependent calcium currents are detected in different cell types and can be distinguished using physiological and pharmacological criteria. Electrophysiological studies provide phenotypic evidence for the existence of both high voltage-activated (HVA) and low voltage-activated (LVA) calcium currents (Figure 1). HVA currents are slowly inactivating whereas LVA currents exhibit rapid inactivation and a negative inactivation range. Thus, LVA currents are "transient" or T-type. All T-type currents are sensitive to organic (dihydropyridine) antagonists. In contrast, HVA currents are further subdivided by their resistance (A-type, B-type, E-type) or sensitivity (L-type) to dihydropyridine channel blockers. Dihydropyridine-resistant HVA currents can be distinguished using different peptide toxins from snail and spider venoms.

3.2. VDCC molecular subunits

The molecular biology of VDCCs is well characterized and complex (28,28,29). Voltage-dependent calcium currents are produced by heteromultimeric ion channels. VDCCs are composed of an alpha$_1$ subunit with generally three auxiliary subunits, alpha$_2$delta, beta, and gamma in a 1:1:1:1 stoichiometry. Cloning and heterologous expression of the alpha$_1$ subunit demonstrated that this subunit in isolation can induce calcium currents

Table 1. Voltage-gated or voltage dependent calcium channels (VDCCs) alpha$_1$ subunits

Alpha$_1$ subunit	Class	Activation	DHP	Tissue distribution
$Ca_V1.1$ (alpha$_{1S}$) (skeletal muscle L)	L	High voltage	sensitive	skeletal muscle
$Ca_V1.2$ (alpha$_{1C}$) (cardiac or smooth muscle L)	L	High voltage	sensitive	heart, cardiac, smooth muscle, brain, neuronal cells, pancreas, endocrine cells, **testis, sperm**
$Ca_V1.3$ (alpha$_{1D}$)	L	High voltage	sensitive	sensory cells, endocrine cells, heart, smooth muscle, brain, neuronal cells, **testis**
$Ca_V1.4$ (alpha$_{1F}$)	L	High voltage	sensitive	retina
$Ca_V2.1$ (alpha$_{1A}$)	P/Q	High voltage	insensitive	brain, neuronal cells, heart, pancreas, pituitary, **testis, sperm**
$Ca_V2.2$ (alpha$_{1B}$)	N	High voltage	insensitive	brain, neuronal cells, **testis, sperm**
$Ca_V2.3$ (alpha$_{1E}$)	R	High voltage	insensitive	brain, neuronal cells, heart, pituitary, **testis, sperm**
$Ca_V3.1$ (alpha$_{1G}$)	T	Low voltage	sensitive	brain, neuronal cells, ovary, placenta, heart, **testis, sperm**
$Ca_V3.2$ (alpha$_{1H}$)	T	Low voltage	sensitive	brain, neuronal cells, adrenal cortex, kidney, liver, heart, smooth muscle, **testis, sperm**
$Ca_V3.3$ (alpha$_{1I}$)	T	Low voltage	sensitive	brain, neuronal cells, **testis, sperm**

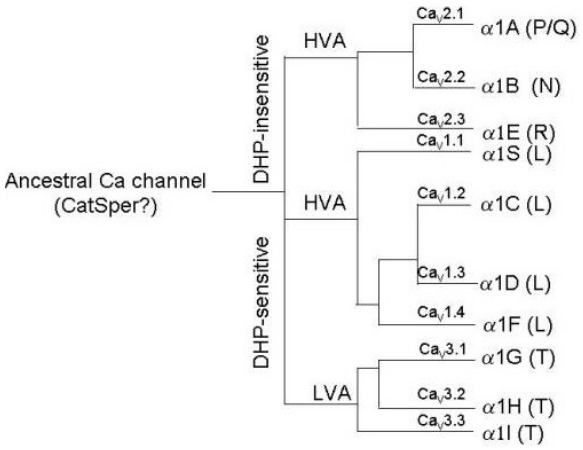

Figure 1. Phylogenetic representation of the sequence similarity of VDCC alpha$_1$ subunits modified from Catterall *et al.* (26). There are three families of VDCC alpha$_1$ subunit genes, 1 to 3. Within a family of HVA or LVA subunits, the amino acid sequences exhibit 70% identity. Between VDCC families, there is less than 40% amino acid sequence identity (26). Sequence divergence is particularly evident in the N and C termini and in the intracellular linkers. The four Catsper subunits exhibit significantly less sequence identity, approximately 22-27% across the ion pore, than do the VDCC families (27).

(review, 1). Thus, the alpha$_1$ subunit forms the ion pore and possesses gating functions that primarily characterizes the VDCC according to their T, L, N, P, Q, or R classification. Ten alpha$_1$ subunits have been identified (Table 1).

3.3. VDCC alpha$_1$ subunit

The structure of the alpha$_1$ subunit has been modeled on structurally homologous voltage-gated channels, such as K^+ and Na^+ channels (30-32). Although smaller alpha$_1$ subunits have been reported (33-37), the typical alpha$_1$ subunit is 190-220 kDa and is composed of four homologous domains (I-IV; Figure 2A) symmetrically arranged around a central pore (Figure 2B). Each domain is composed of six transmembrane segments (S1-S6), with S5, S6 and the extracellular segment between S5 and S6 (P loop = SS1-SS2 segments) surrounding the pore.

In HVA, each P loop contains a highly conserved alpha helix and a glutamate (E) residue that forms the selectivity region or filter for calcium ions (30,38). These P loops in conjunction with the S5 and S6 transmembrane segments form a funnel or "inverted-teepee" on the outer membrane side of the pore that conducts the calcium ions into the cell. Thus, the selectivity filter is likely to be the smallest opening within the pore through which calcium and other ions can pass in an almost dehydrated state (9). The conserved E residues of the selectivity filter are located at position numbers 393, 736, 1145, and 1148 in the alpha$_{1C}$ subunit (40). (Note that two of the conserved E residues are replaced by aspartate residues in LVA alpha$_1$ subunits; 38). L-type and non-L-type HVA differ only in that the amino acid immediately adjacent to the five-member amino acid stretches that center on the E in domain III (38). In L-type channels, this amino acid is phenylalanine while in non-L-type channels, this amino acid is replaced by alanine.

Initial studies with Na^+ channel mutants demonstrated that the S4 transmembrane segment acts as the voltage-sensor for the channel (26,29). Each S4 segment contains at least four highly conserved positively-charged amino acids (arginine or lysine) critical for voltage-sensitivity. Voltage-dependent movement of the S4 segment appears to act as a "paddle" or lever to open and close the pore (41-43). The remaining S1-3 segments also appear part of the voltage sensor (41-44). These segments may serve as a wall to stabilize movement of S4 and/or may have molecular interactions with S4 to stabilize the open and/or closed positions of the pore. The amino terminus contributes to voltage-dependent inactivation while the carboxy terminus to calcium-dependent inactivation (36,45).

On the inner membrane side of the pore, two calcium ion-sensing domains LA and IQ located on the C-terminal cytoplasmic tail also regulate ion entry into the cell (36,45). The LA domain contains one binding site for

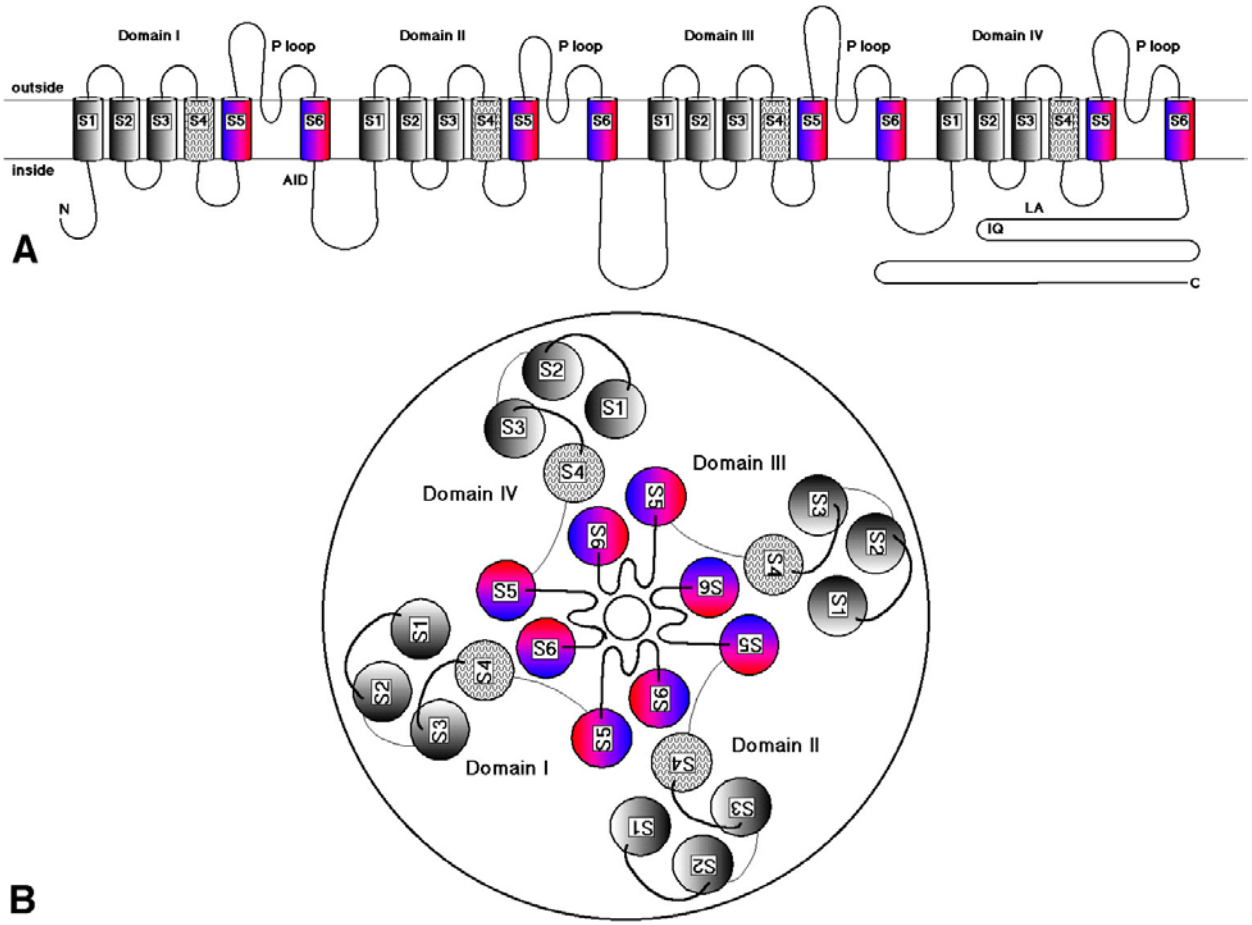

Figure 2. Deduced structure of the VDCC alpha$_1$ pore-forming subunit. A) Schematic of alpha$_1$subunit illustrating its four domains with each domain's transmembrane segments S1-6 shown as cylinders residing in the cell membrane (straight lines) (26). The voltage sensing S4 segment is shaded with wavy lines. The S5 and S6 segments that line the inner pore are shown in color. The P loop between the S5 and S6 segments is shown dipping into the outside membrane because it forms the selectivity region of the outer pore. The P loops in each domain are variable in size and function. The amino (N) and carboxy (C) termini of the alpha$_1$ subunit are labeled. The alpha$_1$ interaction domain (AID) on the loop connecting domains I and II and the Ca^{2+}-sensing domains LA and IQ on the C-terminal cytoplasmic chain are shown. Two cAMP-dependent protein kinase phosphorylation sites found in $Ca_v1.1$ (26) are not sequence conserved in $Ca_v1.2$ (not shown). B) Top view of possible structure of the alpha$_1$ subunit pore from the outside. The S1-6 segments for each domain are shaded as in A. Dark and thin lines indicate extracellular loops and intracellular loops, within each domain, respectively. Intracellular loops connecting each domain are not shown. The P loops, S5 and S6 segments line the inner pore (open circle), with the P loops and S6 segments most adjacent. Each domain's S1-4 segments form a structure separate from its pore segments. Each domain's pore segments are interlaced with the adjacent domain as shown. Movement of the voltage sensing S4 segment may act as a lever to open and close the pore.

calcium and one for calmodulin, a critical cellular calcium-binding protein that is able to inactivate the channel upon calcium binding. Once free calcium ion binds calmodulin, the bound calmodulin slides to the IQ domain, which contains two additional binding sites for calmodulin. Both domains are required for calcium-induced inactivation of the channel.

Secondary messenger systems involved in regulation of alpha$_1$ subunit activity include G-proteins (46) and serine/threonine phosphorylation (47). Further, constitutive phosphorylation on tyrosine residues may be required to maintain the alpha$_1$ subunit in a state that is available for activation by membrane depolarization (48,49). Tyrosine phosphorylation may also serve as a link between G-protein signaling pathways and VDCC (50). In addition, expression and function of this subunit can be modulated by nitric oxide, oxidative stress, hormones and, depending on the specific gene, a large variety of transcription factors (36,51).

3.4. High voltage-activated VDCC auxiliary subunits

It is well documented that the auxiliary beta, alpha$_2$delta and gamma subunits modulate the biophysical properties and membrane targeting of the HVA VDCC (52).

The 52 to 71 kDa beta subunit, which is modulated by cAMP-dependent phosphorylation (53,54),

binds to the $alpha_1$ subunit through the alpha interaction domain (AID; Figure 2A) in the cytoplasmic linker between domains I and II (55,56). From studies of functional co-expression in heterologous systems, it is thought that the beta subunit modulates the level of expression of the $alpha_1$ subunit (e.g., through membrane targeting of the $alpha_1$ subunit; 57), as well as current amplitude and the voltage dependence of activation and inactivation (52,58) and pH_i sensitivity of the calcium current (59). This subunit also contributes to the selectivity of ion permeation, as it stabilizes calcium ion binding to the selectivity filter of the $alpha_1$subunit (60), and to binding of the dihydropyridine class of calcium channel blocking drugs (60-62). The last two effects will be discussed in more detail below in relation to affects of heavy and transition metal ions and to development of new non-steroidal male contraceptive agents.

The 170 kDa $alpha_2$delta complex consists of a membrane spanning delta subunit that is disulfide-linked to a glycosylated extracellular $alpha_2$ subunit. Both $alpha_2$ and delta are encoded by the same gene and produced by post-translational processing of the propeptide (63,64). The complex is inserted transmembrane through an alpha-helical segment in the delta subunit and associates with the $alpha_1$ subunit's carboxy terminus (52). Functions attributed to this complex include membrane trafficking of the $alpha_1$ subunit, increased current amplitude/inactivation kinetics and voltage dependence of activation. The $alpha_2$ subunit also participates in stabilization of drug binding to the $alpha_1$ subunit (51).

The 33 kDa gamma subunit, a glycoprotein with four transmembrane segments, is only recently being appreciated for its role in modulating the voltage gating of the $alpha_1$ subunit (65,66). In general, this subunit reduces the calcium current. It associates with the amino terminus of the $alpha_1$ subunit (52), which may be modulated by glycosylation and/or phosphorylation. There is evidence to suggest that the gamma genes are members of the claudin family, a group of cell adhesion proteins that function at tight junctions by signaling through guanylate kinase homologues (66).

3.5. Low voltage activated VDCC and auxiliary subunits

Exogenously expressed recombinant Ca_v3 subunits encode typical T-type calcium currents, even in the absence of co-expressed auxiliary subunits (e.g., 67-69). Thus, whether or not HVA auxiliary subunits interact with, and modulate expression of, LVA $alpha_1$ subunits has been a matter of some debate. This debate arose from the fact that the LVA $alpha_1$ subunit sequences exhibited low homology with HVA $alpha_1$ subunits (Figure 1) and because of contradictory findings. An example of the former is an interesting feature of LVA $alpha_1$ gene family. The AID sequence (Figure 2A) in the I-II intracellular loop is poorly conserved, leading some investigators to postulate that modulation by interaction with the beta subunit was unlikely (70,71). We believe the latter is the result of differences between laboratories in their choice of gene and splice variant of an auxiliary subunit family to study in exogenous expression experiments, and which tissue to examine in *in vivo* expression analyses. Consider anti-sense targeting of the beta subunit. Anti-sense depletion of beta subunit expression in nodosus ganglion neurons (which lack beta-1 expression) had no effect on T-type current properties (72) while a similar approach in cardiac atrial cells (which express the beta-1 subunit) decreased T-type currents (73). We summarize the existing fragmentary data below. Based on these findings, we currently favor the contention that plasma membrane expression of LVA $alpha_1$ subunits and T-type calcium currents are modulated by auxiliary subunits in a manner similar to that described for HVA calcium channels.

3.5.1. $Alpha_{1G}$ ($Ca_v3.1$)

The potential interactions between auxiliary subunits and LVA channels has best been studied for the $alpha_{1G}$ subunit. Although it was first reported that the $alpha_2$delta-1 (brain-specific) and $alpha_2$delta-3 (ubiquitously expressed) subunits (74) or beta-1b or beta-2a subunits (75) had no effect on co-expressed $alpha_{1G}$ subunits, more recent studies demonstrated that $alpha_2$delta-1 alone or in combination with beta-1 increased the amount of LVA $alpha_1$ protein at the plasma membrane and increased functional expression (T-type current density) without changing the biophysical properties of the calcium currents (69,76). In one study, the effects of the $alpha_2$delta-1 and beta-1 subunits was synergistic (69) while in another the effects of $alpha_2$delta and beta-1b were additive (69). The latter argues for the auxiliary subunits acting as chaperones or through stabilizing mechanisms (69). Co-expression of $alpha_{1G}$ and $alpha_2$delta-2 subunits accelerated the time course of current inactivation, (77-79) while co-expression with the gamma-5 subunit accelerated time course of current activation (79-80). Comparison of $alpha_2$delta and of a truncated delta subunit indicated that the exofacial $alpha_2$ domain was the modulator of $alpha_{1G}$ expression (69). Also, anti-epilepsy drugs inhibit T-type calcium currents (81,82), while both LVA calcium channels and the gamma subunit contribute to the etiology of epilepsy (66). Consistent with this, absence epilepsy gene mutations ablating beta-4 or gamma-2 expression increase peak current densities of what was assumed to be the $alpha_{1G}$ subunit (83).

3.5.2. $Alpha_{1H}$ ($Ca_v3.2$)

Total $alpha_{1H}$ protein expression was up-regulated by all four beta subunits (76). In addition, $alpha_2$delta-1 modulated $alpha_{1H}$ expression in a manner similar to that described for $alpha_{1G}$ (76).

3.5.3. $Alpha_{1I}$ ($Ca_v3.3$)

The amount of $alpha_{1I}$ protein associated with the plasma membrane was up-regulated by the $alpha_2$delta-1 subunit alone or in combination with beta-1subunit (76). Further, the biopshysical properties of the calcium current encoded by the $alpha_{1I}$ subunit were modulated by the gamma-2, but not the gamma-2 or gamma-4, subunit (76,84).

3.6. CatSper subunit

The cation channel of sperm ("CatSper") molecule was initially isolated based on its sequence

similarity to the VDCC alpha$_1$ subunit (9,10). However, unlike the VDCC alpha$_1$ subunit, CatSper is composed of a single six-transmembrane-containing repeat (S1-S6). Thus, four CatSper molecules are thought to associate together to form a functional calcium channel. However, heterologous expression of CatSper molecules have so far failed to form functional calcium channels. Four different CatSper molecules have been identified (CatSper1-4, see Table 2). The lack of functional reconstitution could be due to the requirement of expression of all four CatSper molecules together with or without unidentified auxiliary subunits (27,86). Interestingly, targeted gene disruption of CatSper1 and CatSper2 in mice lead to male infertility, apparently due to sperm motility defects associated with failure of evoked calcium influx (86-88).

3.7. VDCC gene family

Different alpha$_1$ subunits are produced by transcription from at least ten different genes (58,89; Table 2) and potentially at least one more gene may exist (90). Additional variation is generated by alternative splicing of the primary transcripts. Although these genes are located on different chromosomes (Table 2), sequence homologies indicate that these genes are derived from a common ancestor (Figure 1). The CatSper gene family may represent the earliest descendents from the ancestral calcium channel, because the CatSper family was found to originate well before the divergence of the four-repeat channels (91). The various alpha$_1$ subunits and their splice variants exhibit marked differences in their tissue distribution of expression.

In addition, multiple genes have been reported to encode the auxiliary subunits (Table 2). As with the alpha$_1$ subunit, the transcripts of the auxiliary genes are also alternatively spliced and vary in expression by tissue. The different auxiliary subunit isoforms exhibit differences in modulation of VDCC function and in tissue distribution and have been recently discussed elsewhere (52). Ten alpha$_1$, four alpha$_2$delta, four beta, and eight gamma subunit genes are recognized by the Human Genome Organisation (92) (Table 2). Interestingly, many of these subunit genes have alleles or variants associated with diseases (Table 2). These diseases are mainly related to neurological deficits (93-95). Variants of the alpha$_1$ subunit have been associated with hypokalemic periodic paralysis, malignant hyperthermia type 5, thyrotoxic periodic paralysis, Timothy syndrome (a subset of long QT syndrome patients with a multisystem disorder having features of cognitive abnormalities, intermittent hypoglycemia, hypothermia, immune deficiency and congenital heart disease with life-threatening cardiac arrythmias; 96,97), X-linked congenital stationary night blindness type 2, episodic ataxia type 2, familial hemiplegic migraine, spinocerebellar ataxia type 6, idiopathic generalized epilepsy, and childhood absence epilepsy. Variants of the alpha$_2$delta subunit have been associated with malignant hyperthermia susceptibility type 3. Variants of the beta subunit have been associated with Lambert-Eaton myasthenic syndrome, juvenile myoclonic epilepsy, episodic ataxia type 5, and generalized idiopathic epilepsy. In addition, notable mouse variants of $Ca_v2.1$, alpha$_2$delta-2, and gamma-2 exhibit absence epilepsy and a mouse knockout of $Ca_v1.3$ results in deafness.

To date, variants in only one VDCC gene have been implicated in impaired male germ cell function. Splicing variants of the alpha$_1$ subunit $Ca_V1.2$ have been associated with poor outcome after intracytoplasmic sperm injection (98) and with male infertility due to varicocele (12,13).

3.8. CatSper gene family

The four different CatSper molecules, CatSper1 (10), CatSper2 (9), CatSper3 (27,99) and CatSper4 (27), are encoded by separate genes located on different chromosomes (see Table 2).

CatSper1 is the best-studied member of this family. CatSper1 is expressed in rodents and in all primate species tested (10,91). CatSper1 was identified through a search of the EST database with HVA calcium channel sequences. Testis-specific expression of CatSper1 in the mouse has been confirmed by molecular cloning, Northern blotting and immunocytochemistry. Gene expression in the mouse correlates with the appearance of round spermatids (100). Insertions and deletions in the amino terminal sequence encoded by exon 1 are under high positive selection to maintain protein length, supporting findings in related ion channels that this region plays an important role in channel inactivation (91; see below). CatSper1 also has a histidine-rich amino terminal sequence, which suggests that it participates in pH-regulation of sperm motility (10). Consistent with this, the CatSper1 protein localizes to the principal piece of the sperm tail, although lesser amounts also found in the mid-piece (10,87). The only defect detected in association with targeted disruption of this gene in mice was male infertility. Sperm from homozygous knockout animals were unable to fertilize zona-intact eggs but fertilized well zona-free oocytes. Initial studies indicated that sperm from the knockouts had a motility defect with decreased swimming speeds, associated with loss of cAMP-induced calcium influx (10). Further studies on different genetic backgrounds demonstrated that basal sperm motility was unaffected in CatSper1 -/- mice. However, sperm from the knockouts were unable to undergo depolarization-evoked calcium influx in the presence or absence of bicarbonate ions and did not develop hyperactivated motility (87,88). Interestingly, CatSper1 protein co-localizes with an calcium-efflux protein, plasma membrane Ca^{2+}-ATPase4 (PMCA4, ATP2B4), whose function is also required for the development of hyperactivated motility (101). However, heterologous expression of CatSper1 alone or in combination with auxiliary subunits from cyclic nucleotide gated calcium channels failed to produce calcium currents. Consistent with the latter finding, response of mouse sperm to cyclic nucleotides and cyclic nucleotide analogs suggests that CatSper1 is not directly gated by these compounds (87).

Expression of CatSper2 is also testis-specific, is observed in mice, rats and man, and is localized to the meiotic and post-meiotic stage of spermatogenesis (9).

Table 2. Disease association with human VDCC subunit and CatSper genes

Subunit	Gene name	Location[1]	Disease[1]
$Ca_V1.1$ ($alpha_{1S}$)	CACNA1S	1q32	Hypokalemic periodic paralysis, Malignant hyperthermia-5, Thyrotoxic periodic paralysis
$Ca_V1.2$ ($alpha_{1C}$)	CACNA1C	12p13.3	Timothy syndrome, Male infertility
$Ca_V1.3$ ($alpha_{1D}$)	CACNA1D	3p14.3	(Mice: Deafness, bradycardia and arrythymia)
$Ca_V1.4$ ($alpha_{1F}$)	CACNA1F	Xp11.23	X-linked congenital stationary night blindness-2
$Ca_V2.1$ ($alpha_{1A}$)	CACNA1A	19p13.1-p13.2	Episodic ataxia-2, Familial hemiplegic migraine, Spinocerebellar ataxia-6, Idiopathic generalized epilepsy, (Mice: Absence epilepsy ["tottering", "leaner", "roller" and "rocker"])
$Ca_V2.2$ ($alpha_{1B}$)	CACNA1B	9q34	Abnormal axonal distribution in multiple sclerosis (Mice: Disorders of the sympathetic nervous system)
$Ca_V2.3$ ($alpha_{1E}$)	CACNA1E	1q25-q31	(Mice: Altered pain responses)
$Ca_V3.1$ ($alpha_{1G}$)	CACNA1G	17q22	(Mice: Altered pain response)
$Ca_V3.2$ ($alpha_{1H}$)	CACNA1H	16p13.3	Childhood absence epilepsy (Mice: Abnormal coronary function)
$Ca_V3.3$ ($alpha_{1I}$)	CACNA1I	22q13	-
$Ca_V alpha_2 delta$-1	CACNA2D1	7q21-q22	Malignant hyperthermia susceptibility-3
$Ca_V alpha_2 delta$-2	CACNA2D2	3p21.3	(Mice: Absence epilepsy ["ducky"])
$Ca_V alpha_2 delta$-3	CACNA2D3	3p21.1	-
$Ca_V alpha_2 delta$-4	CACNA2D4	12p13.3	-
$Ca_V beta$-1	CACNB1	17q21-q22	-
$Ca_V beta$-2	CACNB2	10p12	Lambert-Eaton myasthenic syndrome antigen
$Ca_V beta$-3	CACNB3	12q13	-
$Ca_V beta$-4	CACNB4	2q22-q23	Episodic ataxia-5; Juvenile myoclonic epilepsy, Generalized idiopathic epilepsy (Mice: Absence epilepsy ["lethargic"])
$Ca_V gamma$-1	CACNG1	17q24	Candidate gene for multiple sclerosis
$Ca_V gamma$-2	CACNG2	22q13.1	(Mice: Absence epilepsy ["stargazer" and "waggler"])
$Ca_V gamma$-3	CACNG3	16p12-p13.1	-
$Ca_V gamma$-4	CACNG4	17q24	Candidate gene for multiple sclerosis
$Ca_V gamma$-5	CACNG5	17q24	Candidate gene for multiple sclerosis
$Ca_V gamma$-6	CACNG6	19q13.4	-
$Ca_V gamma$-7	CACNG7	19q13.4	-
$Ca_V gamma$-8	CACNG8	19q13.4	-
CatSper1	CATSPER1	11q12.1	Asthenozoospermia, Male infertility? (Mice: Male infertility)
CatSper2	CATSPER2	15q14	Asthenozoospermia (Mice: Male infertility)
CatSper3	CATSPER3	5q31.1	-
CatSper4	CATSPER4	1p35.3	-

[1]The majority of references to these disorders can be accessed online at Mendelian Inheritance in Man (OMIM™) (85).

Multiple candidate tyrosine phosphorylation sites in the CatSper2 cytoplasmic carboxy terminus suggest a mode of modulation of channel function. Heterologous expression of CatSper2 alone or in combination with CatSper1 and/or with a cyclic nucleotide-gated channel subunit (CNG4) failed to elicit calcium currents. Like CatSper1, targeted gene disruption of CatSper2 in mice leads to male infertility (88) with similar defects in hyperactivated sperm motility and evoked calcium influx (86). As the hyperactivation-negative phenotype can be rescued by application of local anesthetics, it is suggested that the CatSper2 -/- phenotype is produced by the failure to deliver calcium to the sperm tail, rather than by preventing a localized response to calcium (86). Although suggested to form heteromultimers, attempts to co-precipitate CatSper1 and CatSper2 after heterologous expression failed. However, recent data from the CatSper1 and CatSper2 knockout models indicates that there is a reciprocal requirement for co-expression in order to stabilize both proteins in sperm from the cauda epididymis (86). This is despite the fact that CatSper1 -/- testes have normal amounts of CatSper2 mRNA and visa versa.

CatSper3 was identified by both direct large-scale screening of cDNA clones (99) and by *in silico* gene identification and prediction techniques (27). Although expressed predominantly in the testis, low-level expression is observed in many other tissues. The gene encoding CatSper3 overlaps with encoding dimerization factor of hepatocyte nuclear factor 1alpha (DCOHM) (27,99). This overlap apparently interferes with splicing of both genes and results in transcripts containing exons from each and also containing intronic sequences (Arias *et al.*, 2003). In addition, the ion selectivity filter in the P loop of CatSper3

is analogous to those to LVA (e.g., EEDD), not HVA (e.g., EEEE), VDCC (Arias *et al.*, 2003). As with CatSper1 and CatSper2, heterologous expression of CatSper3 did not lead to induction of calcium currents.

CatSper4 has been identified solely by applied bioinformatics tools and is found in the mouse and human genomes (27). Real-time PCR confirms high expression in testis and low-level expression in other tissues.

To explain the inability to directly demonstrate by heterologous expression and electrophysiology that CatSper proteins function as cation channels, it has been hypothesized that the functional CatSper channel in sperm is a heterotetramer composed of all four CatSper proteins, interacting directly through a coiled-coil domain in their carboxy termini or indirectly via auxiliary subunits (27). Investigations of *in situ* calcium channel activities of CatSper are in the initial phase. By patch-clamping the cytoplasmic droplet of cauda epididymal sperm, Clapham and co-workers recently demonstrated that expression of CatSper1 is associated with a high-affinity calcium binding site in its pore region and a constitutively active calcium current that is alkaline potentiated, voltage-activated and sensitive to cadmium, nickel and ruthenium red but not L-type calcium channel blockers (see 102,103). Further, cAMP had no effect on the alkaline-induced calcium currents, supporting earlier studies that the relationship between CatSper and cAMP is indirect (87). The calcium current, which is potentiated by intracellular alkalinization, localizes to the principal piece of the sperm tail as does CatSper1 protein. It thus appears that CatSper1 encodes one of the calcium currents that are regulated by capacitation-associated changes in intracellular pH (104,105). The only caveate to these studies is that they were performed with immature spermatozoa and the possibility exists that the encoded calcium current could change, as noted by the authors, at ejaculation, the time at which the cytoplasmic droplet is shed (106).

The concept that one or all of CatSper genes function in calcium-mediated regulation of sperm motility is supported by other evidence. Both CatSper1 and CatSper2 knockout mice have irregular sperm motility associated with a lack of depolarization-evoked calcium influx (86-88). Notably, both CatSper1 and CatSper2 knockout male mice are infertile due to this defect. In humans, deletion of part of CatSper2 has been observed in a family that suffers from asthenoteratozoospermia, nonsyndromic deafness and congenital dyserythropoietic anemia type 1 (107). A large fraction of infertile men with sperm motility defects exhibit markedly reduced levels of CatSper1 mRNA in their testis biopsies (100).

Thus, of the VDCC alpha$_1$ subunit related CatSper molecules, CatSper1 and CatSper2 have been shown to be required for male fertility, whereas CatSper3 and CatSper4 remain to be tested. Information on the alpha$_2$delta, beta and gamma subunits has also been accumulating. However, the VDCC alpha$_1$ subunit remains the best studied with regard to subcellular localization, kinetics, voltage-dependence of activation and inactivation, and the effects of dihydropyridine calcium channel antagonists. Therefore, the remainder of this review will concentrate on the role of alpha$_1$ gene expression in the regulation of spermatogenesis and of the biological and biophysical characteristics of mammalian sperm. In many cases, the analysis of the cardiac isoform (alpha$_{1C}$ = $Ca_v1.2$) will be used as an example, as it has been well studied. It is also of particular interest to the authors as it has we have observed that it plays a significant role in human testicular pathologies.

4. VDCC IN TESTIS AND SPERMATOZOA DEFINED BY THEIR ALPHA$_1$ (PORE-FORMING) SUBUNIT

Early studies of mammalian sperm membrane potential initially suggested that sperm did not express calcium channels (e.g., 108). However, the advent of more refined technology clearly demonstrated that sperm indeed express a variety of calcium transport systems. Although calcium transport in relation to the induction of the acrosome reaction has been studied in a large variety of species including guinea pig (109), bovine (110) and ram sperm (111), the examination of the expression of VDCCs in mammalian testis and sperm by electrophysiology, by pharmacology and by molecular techniques has primarily been restricted to three species: mouse, rats and man. The results from these species will be discussed in detail below.

Electrophysiological analyses have generally been performed on immature male germ cells, as the asymmetry, size and stiffness of the plasma membrane of mature sperm precludes fine control of the position of pipette and the formation of tight seals. Extrapolation of findings from immature germ cells to sperm is based on the argument that ejaculated sperm are transcriptionally inactive. Unfortunately, not all results from immature germ cells are supported by analysis of ion channels from mature sperm after transfer to planar lipid bilayers. In addition, early interpretations of the exact nature of the ion channel recordings that deviated from the norm as defined in somatic cells (e.g., L-like rather than L-type) or in heterologous systems (e.g., discrepancies between currents detected in the presence or absence of auxiliary subunits) complicated these analyses.

Pharmacological analyses focused on the effect of organic and peptide antagonists and agonists on calcium currents, on sperm capacitation and on the induction of the acrosome reaction. Such studies have been complicated by the concentration and voltage-dependence of channel selectivity of these agents.

Northern blot analyses of VDCC mRNAs have been limited and may have detected only the more abundant sequences. RT-PCR results depended upon the choice of primers and were generally employed to amplify only partial sequences. Thus, it could be argued that what was detected were portions of transcripts from related genes and/or the remains of non-functional nuclear transcripts.

Until recently, immunohistochemistry and Western blotting has been hampered by the relative paucity of commercially available specific antibodies.

Studies of VDCCs expressed in the male germ line have been further hampered by species differences in expression, equivocal results from knockout animal models possibly associated with the existence of redundant pathways or compensatory elevations of related proteins, and only a few identified links to human male infertility.

As a result, there are many equivocal findings with the nature and function of the male germ cell VDCC(s) remaining a matter of considerable debate.

4.1. High voltage-activated, dihydropyridine-insensitive channels

4.1.1. Alpha$_{1A}$ ($Ca_v2.1$)

The alpha$_{1A}$ subunit has been detected in epididymal sperm by immunostaining (112,113) and Western blotting (113), in testis by RT-PCR (114) and in germ cells by RT-PCR, Northern blotting and immunostaining (112,114,115). Regional localization on mouse epididymal sperm was accomplished using anti-peptide antibodies (113). In testicular spermatogenic cells (pachtyene spermatocytes, round and condensing spermatids), the alpha$_{1A}$ subunit was found on surface membranes but was also diffusely distributed throughout the cytoplasm. The alpha$_{1A}$ subunit protein was widely dispersed in sperm, being present at the apical tip and equatorial segment of the sperm head as well as in the midpiece and flagellum. However, no evidence has been obtained to support a major role for P/Q-type calcium currents in depolarization evoked calcium entry into mouse sperm (116). Although mice with a disrupted alpha$_{1A}$ gene die by the fourth week of life (117), mice with spontaneous mutations in the alpha$_{1A}$ gene can survive to adulthood with no negative effects on fertility reported (118).

The alpha$_{1A}$ subunit has also been detected in sections from immature rat testis by immunostaining, Western blotting and *in situ* hybridization (119), where expression was limited to Sertoli cells membranes. Anti-alpha$_{1A}$ subunit antibodies recognize two bands, 250 kDa and 95 kDa, on the Western blots.

Transcripts encoding alpha$_{1A}$ subunits were not detected in RNA populations from motile ejaculated human sperm when RT-PCR was performed with degenerate primers (120). As in the mouse, spontaneous mutations in the alpha$_{1A}$ subunit have been identified in humans (121,122). These mutations have not been associated with male infertility.

4.1.2. Alpha$_{1B}$ ($Ca_v2.2$)

In mouse, immunostaining to detect the alpha$_{1B}$ subunit in the mouse has produced conflicting results. Serrano *et al.* (112) did not detect alpha$_{1B}$ in germ cells and mature sperm while Wennemuth *et al.* (116), employing anti-peptide antibodies, reported it localized to the acrosomal crescent, the postacrosomal segment and the dorsal and ventral surfaces of the principal piece of the tail. Western blotting of sperm extracts revealed a major band at approximately 210 kDa and a minor band at 240 kDa (116), consistent with prior findings on the size of the alpha$_{1B}$ subunit (123). The presence of an active alpha$_{1B}$ subunit protein on mouse sperm was demonstrated by examination of the effect of calcium channel blockers, venom toxins, nickel and cadmium on depolarization-evoked calcium entry, which also indicated N-type currents were the major currents detected (116). However, alpha$_{1B}$-deficient mice demonstrate normal fertility (124).

The alpha$_{1B}$ subunit has also been detected in sections from immature rat testis by immunostaining, Western blotting and *in situ* hybridization (119). The alpha$_{1B}$ subunit was localized to Sertoli cell membranes and to blood vessels in the interstitium. The alpha$_{1B}$ subunit in Sertoli cells modulates calcium-dependent protein secretion (125).

In motile human sperm, expression of alpha$_{1B}$ was detected by RT-PCR (120).

4.1.3. Alpha$_{1E}$ ($Ca_v2.3$)

In the mouse, the alpha$_{1E}$ subunit has been detected in testis and germ cells by RT-PCR (114) and in epididymal spermatozoa by immunostaining and Western blotting (113,116). Anti-peptide antibodies demonstrate alpha$_{1E}$ protein in the acrosomal region and in the principal piece of the sperm tail (113). This subunit was originally proposed to carry LVA currents in spermatogenic cells (114), as this alpha$_1$ subunit exhibited calcium permeation properties similar to those ascribed to LVA channels (126-128). However, anion channel blockers, that are able to influence calcium channels as well block T-type currents in immature spermatogenic cells and also block the zona-induced acrosome reaction, have essentially no effect on calcium currents encoded by the alpha$_{1E}$ subunit expressed in oocytes (115). Channels encoded by this subunit were non-responsive to progesterone (129). Additionally, immature spermatocytes from alpha$_{1E}$ knockout mice still express LVA calcium currents (130). Further, although consistent with the initial characterization of this subunit in neurons as being transiently activated at negative membrane potentials and nickel-sensitive (126) and with T-type currents being blocked with antisense oligonucleotides against this subunit (131), it is now known that this subunit specifies R-type calcium currents that have been reported to contribute to depolarization-evoked calcium entry in mouse sperm (116,132), although other alpha$_1$ subunits may also contribute to production of R-type currents at least in somatic cells (133). Alpha$_{1E}$ knockout mice are fertile but exhibit only limited calcium transients after exposure to mannosylated-BSA (130,133), an agent that increases calcium influx by human sperm (134) and induces acrosome exocytosis (135,136). Sperm from these knockouts also display increased linear movement, suggesting that alpha$_{1E}$ channels contribute both to calcium transients in response to physiological stimuli and to control of sperm motility (137).

In contrast, the alpha$_{1E}$ subunit was not detected by *in situ* hybridization in rat testis tissue (119).

Expression of the $alpha_{1E}$ subunit has been demonstrated in motile human sperm by RT-PCR (120). The protein has been shown to be distributed over the equatorial segment of the sperm head and over the principal piece of the sperm tail (138).

4.2. High voltage-activated, dihydropyridine-sensitive channels

A series of studies from different laboratories demonstrated that the mammalian sperm had high affinity binding sites on their plasma membranes for 1,4-dihydropyridine calcium channel antagonists such as nifedipine (111). These drugs were developed for blockade of L-type VDCC in the cardiovascular system (51). Transfer of sperm ion channels to planar lipid bilayers with subsequent electrophysiology indicated that mammalian sperm expressed L-type VDCC (139,140), although some of the characteristics of these channels differed from prototype L-type VDCC expressed in somatic cells (1415). Further, abnormal biophysical properties of these channels from some infertile donors as compared to known fertile donors suggested the existence of conformation defects associated with infertility (140).

The dihydropyridine-sensitive calcium channels of mammalian sperm were shown to be functional only in capacitated cells (110,142,143). The mammalian sperm acrosome reaction, inducible in capacitated sperm by the biological stimuli of zona pellucida glycoproteins or progesterone, could be inhibited by these calcium channel blockers (111,143) as well as by pertussis toxin, indicating modulation by G-proteins (110,111,144,145). Thus, it was initially thought that L-type VDCC were the main ion channels regulating the acrosome reaction and responsible for global increases in sperm calcium concentrations initiated by the zona pellucida (110). However, subsequent studies in man disputed these findings with data indicating that nifedipine had no effect on depolarization induced calcium currents (146) and that L-type calcium channel blockers had no effect on agonist-induced calcium influx or the acrosome reaction (e.g., 134,147,148). Interestingly, heterologous expression of a cloned $alpha_{1C}$ subunit was correlated with expression of both L-type and T-type calcium currents depending upon whether or not low depolarization was employed and whether or not the $alpha_2delta$ subunit was co-expressed (149). While the latter publication is considered anecdotal, it is cited to further emphasize problems in defining $alpha_1$ subunit expression solely based on electrophysiology.

It is important to note that there is a distinction between the results in human and in mouse regarding the involvement of LVA channels in the acrosome reaction. In humans there are conflicting results and very sparse electophysiological data. In mouse many groups have reproduced the findings that the main Ca_V currents are of the T-type and their pharmacology, with the exception of one paper, agrees with the participation of these channels in the acrosome reaction. These points are discussed in detail below and in the following section. Nonetheless, evidence continues to accumulate for a pivotal role of L-type VDCC in this process. For example, although T-type VDCCs contribute to the nickel-sensitive transient peak of depolarization induced calcium influx (146), dihydropyridine-sensitive VDCCs accelerate the loss of the secondary sustained increase in internal calcium concentration (150).

4.2.1. $Alpha_{1S}$ ($Ca_v1.1$)

A spontaneous loss of function mutation in the $alpha_{1S}$ gene of the mouse has been described (mdg = muscular dysgenesis; 151,152). However, homozygous mice die perinatally, as they are unable to breathe. Thus, this mutant provides no information as to whether the $alpha_{1S}$ subunit plays a role in male fertility.

Expression of $alpha_{1S}$ was not detected in RNA from ejaculated human sperm when assessed using RT-PCR and degenerate primers (120). Spontaneous mutations in the $alpha_{1S}$ gene have been described in human pedigrees (153,154). However, in contrast to the mouse, viability appears unaffected and no relationship between these mutations and infertility have been reported.

4.2.2. $Alpha_{1C}$ ($Ca_v1.2$)

In the mouse, fragments of the mRNA sequence for the $alpha_{1C}$ subunit has been identified in mouse testis and immature germ cells by RT-PCR (114,115,155) and in germ cells by Northern blotting (115) while the encoded protein has been detected by immunostaining in germ cells and sperm (112,113) and in sperm by Western blotting (113). Western blotting further revealed the presence of both a band of expected size (approximately 200 kDa) as well as a smaller band migrating at approximately 110 kDa (113). Use of anti-peptide antibodies demonstrated that the $alpha_{1C}$ subunit was located throughout the cytoplasm in immature spermatogenic cells as well as in plasma membranes (112). In mature sperm, anti-peptide antibodies localized $alpha_{1C}$ subunit protein to dorsal and ventral aspects of the acrosomal cap and of the proximal segment of the principal piece of the sperm tail (113). However, despite these findings, examination of depolarization-evoked calcium entry did not support a major role of L-type calcium currents in this process (116). Examination of the phenotype of the $Ca_v1.2$ -/-mouse was of no help in elucidating the role of the $alpha_{1C}$ subunit in male reproduction, as this knockout is an embryonic lethal (90).

Full length $alpha_{1C}$ mRNA has been identified in rat testis by RT-PCR and molecular cloning (6,156,157). This subunit has also been detected in rat testis and sperm by RT *in situ* PCR, Western blotting and immunocytochemisty (6,156,158). In contrast to findings in the mouse, only one band migrating at approximately 175-220 kDa is observed on Western blotting of lysates from rat epididymal sperm and testis (156).

In man, the full length $alpha_{1C}$ mRNA has been cloned from testis (S. Benoff, C. Chu and L. Goodwin, unpublished observations), the mRNA is present in ejaculated sperm (detected by RT-PCR; 120,158) and the protein is detectable in testis and ejaculated sperm by immunostaining (6,158). Western blotting also revealed that, analogous to findings in the mouse, two

immunoreactive bands of approximately 165-175 kDa and 60 kDa are detectable in plasma membrane extracts from ejaculated human sperm (1,2). We originally proposed that these protein bands were the result of proteolysis. Particularly because the 60 kDa band was absent in sperm mRNAs from infertile men with an acrosome reaction insufficiency, proteolysis may be required for the formation of an active channel (1,2). However, based on subsequent observations (12,35,159), another possibility is that these protein bands are truncated proteins produced by alternative splicing (see below for further discussion).

In human sperm, the alpha$_{1C}$ subunit was localized to the equatorial segment and the post-acrosomal region of the sperm head by immunocytochemistry (6). It co-localized with the sustained calcium elevations in the sperm head in response to progesterone and zona pellucida (150,160). More recently, expression of the alpha$_{1C}$ subunit was reported on the sperm tail (138). The alpha$_{1C}$ subunit is also thought to contribute to the rise in internal calcium that occurs in conjunction with sperm capacitation (6). Deletions in the alpha$_{1C}$ mRNA in sperm in exons encoding the domain IV S4 and the linker between S3 and S4 correlated with an inability to undergo an acrosome reaction induced by mannose ligands (161). Mannose ligands serve as a surrogate for authentic human zonae pellucidae (135,136). Further, the immunostaining patterns for alpha$_{1C}$ protein and for phosphotyrosine-containing proteins co-localize on the human sperm head (2,7). The intensity of anti-phosphotyrosine antibody binding in the equatorial region increases in association with induction of human sperm acrosome reaction by mannose ligands and is markedly reduced by sperm exposure to tyrosine kinase inhibitors, which also block the induced acrosome reaction (2,3). These data suggest that the human sperm alpha$_{1C}$ subunit participates in the human sperm acrosome reaction and is regulated by tyrosine phosphorylation. The existence of a functional alpha$_{1C}$ subunit in human sperm has recently been confirmed using a high-resolution scanning patch clamp technique, developed to study ion channels in small cells (162), and demonstrating a large number of L-type VDCC on the human sperm head (163).

4.2.3. Alpha$_{1D}$ (Ca$_v$1.3)

Expression of the alpha$_{1D}$ subunit has been detected in mouse testis and germ cells by RT-PCR with evidence for at least two alternatively spliced transcripts (114,155). However, this subunit does not appear to play a role in external calcium influx (116) and targeted ablation of the alpha$_{1D}$ subunit does not impair male fertility (164).

RT-PCR has failed to provide evidence of expression of alpha$_{1D}$ mRNA in ejaculated human sperm (120).

4.2.4. Alpha$_{1F}$ (Ca$_v$1.4)

The alpha$_{1F}$ subunit has not yet been reported to be expressed in mouse germ cells when assessed by electrophysiology (116).

Messenger RNA encoding the alpha$_{1F}$ subunit was not detected using RT-PCR and human sperm RNA as template (120). To date, none of the numerous spontaneous mutations identified in the human alpha$_{1F}$ (e.g., 165,166) subunit have been linked to male infertility.

4.3. Low voltage-activated, dihydropyridine-sensitive channels

LVA T-type currents were characterized by their biophysical properties (e.g., distinctive voltage dependent) and pharmacology long before the first T-type alpha$_1$ subunit was identified by Perez-Reyes and coworkers in 1998 (68). Ultimately, the T-type VDCC family was determined to be composed of three alpha$_1$ subunits (167,168; see Tables 1 and 2 and Figure 1).

Within recent years, thinking about the nature of the calcium channels participating in the mouse and human sperm acrosome reaction has shifted from L-type HVA to LVA and store-operated channels (169). T-type calcium channels have been proposed to be responsible for the large calcium transient in mouse sperm occurring immediately after zona binding (170) with the store-operated channels mediating sustained calcium flux associated with induction of the acrosome reaction (171,172). A T-type calcium channel blocker, mibefradil, inhibited the human sperm acrosome reaction induced by mannosylated-BSA (134). An inhibitor of cyclic nucleotide-gated channels that blocked heterologous expression of T-type calcium currents also blocked the zona pellucida-induced acrosome reaction by caudal epididymal mouse sperm (173).

Patch-clamp studies from a number of laboratories indicate that T-type calcium currents are the main calcium currents detected in immature mouse spermatogenic cells (114,174-176). The channels responsible for these currents are primed for activation, first by membrane hyperpolarization (177) and then, in contrast to L-type VDCC, by tyrosine dephosphorylation during capacitation (178). In addition, these currents are modulated by serum albumin by a mechanism unrelated to the albumin's capacity to deplete the cholesterol from the sperm plasma membrane (179). Rather, one tentative explanation is that the facilitation of T-type calcium currents by albumin is due, at least in part, to removal of beta-estradiol from the plasma membrane (179), although other explanations are also possible. Although activation of the sperm surface estrogen receptor has been correlated with a sustained increased in sperm intracellular calcium (180), beta-estradiol is a known inhibitor of T-type currents (181) (and L-type currents [181-183]) in somatic cells. VDCC in general (148) and T-type channels in particular (184) are activated by sperm capacitation, moving from an inactivated state to a closed state in response to membrane hyperpolarization (170) and then an open state by depolarization induced by contact with the zona pellucida (185). However, again in contrast to L-type VDCC, they apparently do not participate in the capacitation process itself (186). In addition, the channels encoding the T-type currents in spermatogenic cells are, again in contrast to L-type VDCC, G-protein independent (178). Calmodulin antagonists inhibit both T-type calcium currents and the zona-induced acrosome reaction (187). Scorpion toxins, related to kurtoxin (188), have effects similar to calmodulin antagonists, but a toxin-resistant, nickel-resistant calcium current was also detected (189).

Some studies of T-type VDCC in human sperm have shown these channels to be responsive to zona pellucida but not progesterone (134,186) while other studies provide evidence for responsiveness to progesterone (147,148) or have reported inconclusive findings (190). Such conflicting observations may be due, at least in part, to methodological differences or to the possibility that the progesterone-induced acrosome reaction is a laboratory artifact. Our data support the latter, as we have previously published a study demonstrating that a response to progesterone is required for human sperm to undergo an acrosome reaction induced by mannose-containing model zona ligands amd correlates with fertilization in conventional IVF (191). Nonetheless, calcium entry through LVA is thought to sequentially activate a receptor for inositol 1,4,5-triphosphates (IP3R) and a transient receptor potential 2 (TRP2) channel, thereby effecting acrosome exocytosis (172) and inactivating the LVA (177). The latter suggests a molecular proximity between the three calcium channel types (177).

One problem with these studies is that only subtle differences are detected between the calcium currents induced by expression of the three genes encoding T-type alpha$_1$ subunits and their splice variants (71,192-195). Results from various laboratories differ because they employed, for example, different species or different transfection systems. The majority of publications concerning T-type calcium currents in mammalian spermatogenic cells do not discriminate between the three current types. In addition, at least one group has reported that T-type calcium currents do not play a major role in depolarization-evoked calcium entry into mouse sperm (116). More importantly, indirect measurements of T-type calcium currents in mature human spermatozoa indicates that calcium influx differs from that of animal spermatozoa in their voltage-dependence of inactivation, enhancement by intracellular alkalization and insensitivity to nifedipine (146). In addition, the pharmacological properties of the T-type calcium currents in dissociated immature spermatogenic cells differ from those of somatic cells (175).

Several other issues still need to be resolved. For example, the structure of their selectivity filters differs from that of HVA VDCC (38,70,99,196), suggesting a reduced affinity for calcium, and one feature used to distinguish T-type from L-type currents in mammalian sperm is their differential sensitivity to inhibition by nickel. Many T-type currents are more sensitive to nickel than cadmium or cobalt (11,170), the reverse of what is observed for somatic L-type currents (e.g., 197). However, subsequent studies employing heterologous expression of the three T-type VDCC alpha$_1$ subunits revealed that only calcium currents encoded by the alpha$_{1H}$ subunit were blocked to low concentrations of nickel (198). Further, nickel produced a dose-dependent inhibition of the mannose-induced human sperm acrosome reaction (199,200) without affecting mannose-induced tyrosine phosphorylation of the alpha$_{1C}$ subunit (199), an event also correlated with acrosome exocytosis (2,3). Thus, the relative contributions of HVA and LVA VDCC to induction of the acrosome reaction are still a matter of some controversy.

4.3.1. Alpha$_{1G}$ ($Ca_v3.1$)

Messenger RNA encoding the alpha$_{1G}$ subunit was not detected in mouse testis by Northern blot analysis (68). Nonetheless, fragments of the alpha$_{1G}$ subunit can be amplified by RT-PCR from RNAs from immature mouse spermatogenic cells (115,130). Specific immunostaining localized the alpha$_{1G}$ subunit protein to the head and principal piece of the sperm tail (138). Evidence for alpha$_{1G}$ subunit-encoded calcium currents in immature germ cells has also been presented (138). Despite these findings, T-type calcium currents were unaffected in spermatogenic cells from alpha$_{1G}$ null mice (195) and these mice are fully fertile (201).

Full-length alpha$_{1G}$ mRNA sequences have not been detected by RT-PCR employing rat tests RNA as template (199,202).

Results from different laboratories on expression of the alpha$_{1G}$ subunit in the human male germ line do not agree. Some reported that alpha$_{1G}$ transcripts were not identified in RNA isolated from human testis (200) or sperm (202) while results from another group indicated that mRNA encoding the alpha$_{1G}$ subunit is present in human spermatogenic cells and sperm (25,203). Immunocytochemistry has demonstrated that alpha$_{1G}$ subunit protein is found on the human sperm head and on the mid- and principal pieces of the sperm tail (138).

4.3.2. Alpha$_{1H}$ ($Ca_v3.2$)

In the testis of immature and adult male mice, expression of the alpha$_{1H}$ subunit was detected in developing germ cells and Sertoli cells by *in situ* hybridization (155). These results were confirmed using RT-PCR, where it was demonstrated that immature mouse spermatogenic cells express the mRNA encoding the alpha$_{1H}$ subunit. The alpha$_{1H}$ subunit protein was immunolocalized to the sperm head (138). The biophysical and pharmacological properties of T-type calcium currents in mouse spermatogenic cells suggest that the major carrier of these currents is the alpha$_{1H}$ subunit (195). Results from another lab are concordant, with the alpha$_{1H}$ subunit contributing about 60% of the calcium currents in immature spermatogenic cells (138). Consistent with this, the level of alpha$_{1H}$ mRNA is not decreased in testicular spermatogenic cells from alpha$_{1G}$ knockout mice (195). Despite these findings, reproduction by alpha$_{1H}$ subunit knockout male mice is unaffected (204).

RT *in situ* PCR demonstrated expression of alpha$_{1H}$ mRNA throughout the seminiferous epithelium of rat testis, with the density of the signal decreasing with increasing differentiation of the male germ line (199).

In terms of the human male germ line, one group reported that only alpha$_{1H}$ mRNA, and not alpha$_{1G}$ or alpha$_{1I}$, was expressed in testis (200) while others have published that alpha$_{1G}$, alpha$_{1H}$ and alpha$_{1I}$ are co-expressed

Table 3. Variability in $alpha_{1C}$ subunit sequence in human testis (161)

Seq.#		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
# of Clones		14	10	7	6	3	22	1	1	1	1	2	1
Segment	**Exons**												
IIIS2	21	-	+	-	-	-	-	-	-	+	+	+	+
	22	+	-	+	+	+	+	+	-	+	-	-	-
IVS3	31	+	-	+	-	-	+	-	+	+	-	+	+
	32	-	+	+	+	-	-	-	-	-	-	-	+
Linker	33	+	+	+	-	-	-	-	-	-	-	-	+
IVS4	34	+	+	+	+	+	+	-	-	+	+	+	+

Commercially available pooled testis polyA+ mRNA served at template in RT-PCR amplifications of $alpha_{1C}$ exons 20 through 35. PCR products were cloned and sequenced. MacVector 5.0 program was used for sequence alignments. Sequence 1 encodes the 1650 bp fragment of the testis-specific $alpha_{1C}$ isoform and sequence 2 represents the 1650 bp fragment of the cardiac isoform. The remaining sequences contain deletions. The most common deletion was in exon 33, which results in the loss of the hinge region between IVS3 and IVS4 (see Figure 2A). Deletions in exon 34 were not anticipated. These findings indicate that the human testis $alpha_{1C}$ subunit mRNA is polymorphic. That the exons examined are hot spots for $alpha_{1C}$ mRNA alternative splicing were subsequently confirmed transcript scanning of the $alpha_{1C}$ mRNAs expressed in cDNA libraries from fetal and adult brain and fetal and adult human heart (209).

(25,120,203), and that $alpha_{1H}$ is the most abundant VDCC $alpha_1$ subunit mRNA in human sperm (120). In contrast to findings in the mouse, specific antibodies localized the $alpha_{1H}$ subunit protein primarily to the principal piece of the sperm tail, with faint reactivity in the post-acrosomal region of the sperm head (138).

4.3.3. Alpha$_{1I}$ ($Ca_v3.3$)

Calcium currents carried by the $alpha_{1I}$ subunit, which differs from those of the $alpha_{1G}$ and $alpha_{1H}$ subunits both in activation/inactivation kinetics and in pharmacological sensitivities, was not detectable in spermatogenic cells from wild-type or $alpha_{1G}$ knockout mouse testes (195). Despite these findings, mRNA encoding the $alpha_{1I}$ subunit was amplifiable by RT-PCR (138). The encoded protein localized to the principal and midpiece of the sperm tail but sperm motility was unaffected by drugs that block T-type calcium currents, mibefradil and gossypol (138). To the best of our knowledge, the $alpha_{1I}$ subunit is the only $alpha_1$ subunit for which a spontaneous loss of function/expression mutation and/or a knockout mouse model has not yet been reported.

Full length $alpha_{1I}$ transcripts have been detected in rat testis using RT-PCR (199).

Results on expression of the $alpha_{1I}$ subunit in the human male germ line are contradictory. Son *et al.* (200) presented data suggesting that the $alpha_{1I}$ subunit was not detected in human testis while Park *et al.* (120) reported that the $alpha_{1I}$ transcript was present in low abundance in motile human sperm. As in mouse sperm, use of specific antibodies demonstrated that $alpha_{1I}$ protein localizes the midpiece of the sperm tail (138).

5. EFFECTS OF ALTERNATIVE SPLICING

It has been estimated that as many as 74% of human pre-mRNAs are alternatively spliced (205). Further, in the examination of tissue-specific splicing of the human transcriptome, it was observed that the testis shows the sixth highest rate of enrichment among thirty tissues tested, behind brain, skin, retina, muscle and lymph nodes (206). This is particularly true for VDCC mRNAs. Alternative splicing was first described in 1990 for HVA VDCC transcripts (207) and was subsequently reported for LVA VDCC transcripts as well (25,203). Such alternative splicing produces additional complexity and variability in VDCC structure and function (208).

5.1. VDCC $alpha_1$ subunit alternative splicing: $Ca_v1.2$ case study

As a case in point, extensive splice variations (> 40) have been reported for $Ca_v1.2$ (Figure 3) (12,35,36,159,161,207,209-211). Thus, the sizes of full-length $Ca_v1.2$ transcripts are variable, but are on the order of 8 kb. Human $Ca_v1.2$ transcripts are encoded from 55 possible exons spread out over 700 kb of genomic DNA from chromosome 12. These exons have been numbered 1 to 50, with an additional five exons labeled as 1A, 8A, 9*, 10* and 45* (36,209). Not all 55 exons are found in a single transcript. Five pairs of exons are alternatively spliced in a mutually exclusive manner: 1/1A, 8/8A, 21/22, 31/32 and 45/45*. Some exons are optionally included by alternative splicing, such as exons 9*, 10*, and 33, which encode loop sequences between the transmembrane structures. In addition, the alternative exon pair 45/45* is also included optionally (i.e. exons 45/45* may be deleted from the spliced transcript) and would therefore alter the C-terminal tail. Such alterations would affect the ability of the $alpha_{1C}$ subunit to be phosphorylated by cAMP-dependent protein kinases A and C, and calcium and calmodulin-dependent protein kinase II (212), thereby changing the functional role of the channels (213). More drastic alternative splicing that remove exons encoding P loops or transmembrane structures or result in premature translation termination have also been documented. These include deletion of exon 7, exons 7 and 8/8A, exon 8/8A, exons 8/8A and 9, exons 17 and 18, exon 19, exons 19, 20, 21, and 22, exons 21 and 22, exons 31 and 32, exons 31, 32, and 33, exons 31, 32, 33, and 34, exons 32 and 33, or exons 32, 33, 34 (12,35,45,159,161,209; Table 3). The deletions of exon 7, exons 7 and 8/8A, exon 8/8A, exons 8/8A and 9,

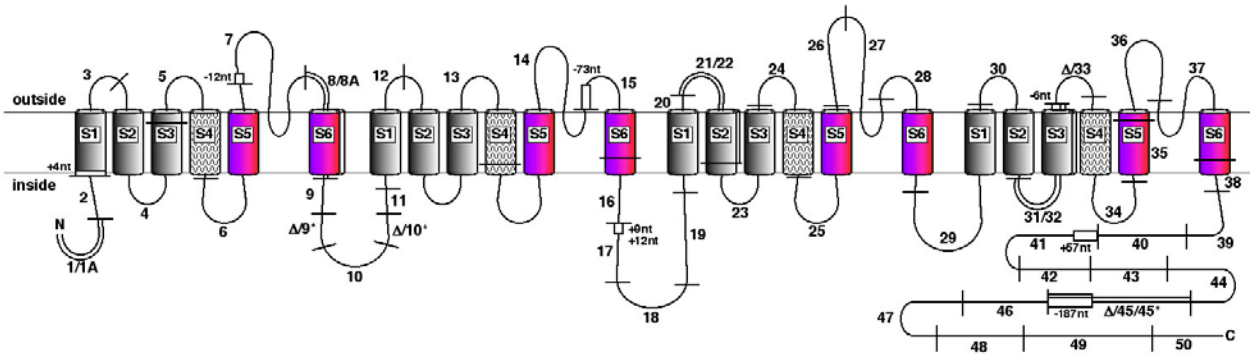

Figure 3. Splicing of VDCC $Ca_v1.2$ subunit. Schematic of $alpha_1$ subunit as in Figure 1, but with lines crossing schematic indicating splice sites for $Ca_v1.2$. Exons are numbered between the splice sites and ends of protein by convention (36,209). Doubled lines and cylinders indicate alternative exons (1/1A, 8/8A, 21/22, 31/32, 45/45*). Open rectangles indicate alternative splice junctions (exons 3, 7, 15, 17, 32, 41, 45*) with +/- nucleotides (nt) contributed by alternative junction. Exons labeled with "Delta/" indicate optional exons that may be individually spliced out (9*, 10*, 33, 45/45*) without major disruption of illustrated subunit transmembrane structure. The AID site is encoded by exon 9. Ca^{2+}-sensing domains LA and IQ are encoded by exons 40-41 and 41-42, respectively.

exons 17 and 18, exon 19, or exons 19, 20, 21, and 22 lead to premature translation termination, whereas the other deletions do not. Finally, additional variability is generated by alternative splice junctions, either at the splice acceptor site as in exons 3, 7, 15, 17, 32, and 41, or the splice donor site as in exon 45*. Use of alternative splice junctions in exons 3, 15, 17 (+12 nucleotides), and 45* lead to premature translation termination, whereas alternative splice junctions in exons 7, 17 (+9 nt), 32, and 41 do not and result in the insertion or deletion of amino acids. Premature translation termination does not necessarily produce useless proteins targeted for degradation. Novel truncated isoforms could potentially combine with each other to form new channels with different functions or combine with full-length isoforms and/or auxiliary subunits to interfere with their normal structure and negatively regulate channel activity (35,208).

5.2. $Ca_v1.2$ alternative splicing in testis and sperm

In the testes, many of these $Ca_v1.2$ alternative splicing isoforms have also been observed. Alternative splicing of mutually exclusive exons 1/1A, 8/8A, 21/22 and 31/32 occurs in testes (1,6,156,157,161). Exon 1 is always present in testes $Ca_v1.2$ transcripts, indicating that transcription initiates from the same promoter region as found in fibroblasts, neurons and smooth muscle, but not from the promoter used in cardiac muscle for exon 1A (36,214,215). The other mutually exclusive exons 8A, 22, and 31 are predominantly observed in testes. These exons encode transmembrane segments and loops between segments, which could profoundly affect pore function based on alterations of the structure. Indeed, alterations in calcium channel characteristics correlate with use of these alternative exons (157,161,209,216-218). Curiously, the strict rule of mutual exclusivity of exons 21/22 or 31/32 is occasionally broken (209; Table 3). In the testes, occasional transcripts are detected with both exons 21 and 22 or exons 31 and 32, as well as occasionally deleted for both exons (1,161; Table 3). Transcripts containing both exons 31 and 32 will result in a duplicate loop and S3 transmembrane segment in domain IV. Transcripts deleting both exons 31 and 32 will delete the S3 transmembrane segment. Both these transcripts could potentially encode proteins that flip the extracellular and intracellular loops to the opposite sides of the membrane after the S3 segment in domain IV and would therefore have a drastic effect on pore structure and function. A similar drastic effect may occur with alternative transcripts containing or deleting both exons 21 and 22. These exons encode most of the S2 segment in domain III (IIIS2). Thus, these alternative transcripts may result in a duplication or deletion of IIIS2, respectively, resulting in the potential flipping of the downstream extracellular and intracellular domains to the opposite side of the membrane.

Deletions of optional exons 45/45* and occasionally of exon 33 have also been reported in testes (1,6,156,157,161; e.g., see Table 3). Inclusion or deletion of optional exons can affect voltage-dependent channel characteristics (219). Inclusion or deletion of exon 33, which encodes a large portion of the extracellular loop linking S3 and the voltage-sensing S4 transmembrane segments in domain IV, affects voltage-dependent activation (6,209) as well as ability to undergo an induced acrosome reaction (161). Inclusion or deletion of optional exons 45/45* affects oxygen-sensing characteristics, minor voltage-activation characteristics, and possibly calcium/calmodulin kinase II phosphorylation (220-222).

Alternative splicing between exons 6 and 9 has been extensively studied in testes (12,159) and serves as a good illustration of the varieties of alternative splicing (Figure 4). One full-length and four smaller splice variants have been identified. The full-length sequence and the four splice variants are co-expressed in different combinations in the testis and sperm of different men (12,159). Such co-expression was not unexpected, as it has also been reported for gonadotropin receptor mRNAs in testis (223,224).

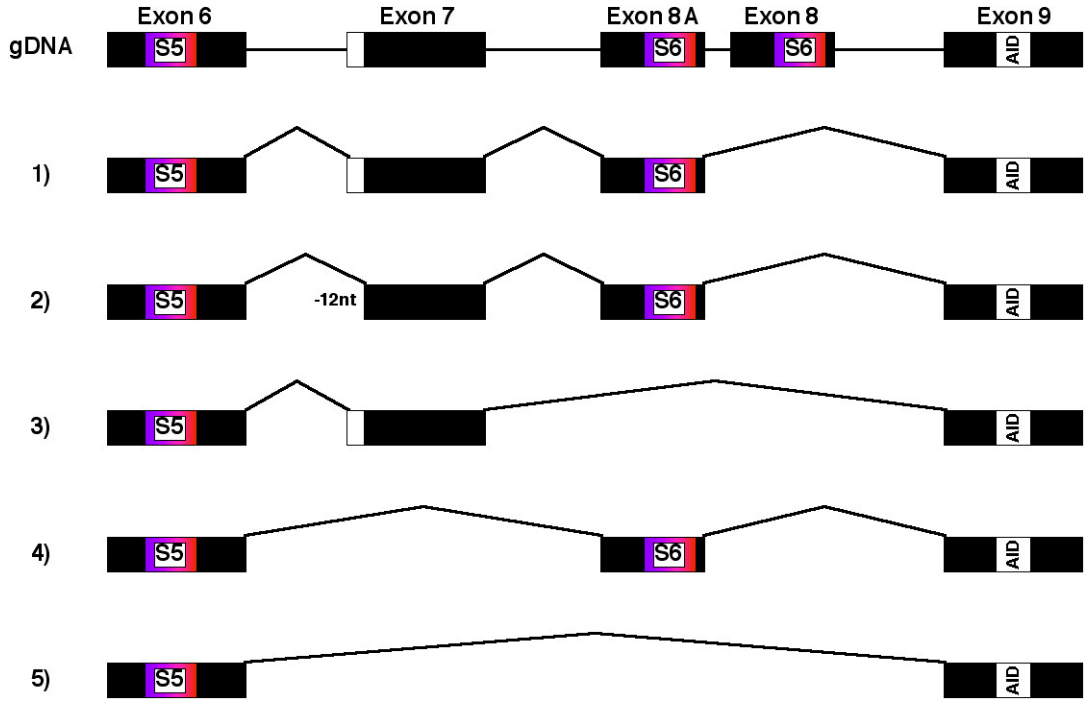

Figure 4. $Ca_v1.2$ splicing variants from exon 6 to 9 in testes (12). Top line illustrates $Ca_v1.2$ exon 6 to 9 structure in genomic DNA (gDNA). Coding regions for domain I transmembrane segments S5 and S6 are labeled in color. Exons 8 and 8A are mutually exclusive exons. P loop between S5 and S6 is encoded by exons 6, 7, and 8/8A. Coding region for AID is indicated in exon 9. Alternative splice junction in exon 7 resulting in a 12 nucleotide deletion is shown by a white box. Five splicing variants are shown below (1-5) with lines joining spliced exons.

In testes, the typical transcript is splice variant 1, which includes exons 6, 7, 8A, and 9, and illustrates the use of alternative exons. In testes, exon 8A is predominantly used instead of alternative exon 8 in $Ca_v1.2$ transcripts. In other tissues, exon 8 is generally expressed in smooth muscle, neurons and fibroblasts, whereas exon 8A is generally expressed in cardiac muscle (45,210,218). The use of these alternative exons may play a key role in calcium channel function, because exons 8/8A encode part of the P loop and all of transmembrane segment 6, which both structurally line the pore of the channel. Indeed, different sensitivities to DHP and voltage-dependent inactivation can be attributed to the alternative use of exons 8/8A (218,225,226) and missense mutations in these exons result in failure of voltage-dependent channel inactivation found in Timothy syndrome (97).

Testes splice variant 2 is similar to the typical transcript, but utilizes an alternative splice acceptor site in exon 7 resulting in the deletion of 12 nucelotides (Figure 4). This in-frame deletion results in the loss of 4 amino acids in an area of the P loop near the conserved alpha helix and glutamate that forms the selectivity region (31,38). Thus, this deletion could conceivably modify calcium ion permeation, but this idea remains to be tested (209).

Testes splice variants 3, 4, and 5 are deletions of exon 8/8A, exon 7, or both exons, respectively (Figure 4). Deletions in splice variants 4 and 5 are new alternative splice patterns that have not been reported elsewhere (12). All three deletions result in frameshifts causing premature translation termination that could potentially produce a truncated protein containing the amino-terminus plus a shortened domain I fragment ending at transmembrane segment S5 plus a variable portion of the P loop with some additional amino acids introduced by the frameshift. These truncated isoforms could potentially combine to form a tetramer with a new channel function, which would probably be very different from the original $Ca_v1.2$ subunit because of the loss of the S6 transmembrane segment. By analogy with the gonadotropin receptors (223), another possibility is that these truncated isoforms could negatively regulate the full-length isoforms by association with the full-length $Ca_v1.2$ subunit and disrupting its structure, or by competing for binding with the auxiliary subunits. Consistent with this possibility, both full length and potentially truncated proteins encoded by mRNAs with deletions in exons 7 and/or 8/8A localize to the plasma membrane of testicular germ cells (12; see Figure 5).

5.3. Susceptibility to environmental toxicants

To understand the mechanisms underlying the action of some toxicants on spermatogenesis and sperm function, it is necessary to understand how calcium permeates the ion-conducting pore of the $alpha_1$ subunit. A calcium binding site is formed by carboxyl side chains of the four glutamate residues, one each in the P loop of the S5-S6 linkers in each repetitive domain, projecting into the lumen of the pore (40; see Figure 2). The VDCC selectivity filter binds one calcium ion with high affinity or two with

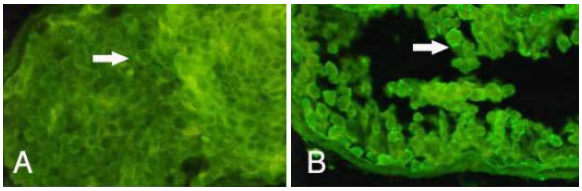

Figure 5. L-VDCC alpha$_{1C}$ antigenic epitopes detected in human testicular biopsies encoded by full length transcripts and those deleted in exons 7 and 8/8A (12). Testis biopsies were obtained by single stick percutaneous aspiration and fixed in formalin (13,22). Biopsy material was processed as previously described (12) and reacted with primary antibodies (IIF7 prepared against the L-VDCC alpha$_1$ dihydropyridine receptor; 6,227) after microwave digestion in 0.01M citrate buffer to reduce non-specific background (12,228). Anti-actin antibodies (A-2668, Sigma Chemical Co., St. Louis, MO; 6) were used as a positive control (not shown). Negative controls were comprised of sections stained with secondary antibodies alone (for IIF7 = F-3008, anti-mouse IgG, whole molecule, affinity isolated and absorbed with human serum proteins, Sigma; for actin = F-0511, anti-rabbit IgG, whole molecule, affinity isolated and absorbed with human IgG to reduced background staining with human samples, Sigma) (not shown). (Note that both secondary antibodies were also pre-absorbed with human sperm to further reduce any non-specific binding.) Stained sections were examined at X600 magnification with an Olympus BX50 microscope (Olympus Corporation, Lake Success, NY) and photographed using an Olympus DP-12 Digital Camera System. [A] Testis biopsy section from a patient with obstructive azoospermia and normal spermatogenesis. L-VDCC encoded by full-length alpha$_{1C}$ mRNAs localize to cell membranes within the seminiferous epithelium (arrow). As observed in animal models (112), the cytoplasm is also diffusely stained but the antigenic epitopes are absent from the nucleus. [B] Testis biopsy section from an infertile male with varicoceles, hypospermatogenesis and elevated testicular cadmium levels. Proteins encoded by a deleted transcript similarly localize to the plasma membrane (arrow) and to the cytoplasm.

low affinity. The former provides the basis for ion selectivity while the latter, when the channel is in the open state, allows one calcium ion to enter into the cytoplasm (51,229). (The ion-binding site thus differs from that of potassium channels, which is formed by backbone carbonyl oxygens and aligns multiple ions in single file; review, 38). Futher, the four E residues are functionally asymmetric (38). Mutational analyses revealed that replacement of E in domain III (E_{III}) produced strongest alterations in channel characteristics (ion-binding affinity, reversal potential, unitary conductance) followed by changes in E_{II}. In constrast, changes in E_I and E_{IV} were less significant.

However, the alpha$_1$ subunit is incompletely ion-specific and can transport cadmium, lead, zinc, nickel, cobalt, aluminum and manganese in addition to calcium (14,15,230,231). As an example, cadmium competes with calcium for the high-affinity binding site (232-235). Cadmium binding can occlude calcium entry while at the same time facilitating cadmium entry upon membrane depolarization (236). These data are supported by findings that calcium channel blockers, such as nifedipine, can protect against the negative effects of metals by blocking their intracellular accumulation (14,237-241) while channel agonists enhance metal uptake (14).

Therefore, we find the alternative splicing between exons 6 and 9 of great interest. The P-loop between IS5 and IS6 is encoded by exons 6, 7 and 8/8A (see Figure 3). Interestingly, exon 7 encodes the P-loop binding sites for metal ions whereby single amino acid changes in this exon alter the ion-selective properties of the alpha$_1$ subunit of voltage-dependent ion channels (89,235,242-245). Exon 8/8A encodes the carboxy terminus of this P-loop and also IS6 (see Figure 3). IS6 controls voltage-dependent channel inactivation (225,246). Based on the need for coordination of calcium with IS6 and the beta subunit to affect high affinity dihydropyridine drug binding (60,61), exon 8/8A may also contribute to the selectivity of ion binding. Thus, both exons 7 and 8/8A impact on ion transport.

Transport of other metal ions (e.g., cadmium and lead) through VDDC alters calcium homeostasis and can trigger apoptosis in somatic cells (e.g., 247). Therefore, the role of the contribution of alternative splicing in exons 6-9 in the alpha$_{1C}$ subunit expressed in mammalian testis to elevations in apoptosis was examined in two situations where reproductive failure was associated with testicular accumulation of heavy or transition metal ions.

First, alpha$_{1C}$ subunit splicing was examined in human testis biopsies from infertile men with varicoceles. Varicoceles are a clinical condition caused by abnormal blood flow in the testes due to dilation of veins of the pampiniform plexus and cause infertility in about 17% of males (248). The obstructed blood flow is thought to increase intratesticular heat leading to abnormal sperm production and infertility. However, surgical repair of varicoceles to restore blood flow does not restore fertility in all cases. Therefore, we have hypothesized that something else is interacting with the varicoceles to produce a profound infertile state ("2nd Hit" Hypothesis; 11,249).

Unexpectedly, we found that infertile men with varicoceles frequently present with elevated levels of cadmium in their seminal plasma (250,251) and cadmium entry sites on the human sperm surface colocalize with L-type VDCC (17). Further examination revealed that cadmium levels were also markedly elevated in the testes of 40% of infertile men with varicoceles (11,22,249). This same subpopulation exhibited elevated germ apoptosis and deletions of exons 7 and/or 8/8A of the alpha$_{1C}$ subunit (12,22). We then proposed that a defect in the L-type VDCC alpha$_{1C}$ subunit in cells of the male germline may contribute to this type of varicocele-induced infertility. Indeed, the presence of the typical alpha$_{1C}$ subunit splice variant 1 (Figure 4) correlated with better fertility outcome after surgical repair of varicoceles (12). Whereas, the absence of splice variant 1 and the presence of the other variants (2 to 5; Figure 4) deleted in exons 7 and/or 8/8A

correlated with infertility after surgical repair and increased cadmium levels. Thus, the lack of a proper $Ca_v1.2$ subunit calcium channel could result in improper clearance of cadmium leading to cell damage and infertility after varicocele repair (12,159,252,253).

Second, as L-, N- and R-type VDCC can transport lead and also differentially blocked by lead (231), $alpha_{1C}$ subunit splicing was compared in testes from lead-sensitive and lead-resistant rat strains. Our microarray studies indicated that resistance to lead-induced testicular toxicity was associated with biphasic changes in genes whose expression is regulated by calcium (254). Interestingly, the splicing patterns of the mRNA encoding the $alpha_{1C}$ subunit in rat testes paralleled those described in man (255) and development of testicular resistance was associated with a transient increase in germ cell apoptosis as well as decreased levels of $alpha_{1C}$ transcripts and transient production of deleted $alpha_{1C}$ transcripts.

We suggest that the observed splicing changes in testicular L-VDCC $alpha_{1C}$ mRNAs may represent a regulatory or "protective" response in testis adjusting calcium levels in response to cadmium or lead entry. This suggestion is based on two observations. First, in patients with varicoceles, deletion of exon 8/8A was associated with a decrease in apoptosis despite elevated cadmium levels and an improved response to varicocele repair (253). Second, in contrast to findings in testes from a lead-resistant rat strain, total $alpha_{1C}$ transcript levels increased without an increase in $alpha_{1C}$ mRNA alternative splicing in the testes from a lead-sensitive rat strain (S. Benoff and R.Z. Sokol, unpublished observations).

A mechanism regulating this alternative splicing is also suggested from the study of calcium homeostasis in somatic cells. In brain, protection against changes in calcium homeostasis involves, at least in part, altering splicing of calcium transporters (256,257). Regulation of such alternative splicing involves calcium/calmodulin-dependent serine/threonine protein kinase IV (CaMK IV). Changes in intracellular calcium levels alter the CaMK IV activity (e.g., 258,259). CaMK IV responsive RNA elements (260) within mRNAs encoding calcium channels permit calcium to modulate splicing of its own transporters (261). This splicing switch can favor formation of truncated transcripts (261). Since we have identified potential CaMK IV responsive RNA elements in testes L-VDCC $alpha_{1C}$ mRNA sequences (252,253) and testicular CaMK IV levels are transiently elevated in response to chromic lead exposure in a lead-resistant rat strain (254), this can explain how a change in intracellular calcium levels in response to cadmium or lead could create variant L-VDCC transcripts.

6. CONTRACEPTION

We were the first to suggest that calcium channel antagonists might be developed into non-steroid, reversible and safe male contraceptives. This suggestion was based on strong evidence from studies of patients undergoing *in vitro* fertilization, demonstrating that all three major classes of calcium channel blockers (1,4-dihydropyridines [prototype: nifedipine], phenylalkylamines [prototype: verapamil], benzothiazepine [prototype: diltiazem]), which inhibit depolarization-induced calcium flux in somatic cells (262), produced a reversible infertile state (263-267) and was supported by at least one other study (268). Therefore, our initial goal was to target the $alpha_{1C}$ subunit expressed in testis and sperm (6). With the subsequent characterization of other $alpha_1$ subunits expressed in the male germ line additional potential targets for drug action have emerged. Nonetheless, eleven years later this approach still remains essentially at the theoretical stage for a number of reasons.

6.1. Funding

Although men and women have similar attitudes and knowledge levels of contraception, the primary responsibility for prevention of pregnancy is borne by women. This is due, in part, to the disadvantages of the limited male contraception options (hormones to suppress spermatogenesis; vasectomy or condoms to prevent sperm transport in the female tract). What was perceived as lack of public interest by the public sector had deterred drug development by the pharmaceutical industry. More recent concerns about adverse endpoints associated with drug administration make the likelihood of pharmaceutical company-sponsored clinical trials quite poor (269,270). As such, the main source of new, non-steroidal male contraceptive protocols are reports on the unexpected anti-fertility effects of drugs created for other purposes (review, 1) and studies of Chinese traditional medicine (271,272).

6.2. Complexity of drug binding site(s) and drug interaction

The interaction between $alpha_{1C}$ subunit and dihydropyridine antagonists will be used an example, as dihydropyridines are thought to be the most efficacious and potent L-type calcium channel blockers.

Binding sites for the three major classes of calcium channel blockers have been identified using photoaffinity labeling, antibody mapping, gain-of-function chimeric $alpha_1$ subunits, and loss of function site-directed mutagenesis (51,273,274). Initially, it was thought that the three classes of drugs bound to independent but allosterically interacting sites. These sites were thought to be in close proximity with the potential for some overlap. Current thinking, however, envisages a domain interface model where all three classes of drugs can bind at the same time (273). It is composed of transmembrane helices IIIS5 (exon 25), IIIS6 (exon 28) and IVS6 (exons 37 and 38). These regions form part of the central pore of the $alpha_1$ subunit (see Figure 2B). Thus, drug binding occurs in reasonable proximity to the selectivity filter of the ion channel and is consistent with observations that high-affinity drug binding is stabilized by binding of calcium within the channel pore (274). Further, the finding that the drug binding domains lay within the plane of the plasma membrane raises the issue of how the calcium antagonists access their binding domains (273; see below).

Although transmembrane segments IIIS5, IIIS6 and IVS6 are thought to be the primary site for dihydropyridine binding, other sites within the $alpha_1$

subunit also contribute to binding. For example, site-specific mutagenesis and homology modeling of agonist and antagonist binding revealed that the glutamates of the selectivity filter in domains III and IV contribute to high affinity dihydropyridine binding (273,275). As another example, using both photoaffinity labeling and chimeric constructs, it was determined that amino acid sequences within alternatively spliced transmembrane helix IS6 (exons 8/8A) contribute to the sensitivity of the $alpha_{1C}$ subunit to dihydropyridines (218,226,276). This was not completely unexpected, as this is also true for IS6 segment in the HVA $alpha_{1S}$ subunit (277) and for drug binding sites within the $alpha_1$ subunit of related sodium channels (278,279). Alternative splicing of exons 21/22, which encode transmembrane segment IIIS2, modulated the voltage-dependence of channel blockade by dihydropyridines (217). Site-directed mutagenesis of two amino acids within the IIIS5-S6 linker altered binding of dihydropyridines and the action of calcium channel anatagonists (280). Co-expression experiments demonstrated that binding of the beta and $alpha_2$delta subunits affects the affinity of dihydropyridine binding to the $alpha_{1C}$ subunit (51,60-62).

VDCCs exist in three distinct states: [1] resting, [2] open/activated, and [3] inactivated (281). Channels move from the resting to the open/activated via depolarization and then to the inactivated state. Repolarization again moves the channel to the resting state. All three major classes of calcium channel blockers exhibit state-dependent interactions with the $alpha_1$ subunit. Thus, an additional complexity is derived from the voltage dependence of dihydropyridine inhibition of calcium flux, favoring binding to an inactive $alpha_{1C}$ channel (51). Examination of naturally occurring splice variants with subsequent site-directed mutagenesis identified alternately spliced transmembrane segment IIIS2 (exons 21/22) as an important modulator of this effect (217).

Finally, issues exist about absolute drug specificity, as antagonists of L-type channels can block T-type channels and visa versa (e.g., 8,282).

6.3. Tissue-specific drug targeting

We initially thought that the alternative splicing of the $alpha_{1C}$ subunit expressed in human testis and sperm would aid in the development of calcium entry antagonists targeted specifically to the male reproductive tract (1), as this is a well traveled route of drug development, e.g., structure-activity requirements have been defined for drugs that block related sodium channels (283). Further, we considered using dihydropyridine analogs with nonidentical ester groups as a starting point, as this class of drugs demonstrates tissue selectivity in somatic organs (284). This approach needed to be revisited as the $alpha_{1C}$ splice variants detected in the human germ line also occur in various somatic cells (e.g., 209) and as multiple splice variants co-exist in testis and sperm specimens from individual human subjects (12,159).

6.4. Mode of anti-fertility effects of existing drugs

Our initial studies demonstrated that the anti-fertility effects of calcium antagonists were not directly associated with blockade of calcium transport and the acrosome reaction, but rather the result of blocking the decrease in plasma membrane cholesterol content associated with sperm capacitation, inhibiting the normal rise in intracellular free calcium and in zona binding potential (6,263,265,266). We originally attributed this to effects on membrane width and packing. Although a strong body of evidence supports the concept that calcium channel blockers reach their receptor site on the $alpha_1$ subunit via transit through the outer vestibule of the pore (275); there is data supporting an alternate route. The work of Mason and his colleagues suggests that these lipophilic drugs can partition into the membrane before binding to L-type VDCC (285-288), thereby stabilizing the membrane bilayer (288).

Consistent with this, calcium channel blockers have long been reported to alter cholesterol metabolism in somatic cells by inhibiting esterification of free cholesterol (289), stimulating cholesterol esterification (290) or stimulating cholesterol ester metabolism (291,292). These effects differ for each compound tested. Subsequent studies established that these were mediated by antagonist binding to molecules unrelated to calcium channels but involved in cholesterol biosynthesis or metabolism (273,293-295).

As sperm contain the enzymes required to synthesize plasma membrane lipids (296,297) including cholesterol (298,299), we assessed the effects of nifedipine on 2-[^{14}C]-acetate incorporation into cholesterol and on membrane cholesterol content of ejaculated human sperm (300,301). We observed that nifedipine stimulated sperm cholesterol synthesis. These *in vitro* findings clearly paralleled the effects of *in vivo* administration of nifedipine on sperm membrane cholesterol content. Although these findings suggest that compounds like nifedipine that modulate membrane cholesterol levels could be developed as male contraceptives, the considerable variability of sperm cholesterol content between men (300,302) emphasizes the possibility of inter-male differences in contraceptive efficacy, helping to explain why some men medicated with calcium channel blockers retain their fertility (303).

7. PERSPECTIVES

The past few years have been occupied by elaborating on the work recognized by the 2003 Nobel chemistry prize. The bacterial ion channel model structure has proved surprisingly robust and informative. It has provided a framework for understanding a wide variety of experimental results across the whole range of living organisms. We now have some general understanding of how ion channels open and close on a molecular level, why some ion channels are monomeric, while others with similar properties can be hetero-or homomultimeric, how many classes of inhibitors function, and how small changes in protein coding can drastically change gating properties and selectivity. This is fortunate, since the family of voltage gated calcium ion channels continues to grow. While other lineages appear to be approaching maturity, it is anyone's guess how large the CatSper clan will be in five

years time, and we fear that there other clans out there waiting to be discovered. We do not yet know how the auxiliary subunits function (or indeed, if we have identified all these subunits). We do not have a complete list of secondary signals that modulate properties of membrane-embedded ion channels, not to mention signaling systems that must control synthesis and transport of these proteins. We also do not know the purpose and function of the abundant coexisting splice variants.

Looking only at sperm, it appears that as calcium ion channels are vital to motility, capacitation and acrosome reactions, drugs targeted at them should be good contraceptives. But as the T-type/N-type/L-type controversies are giving way to a realization that a number of ion channels are involved in parallel redundant pathways, and a fear that the overall picture will prove to be even more complex than we believe today, the ion channel contraceptive target becomes fuzzier, and other cellular process (for example, cholesterol metabolism) beckon. In our current state of ignorance it is not difficult to develop schemes in which small differences in cellular signal molecules between species could drastically change the relative importance of redundant pathways, so that channels that dominate, say, the acrosome reaction, in one species might be relatively unimportant in another. Investigators persisting in ion channel contraceptive development should take warning from this, and work as much as possible in the human.

From the point of view of a reproductive toxicologist, ion channels have become much more interesting. The demonstration that ion channels are proteins whose levels, structures and functions are modulated by both toxicant exposure and genetic background moves them nearer the center of a toxicologist's universe than when they appeared to be passive portals for toxicant ions. Elucidating their role will be challenging. Neither today's gene arrays nor today's proteomics can exhaustively characterize even relative levels of the various splice variants. Until technology catches up, our understanding of the impact of a heavy metal or endocrine disruptor on cellular ion transport will probably continue to be stitched together from many very focused experiments, with all the limitations inherent in this approach.

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Send correspondance to: Dr Susan Benoff, Fertility

Research Laboratories, The Feinstein Institute for Medical Research, 350 Community Drive, Room 125, Manhasset, New York 11030, Tel: 516-562-1121, Fax: 516-562-1022, E-mail: sbenoff@nshs.edu

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