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Cloning of a novel one-repeat calcium channel-like gene☆

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Abstract

We describe the cloning of a cDNA from a human testis library that encodes a novel protein with similarity to one repeat of voltage-gated Ca^{2+} channels (Ca_v). Northern and dot blot analyses indicate that the novel Ca_v-like gene is expressed predominantly in testis and at lower levels in many other tissues. Heterologous expression of the Ca_v-like protein did not lead to the induction of any detectable ionic current and failed to modify intracellular Ca^{2+} concentrations. Similar one-repeat Ca_v-like proteins have been cloned from *Bacillus*, *Mus*, and *Homo*, and appear to encode ion channels involved in renal function, axis determination, and sperm motility.

Keywords: Calcium channels; Molecular sequence data; Amino acid sequence; Ion channel gating; Sequence homology; Testis

Some ion channels appear to have evolved from a primordial ancestor that contained a pore loop flanked on each side by a transmembrane segment (S1-P-S2). The next evolutionary jump was the addition of 4 transmembrane (TM) segments (S1-S2-S3-S4-S5-P-S6), which play a role in gating the channel. This fundamental repeat is found in a wide variety of ion channels including intracellular release channels, transient receptor potential (TRP) channels, cyclic nucleotide-gated channels (CNG), and voltage-gated K^+ (K_v), Ca^{2+} (Ca_v), and Na^+ (Na_v) channels [1–3]. In Ca_v and Na_v channel proteins this unit is repeated four times, while K_v channels are formed by a tetramer of proteins. Interestingly, there are one repeat channel proteins that show significant sequence identity to the pore loops of Ca_v channels. Such Ca_v-like channels include: PKD2 [4], PKD2-L [5], PKD2-L2 [6], CatSper1 [7], CatSper2 [8], and the bacterial gene, NaChBac [3,9]. The first was identified by positional cloning, as mutations in the PKD2 gene are found in 15% of families affected by autosomal dominant polycystic kidney disease [4]. Recent studies suggest that the PKD2 protein is localized to the endoplasmic reticulum of tubular epithelial cells, where it functions as a calcium release channel [10]. Targeted disruption of the homologous gene in mice also leads to renal cysts, but also to cardiac defects and disruption of left–right axis determination [11]. Similarly, PKD2-L has both been reported to form non-selective cation channels (polycystin-L), and deletion of the homologous gene in mice causes renal and retinal defects [12]. In contrast, expression of the CatSper proteins did not induce ion channel activity, although sperm from transgenic mice lacking the CatSper1 gene were shown to be deficient in cAMP-induced Ca^{2+} influx, to swim slow, and to be unable to fertilize intact eggs [7]. These studies establish the importance of one-repeat Ca_v-like proteins in renal and reproductive physiology.

Here we describe the cloning of a novel member of the one-repeat Ca_v-like family of ion channels. Northern blot analysis indicates that the gene is predominantly expressed in testis, while dot blot analysis indicates that it is expressed at lower levels in many tissues including brain and heart. Similar to many one-repeat Ca_v-like proteins, expression in heterologous systems did not lead to the induction of identifiable cation currents.

☆ The sequences reported herein have been assigned GenBank Accession Nos. AF432876 and AY156951.

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Materials and methods

Tissue expression. Northern and multiple tissue arrays of human poly(A)$^+$ RNA were obtained from OriGene Technologies (Rockville, MD) and BD Biosciences Clontech (Palo Alto, CA), respectively. The radiolabeled riboprobe was synthesized with the Strip-EZ RNA T7/T3 kit (Ambion, Austin, TX) in the presence of [α-^{32}P]UTP. The template was *Eco*RI-linearized plasmid of IMAGE Consortium clone 1841778 [13], whose complete sequence we report in GenBank #AY156951. Blots were hybridized to the probe ($\geqslant 10^6$ cpm/ml) overnight in ULTRAhyb buffer (Ambion). After washing membranes at temperatures of up to 65 °C in 0.25× SSC, 0.1% SDS buffer, they were exposed to film (Hyperfilm MP, Amersham Biosciences, Piscataway, NJ) at −70 °C between intensifying screens.

Molecular cloning. A λTriplEx human testis cDNA library (Clontech, #HL5033t) was screened to clone the full-length cDNA. Probe was generated by PCR amplification using the forward primer m4 (GACAATGGTGACCATGATAA) and the reverse primer m6 (AGCTCTCGCTCAAACTTTCT), and clone 1841778 as template. The gel purified PCR product (240 bp) was radiolabeled using random primers and [α-^{32}P]dATP. Hybridizations ($\geqslant 10^6$ cpm/ml) were carried out overnight at 65 °C in standard hybridization buffer that included 50% formamide. Washes were performed up to 45 °C in 1× SSC, 0.1% SDS buffer. Positive colonies (8 out of 3.4×10^5) were plaque purified and those that contained inserts of the expected size were identified by PCR amplification using the λTriplEx 5′ LD-insert primer and the internal reverse primer m6. Inserts in 3 colonies (called AC2, AC4, and AC8) were converted to pTriplEx plasmids according to manufacturer's instructions. Subsequently, the *Not*I fragments of AC2 and AC8 plasmids were subcloned into pcDNA3-HE2 vector for expression studies.

RT-PCR. Human fetal brain poly(A)$^+$ RNA and testis total RNA were purchased from Clontech. cDNAs were synthesized from either 1 μg of total RNA or 100 ng poly(A)$^+$ RNA, using 5 μM random decamers, 0.5 mM each dNTP, 20 U SUPERase · In (Ambion), and 100 U M-MLV reverse transcriptase (Ambion). Amplification reactions were performed as described previously [14].

Heterologous expression. 293 cells (human embryonic kidney, #CRL-1573, American Type Culture Collection, Manassas, VA) were transiently transfected by the calcium phosphate method (CalPhos Maximizer Transfection Kit, Clontech). To identify transfected cells, a plasmid containing green fluorescent protein (GFP; pGreen Lantern) was co-transfected with pcDNA3-HE2-AC2 at a molar ratio of 1:10. Experiments were also performed with GFP fused to the amino terminus. GFP was either fused at position-6 (full-length Ca_v-like protein) or at position 99 to remove a putative nuclear localization signal in exon 1. After approximately 24 h, GFP positive cells were selected for recordings.

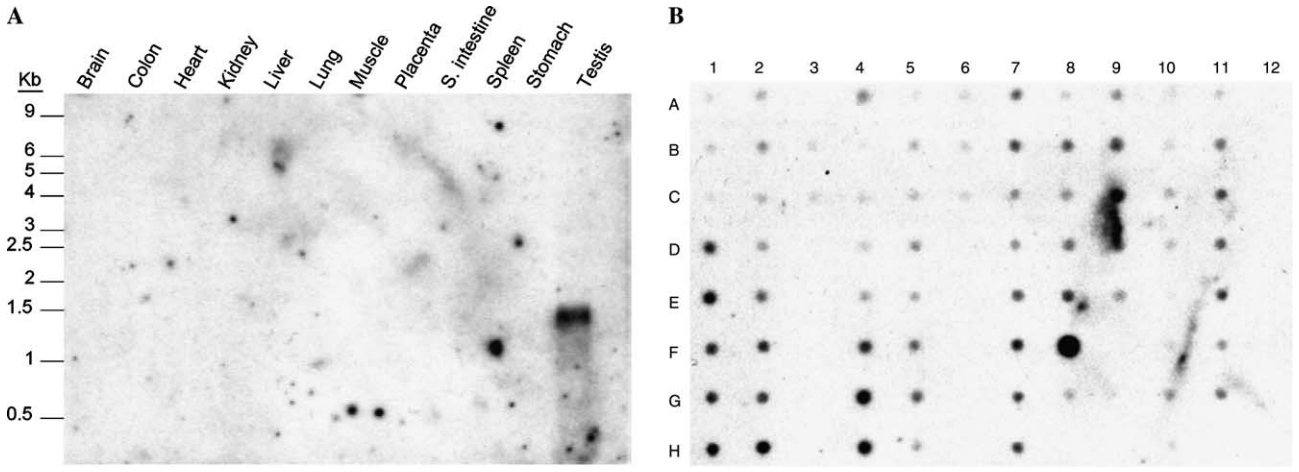
Electrophysiological recordings were made using the whole cell configuration of the patch clamp technique as described previously [14]. Two external solutions were used, one was a typical physiological saline (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 dextrose, and 10 Hepes, pH adjusted to 7.4 with NaOH. The second solution was designed to isolate Ca^{2+} currents (in mM): 5 $CaCl_2$, 155 tetraethylammonium (TEA) chloride, and 10 Hepes, pH adjusted to 7.4 with TEA-OH. The pipettes were filled with a solution that contained (in mM): 125 CsCl, 10 EGTA, 2 $CaCl_2$, 1 $MgCl_2$, 4 Mg-ATP, 0.3 Na_3 GTP, and 10 Hepes, pH adjusted to 7.2 with CsOH. Under these solution conditions the pipette resistance was typically 3–4 MΩ. Series resistance values ranged between 3 and 9 MΩ (5.5 ± 0.2, $n = 27$), and the average cell capacitance was 9.7 ± 0.5 pF. Some experiments were made using the perforated patch clamp configuration with amphotericin B (120 μg/ml) in the internal pipette solution. These recordings began when the series resistance value was below 20 MΩ. Results were obtained from over 50 cells from 15 transfections.

Oocytes from *Xenopus laevis* were injected with 6 or 18 ng cRNA made from the linearized template, pcDNA3-HE2-AC2, and then incubated at least for 3 days before recording. Currents were recorded using a two-microelectrode voltage-clamp amplifier (OC-725B, Warner Instruments). Monovalent and Ca-activated Cl^- currents were measured in SOS solution: 2 mM $CaCl_2$, 100 NaCl, 2 KCl, 1 $MgCl_2$, and 5 Hepes (pH 7.4). This solution was then exchanged with the following solution to test for the presence of Ca^{2+} channel activity (in mM): 40 $BaCl_2$, 36 TEA-Cl, 2 CsCl, and 5 Hepes (pH 7.4). Voltage and current electrodes contained an agarose cushion and were filled with 3 M KCl. All experiments were performed at room temperature (~22 °C).

Results and discussion

Large scale sequencing of cDNA clones has provided a rich source for the discovery of novel genes, and provided the lead for cloning of many ion channels, including T-type Ca^{2+} channels [15] and putative one-repeat Ca^{2+} channels [3,7]. To identify new Ca^{2+} channel genes we monitored new entries into the GenBank database for proteins that were similar to the T-type channel Ca_v 3.3. This led to the identification of a mouse cDNA that encoded a protein with 25% identity to Ca_v 3.3 over 193 amino acids (GenBank #BAB29631). This cDNA was cloned from a mouse testis cDNA library by the RIKEN Genome Exploration Research Group as part of their Mouse Gene Encyclopedia Project [16]. BLAST search of the EST division of the GenBank database with the cDNA sequence of this clone (GenBank #AK014942) identified 6 other mouse clones and 12 human clones that were ~85% identical. The mouse cDNAs were cloned from 3 distinct libraries, including two from testis and one from spermatogenic cells. Although some of the human cDNAs were cloned from testis, many were cloned from other tissues, including colon, ovary, and brain. Interestingly, many of the human clones were derived from tumors, including those of germ cells, colon, uterus, and melanocytes. These electronic Northern blot results indicate that mRNA transcripts of this gene are expressed in a wide variety of tissues.

To determine the human tissues in which this gene is expressed we probed both Northern and dot blots at high stringency (Fig. 1). Northern blot analysis revealed a single 1.4 kb transcript in testis mRNA (Fig. 1A). Similarly, the RNA Master blot indicated a predominant expression in testis. In addition, a wider expression was detected in specific tissue sections by dot blot analysis, including heart (right ventricle, apex, and interventricular septum) > brain (putamen, temporal, occipital, and parietal lobes, hippocampus, and medulla) > adrenal gland ⩾ lymph node, pancreas, placenta, skeletal muscle, prostate, bone marrow, and trachea. Expression was higher in fetal tissues (Fig. 1C, column 11) than the corresponding adult tissue. The probe did not hybridize to any of the negative controls



C

	1	2	3	4	5	6	7	8	9	10	11	12
A	Whole brain	Cerebellum, left		Heart	Esophagus	Colon trans.	Kidney	Lung	Liver	Leukemia HL-60	Fetal brain	Yeast RNA
B	Cerebral cortex	Cerebellum, right	Accumbens	Aorta	Stomach	Colon desc.	Skeletal muscle	Placenta	Pancreas	HeLa S3	Fetal heart	Yeast tRNA
C	Frontal lobe	Corpus callosum	Thalamus	Atrium left	Duodenum	Rectum	Spleen	Bladder	Adrenal	Leukemia K-562	Fetal kidney	*E. coli* rRNA
D	Parietal lobe	Amygdala		Atrium right	Jejunum		Thymus	Uterus	Thyroid	Leukemia MOLT-4	Fetal liver	*E. coli* DNA
E	Occipital lobe	Caudate		Ventricle left	Ileum		Leukocyte	Prostate	Salivary gland	Burkitt's Raji	Fetal spleen	Poly r(A)
F	Temporal lobe	Hippocampus		Ventricle right	Ilocecum		Lymph node	Testis		Burkitt's Daudi	Fetal thymus	C_0t-1 DNA
G	Paracentral gyrus	Medulla		Septum	Appendix		Bone marrow	Ovary		SW480	Fetal lung	DNA 100 ng
H	Pons	Putamen		Apex of heart	Colon asc.		Trachea			Lung A549		DNA 500 ng

Fig. 1. Expression of the novel Ca_v-like gene in human tissues. (A) Autoradiogram of a Northern blot. The film was exposed for 5 days. (B) Autoradiogram of a RNA master blot. The film was exposed for 2 days. Both blots were probed together with a ^{32}P-labeled riboprobe, corresponding to nucleotides 935–1270 of the full-length Ca_v-like cDNA (GenBank #AF432876). (C) Tissues on the RNA master blot.

(Fig. 1C, column 12). To confirm expression of the Ca_v-like gene in brain, we PCR amplified a fragment from cDNA, subcloned the products, and sequenced representative clones. A sequence identical to that cloned from the testis cDNA library was found. We also cloned a variant that appears to include the same intronic sequence as GenBank entry BM804844 (described below). We conclude that the Ca_v-like mRNA is highly expressed in testis and at lower levels in most tissues.

To clone a full-length human cDNA of this novel gene we PCR amplified a fragment, radiolabeled it, and then used it to screen a human testis cDNA library by con-

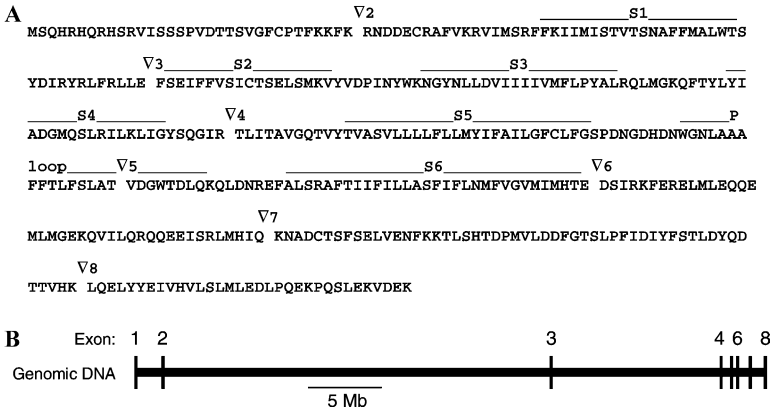


Fig. 2. Deduced amino acid sequence and genomic organization of the novel Ca_v-like gene. (A) The putative membrane spanning regions (S1–S6) and pore (P) loop region are indicated above the amino acid sequence. All 8 exons of the gene encode protein and these regions are indicated with an inverted triangle. (B) A schematic representation of the Ca_v-like gene showing the relative size of the introns.

ventional filter hybridization techniques. The two largest clones (1.3 kb inserts) were subcloned and sequenced. Both clones contain an open reading frame that would encode a 398 amino acid protein with a molecular weight of 46,420 (Fig. 2A). The deduced amino acid sequences of these clones are 100% identical, while the nucleotide (nt) sequence differs at two bases (wobble codon for alanine), and differ by one clone having 15 extra bases at the 5′ end. An in-frame stop codon occurs 21 nt before the presumptive start codon. The 3′ untranslated region is only 76 nt long, but contains a polyadenylation signal (ATTAAA). The sizes of these cDNAs are similar to that of the transcript detected on Northern blots, indicating that they are full-length.

BLAST search of the GenBank allowed identification of the human gene and its intron/exon borders (Fig. 2B, Table 1). The gene contains 8 exons spanning 43.7 kb and is located at chromosome 5q31.2. The 5′ end of the gene lies only 6.6 kb downstream of the gene encoding a muscle isoform of dimerization factor of hepatocyte nuclear factor 1α (DCOHM, GenBank #499009; [17]). Somewhat surprisingly, variants of DCOHM have been reported that overlap with the gene encoding the novel Ca_v-like sequence. In the case of AK098495, a putative 5th exon of DCOHM corresponds to the intronic sequence between the 1st and 2nd exons of the Ca_v-like gene. This prompted us to examine the genomic sequence in greater detail. The program RepeatMasker (http:ftp.genome.washington.edu/RM/RepeatMasker.html) identified 204 repeats, of which 69 were Alu repeats and 62 were LINE repeats. The percentage of repeats in introns 2 and 3 approaches 90% (Table 1), which is considerably greater than the 42% estimated for the entire human genome [18]. We conclude that these repeats interfere with the splicing of the DCOHM and Ca_v-like genes, leading to transcripts that contain exons from both genes (e.g., GenBank AL136721), as well as intronic repeats (e.g., BM804844).

Hydropathy plots of the novel protein suggest that it contains six transmembrane segments (Fig. 3A). A similar structure is predicted for the CatSper proteins, NaChBac, the PKD proteins, and to one repeat of an α1 subunit of voltage-gated Ca^{2+} channels [3,6,7,19]. The PKD proteins show 60% identity to one another and therefore can be considered a related family. The other one-repeat proteins are less than 15% identical to each other, and even when the analysis is restricted to the membrane spanning regions, no direct evolutionary relationships can be deduced (Fig. 3B). The highest level of sequence identity between these proteins occurs in the S5, S6, and pore loops (Fig. 3C). This is notable because these regions are thought to play a central role in forming the channel wall and its selectivity filter [20]. The selectivity filter of high voltage-activated Ca^{2+} channels includes the signature sequence FxxxTxExW in each repeat (EEEE), where the conserved glutamate (E) has been shown to form a Ca^{2+}-binding site [3]. Low voltage-activated Ca^{2+} channels also have similar P loop sequences, except that in two repeats the glutamate has been replaced by an aspartate residue (EEDD). Similarly, the novel channel contains an aspartate residue in its P loop, and due to this similarity we refer to it as a Ca_v-like protein and speculate that it may form a Ca^{2+}-selective channel.

Table 1
Intron–exon boundary sequences of the Ca_v-like Gene

Exon	Length (bp)	Acceptor	Donor	Intron	Intron size (bp)	% Repeats in intron
1	>163		TTT AA[gtaaat] F K		~8800	37
2	154	[tgacag]G AGG AAC R N	CTT GAG[gtaagc] L E	1	1849	73
3	234	[ctgtag]TTC TCG E I	ATC CGG[gtgagt] I R	2	26211	87
4	182	[ttgcag]ACG CTG T L	GCC ACG[gtactg] A T	3	11,444	89
5	145	[gagcag]GTT GAT V D	ACA GAG[gtgagg] T E	4	697	0
6	120	[ctctag]GAC TCC D S	ATA CAG[gtgagt] I Q	5	389	0
7	157	[ctctag]AAA AAT K N	CAC AA[gtcagt] H K	6	924	0
8	>100	[tttcag]G CTT CAA L Q		7	991	32

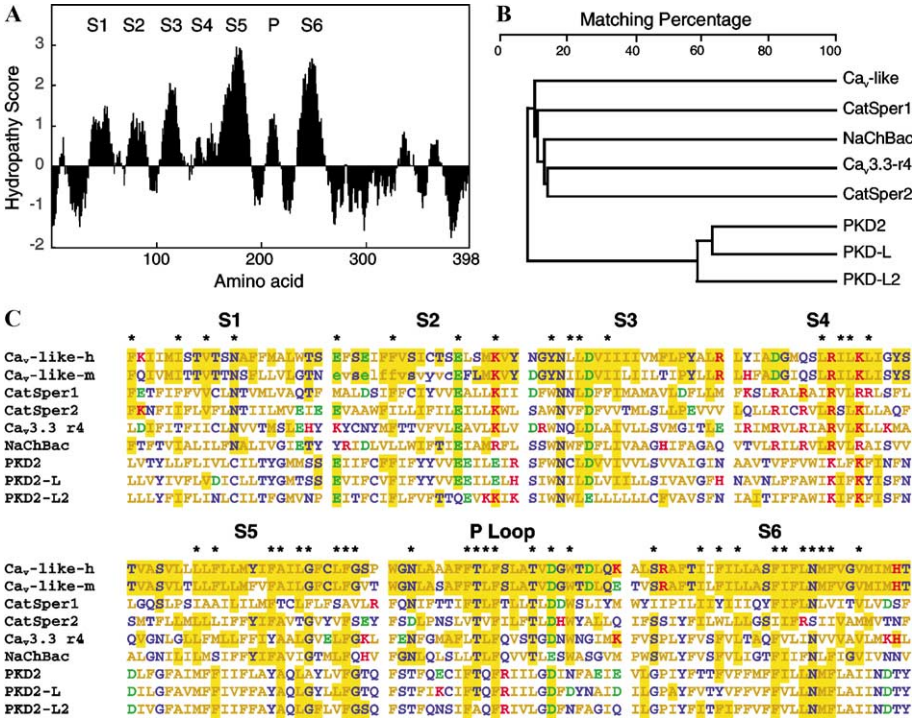


Fig. 3. Similarity of the novel Ca_v-like protein to other proteins. (A) Hydropathy plot of the Ca_v-like protein calculated using the Kyte and Doolittle [22] algorithm and a window of 18 residues. (B) Dendrogram showing the matching percentage calculated by the CLUSTAL algorithm [23] when the input sequence of each protein has been reduced to just the membrane spanning regions (as in C). (C) An alignment of the putative membrane spanning regions of Ca_v-like proteins to the fourth repeat of the T-type Ca^{2+} channel, Ca_v 3.3. Amino acids are represented by the one-letter code and colored by their functional property as follows: hydrophobic, yellow; polar, blue; positively charged, red; and negatively charged, green. Residues in the human Ca_v-like protein that are common with any given sequence are highlighted in yellow, while those found in 4 or more other sequences are marked with an asterisk. The sequences are from the following GenBank entries: Ca_v-like-m, mouse homolog of the human Ca_v-like-h protein described herein, BAB29631; human CatSper1, AA14105; human CatSper2, NP_473361; human Ca_v 3.3, AAM67414; NaChBac, BAB05220; human PKD2, Q13563; human PKD2-L, NP_057196; and human PKD2-L2, NP_055201. The Ca_v-like-m sequence contains 13 amino acids that were deduced from the mouse genomic sequence contained in GenBank entry NW_000081, and these residues are shown in lowercase.

Another region found in all voltage-gated channels (and in some that are not) is the S4 segment [1]. Considerable evidence supports the hypothesis that S4 segments sense the transmembrane voltage field and shift outwards upon depolarization, leading to channel opening [21]. This region typically contains 4–6 positively charged amino acids spaced every third residue and separated by hydrophobic residues. The putative S4 segment of the novel Ca_v-like protein contains 2 positively charged residues with similar spacing. This region diverges from Ca_v channels by containing many polar residues. Typically the S4 sequence is highly conserved across species, with 100% conservation between mice and human for most Ca_v channels. In contrast, 4 of the 24 residues in Ca_v-like S4 segments are not conserved between these two species (Fig. 3C). This has prompted an examination of sequence conservation for all of the one-repeat Ca_v-like proteins. Typically the amino acid sequence of any particular Ca_v channel is conserved at 90–95% between mice and human, e.g., Ca_v 3.3 is 90% identical and PKD2 is 85% identical. In contrast, the other one repeat Ca_v-like proteins are not well conserved: CatSper1, 34%; CatSper2, 57%; and the novel Ca_v-like sequence, 56%. The mouse Ca_v-like sequence is located in a syntenic region on mouse chromosome 13 (between markers Cspg2 and H2afy), indicating that it is the homolog of the human sequence we cloned. This low level of conservation can be interpreted in at least two ways: one, the function of the proteins differs between these species, or two, that functionally important residues are non-contiguous.

To test for ion channel function we expressed the Ca_v-like protein in two commonly used heterologous expression systems, *Xenopus* oocytes and 293 human embryonic kidney cells. Currents were measured with ruptured or perforated patch clamp techniques under the following conditions: after voltage steps in the range from 100 to +100 mV, which were applied from different holding potentials (−50 to −100 mV); after a change in the pH; after application of ATP; and in solutions containing either Na^+ or Ca^{2+} as charge carrier. Although both systems contain endogenous ionic currents, their electrophysiological properties were unchanged by expression of the Ca_v-like protein. As the pore loop sequence suggested that it might conduct Ca^{2+}, we also examined intracellular Ca^{2+} concentrations using Fura-

2 loaded 293 cells. No change was observed in either resting intracellular Ca^{2+} concentration, or the rise in Ca^{2+} concentration produced by treatment with either thapsigargin or ATP. To verify that the protein was expressed, we fused GFP to the amino terminus and expressed the construct in 293 cells. Fluorescent protein was readily detected. Confocal microscopy indicated that the majority of the GFP-tagged protein was expressed in the nucleus. Removal of a putative nuclear retention signal encoded in exon 1 did not alter its intracellular distribution (results not shown).

In conclusion we have cloned the cDNA from a novel gene that encodes a protein with similarity to one repeat of a voltage-gated Ca^{2+} channel. It is similar to the CatSper proteins in the following ways: they share modest sequence similarity, all the three are predominantly expressed in the testis, and that none of them generates measurable ionic currents in heterologous expression systems. Targeted disruption of the mouse CatSper1 gene decreased sperm motility and abolished the ability of cAMP and cGMP to increase intracellular Ca^{2+} concentrations [7]. Since the CatSper1 sequence does not contain any canonical nucleotide-binding regions, it was concluded that it may form a complex with as yet unidentified protein(s) that are only expressed in mature sperm to form a Ca^{2+} selective channel. Similarly the Ca_v-like protein described herein may require auxiliary proteins for proper localization, and under the appropriate conditions may form an ion channel.

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