TRANSIENT AND BIG ARE KEY FEATURES OF AN INVERTEBRATE T-TYPE CHANNEL (LCAV3) FROM THE CENTRAL NERVOUS SYSTEM OF LYMNAEA STAGNALIS
Adriano Senatore and J. David Spafford
From Department of Biology, University of Waterloo, Waterloo, Canada. N2L 3G1
Running head: Features of a novel invertebrate T-type channel
#Address correspondence to: J David Spafford, B1-173, Department of Biology,
University of Waterloo, Waterloo, ON. N2L 3G1
PH: 519-888-4567 x 38186; FAX: 519-746-0614; E-mail: spafford@uwaterloo.ca

Here we describe features of the first non-mammalian T-type calcium channel (LCa,3) expressed in vitro. This molluscan channel possesses combined biophysical properties that are reminiscent of all mammalian T-type channels. It exhibits T-type features such as transient kinetics, but the tiny label, usually associated with Ba\textsuperscript{2+} conductance, is hard to reconcile with the “bigness” of this channel in many respects. LCa,3 is 25% larger than any voltage-gated ion channel expressed to date. It codes for a massive, 322 kDa protein that conducts large macroscopic currents in vitro. LCa,3 is also the most abundant Ca\textsuperscript{2+} channel transcript in the snail nervous system. A window current at typical resting potentials appears to be at least as large as that reported for mammalian channels. This distant gene provides a unique perspective to analyze the structural, functional, drug binding, and evolutionary aspects of T-type channels.

T-type calcium channels open in response to slight depolarizations in the low voltage range. Paradoxically, they are also recruited after membrane hyperpolarization as occurs during rebound burst firing (1). A window current of T-type channels is a feature which permits Ca\textsuperscript{2+} entry at rest (2) and contributes to differentiation and growth promoting functions in both excitable and non-excitable cells (3). T-type channels are also a leading pharmaceutical drug target, and are implicated in a wide range of conditions such as epilepsy, pain, hypertension, cancer and mental disorders (4).

T-type Ca\textsuperscript{2+} currents were first measured in starfish eggs using two-electrode voltage clamp (5). Currents conducted by “Channel I” were evoked by small depolarizations (low voltage-activated, LVA) visible as a small hump in a current amplitude vs. test potential plot, appearing inconsequential beside the “Channel II” currents elicited by larger depolarizations (high voltage-activated, HVA). Ca\textsuperscript{2+} channel types would be discriminated further by Tsien and colleagues on the basis of properties where Ba\textsuperscript{2+} is the charge carrier (6). HVA L-type channels have a Large unitary Ba\textsuperscript{2+} conductance with Long-lasting openings; N-type (or Non-L-type) channels are typically associated with Neurons of iMtermediate unitary conductance and the LVA. T-type channels produce Transient currents that are of Tiny unitary conductance in Ba\textsuperscript{2+}, and close slowly upon membrane repolarization, producing a slowly deactivating Tail current (6).

T-type channels remain as the least understood amongst the Ca\textsuperscript{2+} channel families. While most of the ten mammalian Ca\textsuperscript{2+} channel genes were characterized in the late 1980s, it required an additional decade for a description of the three T-type genes, Ca,3.1 (alpha1G), Ca,3.2 (alpha1H) and Ca,3.3 (alpha1I) (7). Progress in understanding T-type channel functions continues to be hampered by the lack of a highly selective blockers that discriminate between Ca,3 channel types or separate Ca,3 channels from related L-type (Ca,1) and non-L-type (Ca,2) Ca\textsuperscript{2+} channels which usually produce more robust Ca\textsuperscript{2+} entry into the same cells (7).
Here we describe the in vitro expression characteristics of the first non-mammalian, T-type channel, LCa,3 cloned from the pond snail, Lymnaea stagnalis. This structurally distant channel has quintessential features of T-types such as transient kinetics. LCa,3 is big in many respects such as its protein size; it expresses large macroscopic currents in human cells; it is the most abundant Ca\(^{2+}\) channel transcript in the snail nervous system and it generates window currents that appear to be at least as large as those reported for mammalian channels. LCa,3 provides a unique perspective to analyze the structure, function, and drug binding of T-type channels and serves as a useful surrogate in residue swapping experiments. Searches for the fundamental mechanisms that regulate this singleton invertebrate T-type channel will be facilitated by the simple molluscan preparation, where accessible and identified neurons underlying well described behaviours can be studied in isolated Lymnaea neurons, cultured synapses, or within intact, identified networks in situ. Also, LCa,3 provides nourishment for evolutionary speculation. While the first gastropods (500 MYA) are likely quite distant from this ancestral branchpoint, the extant snail homolog, LCa,3 is reminiscent of the gene that predates the speciation that led to the emergence of the three distinct, mammalian T-type channel genes.

Experimental Procedures

Cloning and sequencing of LCa,3- The complete open reading frame for LCa,3 was determined from at least three independent, overlapping DNA fragments from PCR screening of Lymnaea stagnalis central nervous system (CNS) λZAP cDNA libraries to generate a consensus gene. The full-length 9031 bp cDNA transcript is available in DDBJ/EMBL/GenBank databases under accession No. AF484084 and replaced a previous partial coding sequence entry of 5991 bp. The final clone was assembled from four overlapping PCR with sticky ends (numbered by cDNA transcript positions) respectively, XhoI-SpeI (209-2865), SpeI-SalI (2812-4544), SalI-MluI (4503-6874), and MluI-BamHI (6850-8869). Silent mutations were created in a Kozak consensus sequence upstream of the start codon (209-211), an MluI site (6858-6863) and several hairpin structures thought to interfere with the site-directed mutagenesis reaction (5225-5285). The full length LCa,3 coding sequence was assembled between XhoI and BamHI sites in bicistronic vector pIRES2-EGFP (Clonetech). Low frequency of positive recombinants during cloning and the slow rate of growth of the full-length plasmid in bacteria (five full days before a colony appears on a bacterial plate after transformation) suggest that the plasmid insert is toxic to bacteria.

Transfections- HEK-293T cells (M. Calos, Stanford University) were cultured in DMEM (Sigma) with 10% FBS (Sigma) and supplemented with 0.5% (v/v) penicillin-streptomycin solution (Sigma). For electrophysiology, 6μg of the LCa,3 pIRES2-EGFP construct was transfected into cells at 40-50% confluency using the standard Ca\(^{2+}\) phosphate transfection method. After overnight transfection, the cells were washed twice with culture media and incubated at 28°C in a humidified, 5% CO\(_2\) chamber for three days. Following incubation, cells were detached using a trypsin-EDTA solution (Sigma), plated at 10% confluency onto glass coverslips and incubated at 37°C for 4 hours since adhesion to the glass substrate requires warmer temperatures (8).

Whole cell patch clamp recordings- Whole-cell recordings were carried out at 23°C using either a 5mM external Ca\(^{2+}\) solution (5mM CaCl\(_2\), 166 mM tetraethylammonium (TEA)-Cl, 10 mM HEPES pH 7.4) or 5mM external Ba\(^{2+}\) solution (5mM BaCl\(_2\), 166 mM TEA-Cl, 10 mM HEPES pH 7.4) and an internal solution consisting of 125 mM CsCl, 10 mM EGTA, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 4 mM MgATP, 0.3 mM Tris-GTP, and 10 mM HEPES pH 7.2. Recordings were obtained using an Axopatch 200B amplifier, sampled to a PC through a Digidata 1440A A/D converter. Data were filtered at 2 kHz and digitized at 5
kHz and acquired using pCLAMP 10.1 software (Molecular Devices). The pipette resistance was maintained between 3 and 5 MΩ, and the typical access resistance was between 4-5 MΩ. Only recordings with minimal leak (<10%) and small current sizes (< 2 nA) were used for analysis, and offline leak subtraction was carried out using the Clampfit 10.1 software (Molecular Devices). Series resistance was compensated to 70% (prediction and correction; 10 μs lag). A gravity flow system was used to perfuse 5mM Ca²⁺- or Ba²⁺-containing extracellular solution, or 5 mM Ca²⁺ external solution containing solubilized Ni²⁺ (Sigma) or mibefradil (Novagen).

Data analysis- Ca²⁺ current activation curves were constructed by converting the peak current values from each current-voltage relationship data set to conductance using the equation 

\[ g_{Ca} = \frac{I_{peak}}{(V_{command} - E_{Ca})} \]

where \( I_{peak} \) is the peak current, \( V_{command} \) is the command pulse potential, and \( E_{Ca} \) the Ca²⁺ reversal potential as determined by linear extrapolation of the current values in the ascending portion of the current-voltage relationships. Conductance values were then normalized and individually fitted with the Boltzmann equation:

\[ \frac{g}{g_{max}} = \left(1 + \exp\left(-\frac{V_{command} - V_{1/2}}{k}\right)\right) - 1 \]

where \( g \) is the peak conductance, \( g_{max} \) is the maximal peak Ca²⁺ conductance, \( V_{command} \) is the conditioning potential, \( V_{1/2} \) is the half maximal activation, and \( k \) is the activation slope factor. The steady-state inactivation curves were constructed by plotting normalized current (peak test pulse current/peak prepulse current) as a function of the inactivating potential. The data were fitted with a Boltzmann equation:

\[ \frac{I}{I_{max}} = \left(1 + \exp\left(\frac{V_{inact} - V_{1/2}}{k}\right)\right) - 1 \]

where \( I \) is the peak test pulse current, \( I_{max} \) is the peak test pulse current when the conditioning pulse was -110 mV, \( V_{inact} \) and \( V_{1/2} \) are the conditioning potential and the half maximal inactivation, respectively, and \( k \) is the inactivation slope factor. Kinetics of activation, inactivation and deactivation were determined by fitting mono-exponential functions over the growing or decaying phases of each current trace using the software Clampfit 10.1.

Antibody production- LCa₃ I-II linker coding sequence (1976 bp - 2575 bp) was PCR amplified and cloned into the bacterial protein expression vector pET-22b(+) (Novagen) via Ndel and XhoI restriction sites. Expression of each peptide was induced by 1 mM IPTG in Rosetta™ (DE3) cells (Novagen) transformed with the pET22b(+) plasmid-containing construct. Supernatant of lysed bacterial cells containing 6xHis LCa₃ I-II linker expression was run and washed through columns containing Ni²⁺-charged His•Bind® resin (Novagen), then eluted off of the beads, dialyzed and quantified using the Bradford assay. Rabbits were each injected three times with recombinant proteins emulsified with Freund’s complete adjuvant for the first injection and Freund’s incomplete adjuvant (Sigma) for the subsequent injections. IgG rabbit antiserum was tested for immune-reactivity with the antigen by western blotting.

Immunolabeling of recombinant LCa₃ in HEK-293T cells- HEK-293T cells were transfected with either 8 μg of LCa₃ in pIRES2-EGFP alone or with 3.2 μg of LCa₁α₁ subunit in pIRES2-EGFP (9) plus 2.4 μg of rat β₁ subunit in pMT2 and 2.4 μg of rat α₂δ in pMT2. Cells were washed following transfection and incubated at 28°C for one week prior to trypsinization and plating onto glass coverslips. Cells were then fixed with 1% paraformaldehyde in PBS overnight at 4°C, washed twice with PBS, then permeabilized using phosphate-buffered saline containing 0.2% TWEEN-20 (PBS-T) for 10 minutes at RT. 1:500 1° antibody or pre-immune serum was applied to pre-blocked cells overnight in PBS-T containing 3% BSA. 3x-washed cells were incubated with 1:1000 diluted AlexaFluor 594 goat anti-rabbit 2° antibody in PBS-T containing 3% BSA, for 1 hr at 23°C, washed 4x and imaged at 40x magnification with a Zeiss AxioObserver Z1 inverted epifluorescent microscope to detect AlexaFluor 594 antibody and eGFP. Images were captured using Zeiss AxioVision.
software and brightness/contrast was adjusted using Adobe Photoshop.

Southern blot- 15 μg aliquots of genomic DNA isolated from Lymnaea tissue (modified from Richards, Reichardt, & Rogers, 2001), were digested with EcoRV, HindIII, EcoRI, and XhoI and DNA fragments separated through a 1% agarose gel. Digested DNA was transferred onto a positively charged nylon membrane (Roche) and a standard hybridization procedure was carried out using manufacturer’s instructions (EasyHyb, Roche). Membranes were probed with gel-purified 597 bp PCR product of the LCa_3 gene (1979 bp - 2575 bp) incorporated with DIG-11-dUTP (Roche). The probe was localized on the membrane using anti-DIG alkaline phosphatase-conjugated antibody (Roche; 1:5000 dilution) and colour substrate solution NBT/BCIP (Roche).

Semi-quantitative RT PCR- RNA was extracted from the CNS of Lymnaea stagnalis using Tri Reagent (Sigma-Aldrich) and optimized from standard methods (10). RNA was treated with DNAse (Fermentas) to remove contaminant DNA for RT-PCR analysis. RNA was quantified by spectrophotometry, and visualized by gel electrophoresis to confirm lack of degradation. First strand cDNA were synthesized from RNA at 54°C for 80 minutes and 70°C for 15 minutes using either oligo-dT or random hexamer primers and Superscript III reverse transcriptase (RTase; Invitrogen) and controls were prepared that lacked RTase. Primers used for cDNA amplification were designed to have similar melting temperatures, minimal secondary structure and amplified fragments of similar sizes (500-600 bp). PCR primers spanned sequences from the following genes (accession #: cDNA transcript positions): Lactin (DQ206431: 38-628), LNALCN (AF484086, 3149-3761), LCa_1 (AF484079: 5421-6103), LCa_2 (AF484082: 5427-6095), LCa_3 (AF484084: 7816-8546). PCR products generated after 25 cycles in the thermocycler were imaged in ethidium bromide stained gels using a gel documentation system (Alpha Innotech) under UV light. Densinometric analysis of DNA band intensity was performed using Automatic Image Capture software (Alpha Innotech).

RESULTS

Identity of an invertebrate T-type channel (LCa_3). A novel invertebrate T-type channel transcript (9,031 bp) was assembled from cDNA derived from the central nervous system of the freshwater pond snail, Lymnaea stagnalis with a coding region that starts as an almost perfect match to an EST database entry from the marine snail Aplysia californica (Accession # EB302921). The LCa_3 open reading frame predicts a 2886 amino acid protein, with an estimated ~322 kDa MW. The start and end of the LCa_3 amino acid sequence also resemble those of the human Ca_3.1 channel (fig. 1a). Consistent with other related cation channels of this type, LCa_3 has four repeat domains (DI to DIV) with each domain containing six membrane-spanning segments similar to the voltage-gated K channels (fig. 3a). LCa_3 is the largest voltage-gated ion channel expressed to date, being 25% larger than the mammalian T-types, 50% larger than a T-type homologue from C. elegans (cca-1b), and significantly larger than other Ca_2+ channels (Ca_1, Ca_2), Na channels (Nav) and NALCN (fig. 1b). The extra size is mostly due to long cytoplasmic N- and C- termini, and the cytoplasmic I-II and II-III linkers (fig. 1c).

Snails, like most invertebrates appear to have branched before the genomic duplication events that led to the expansion of gene isoforms and as such bear only single representatives for each of the three Ca_2+ channel gene families (Ca_1, Ca_2 and Ca_3) compared to the ten different Ca_2+ channel genes in mammals (fig. 2a). The snail T-type gene diverges dramatically in amino acid sequence from the mammalian genes (mean identity/similarity = 37.6/46.1) while the mammalian homologues are clustered closer together (mean identity/similarity = 50.7/57.9) (fig. 2C). The single copy nature of the
LCα₃ gene in the genome is evidenced by Southern blotting producing a single banding hybridization pattern, except for a gel lane where the restriction enzyme (EcoRV) cuts the genomic DNA within the probe sequence, leading to two bands of weaker intensity (lane a, fig. 2b). The LCα₃ transcript is more abundant than other Ca²⁺ channels (LCα₁,2 and LCα₃) or LNALCN in central nervous system tissue as measured by semi-quantitative RT-PCR (fig. 2d). The higher expression of LCα₃ compared to these others was also found by single cell, quantitative Real-Time PCR of individual VD4 neurons in a previous study using different primer sets (see Spafford et al. 2003 (11), fig. 2).

Fig 3a, illustrates a running window of similarity between aligned LCα₃ and human Ca₃.3 channel protein sequences. The strongest homology is observed in the six transmembrane segments and pore (P)-loops of each domain (fig. 3a). Side chains of conserved negative residues lining the four P-loops contribute to a “DDEE” selectivity filter in T-type channels (7), including LCα₃ (fig. 3b). The pores of LCα₃ and all voltage-gated Ca²⁺ channels also include a highly conserved aspartate (D) adjacent to the selectivity-filter glutamate (E) in Domain II (fig. 3b), which may serve to attract incoming Ca²⁺ ions to the ion selective pore (B. Zhorov, personal communication). One noticeably conserved region outside of the membrane spanning domains is the gating brake present in the proximal I-II cytoplasmic linker (fig. 3c). Comparison of LCα₃ with Cav3.3 residues, suggest conserved elements in the gating brake, including a putative helix-loop-helix hydrophobic core, a putative salt bridge and potential protein-protein interaction sites facing away from the hydrophobic core (fig. 3c). (E. Perez-Reyes, personal communication).

Expression characteristics of LCα₃ in HEK-293T cells. Transient transfection of LCα₃ cDNA contained in pIRES2-EGFP vector reveals membrane-delimited staining of HEK-293T cells (box, fig. 4a) with a rabbit polyclonal antibody generated against the I-II cytoplasmic linker of LCα₃ (see fig. 3a for relative location of the epitope). Antibody staining was not apparent in LCα₁-transfected cells (9) or when pre-immune serum was used to detect LCα₃ (fig. 4a). Channel expression levels generally correspond to the eGFP intensity in HEK-293T cells, as would be expected with the LCα₃ cDNA expressed on the same mRNA as eGFP using the bicistronic expression vector, pIRES2-EGFP. The optimal level of HEK-293T expression for electrophysiological recording (200 pA to 1.5 nA) corresponds to three days after transfection, while larger currents of up to 10 nA were possible by allowing protein expression to continue for up to six days (fig. 4b). Typical transient kinetics of T-type currents are revealed in whole cell recording of LCα₃. Small voltage steps above threshold (-70 to -65 mV) are slow to activate and inactivate (requiring tens of milliseconds). Larger voltage steps elicit currents with progressively faster activation and inactivation kinetics that cross over each other with each successive step, towards a maximal current for a voltage step of -35 mV (fig. 4c). τ of inactivation kinetics follow the change in the time to peak and τ of activation kinetics with increasing voltage steps (fig. 4e). (See Table I for detailed comparison of biophysical parameters between Ca₃ channels).

The pairing of activation and inactivation with LCα₃ is typical of T-type currents, and has led some to suggest that T-type channel inactivation is voltage-independent (12). The voltage sensitivity of LCα₃ approximates a typical low threshold T-type current which peaks at -35 mV (fig. 4d), although technically LCα₃ is slightly lower threshold than mammalian T-types (peak between -30 mV and -25 mV). Channel availability at steady-state was assessed after a 1 s pre-pulse protocol (fig. 5a) and 5 s pre-pulse protocol (data not shown), revealing a surprisingly steep and positively-shifted availability curve compared to mammalian T-type channels (fig. 5b). The combination of the very low threshold of channel activation and the large
fraction of possible available channels creates a potentially large and persistent window current near the resting membrane potential of typical neurons (2) (fig. 5c). An estimated window current was gathered by the product of available channels under steady-state conditions and the relative peak conductance. As many as 1.8% of total T-type channels may contribute to this current at -65 mV (fig. 5c), a value at least as high as that calculated for recombinant mammalian channels gathered under similar conditions. A measure of the persistent, steady-state current amplitude was assessed after 1 second of sustained potentials held from a range of -70 mV to -50 mV in 5 mV increments. The largest, persistent current corresponded to the estimated maximal window current size at resting membrane potentials of -65 mV (fig. 5c, inset).

One of the characteristic features of T-type channels is a slow rate of deactivation (7). Deactivation is measured as the rate of current decay from a tail current generated by the rapid return to lower, more hyperpolarized potentials with maximally opened channels (held at -35 mV) (fig. 6). Deactivation rates of LCa,3 are fastest at hyperpolarized potentials (-110 mV) and quickly slow with depolarization steps to resting potentials (-60 mV) (fig. 6). LCa,3 fits within the faster end of the range of deactivation kinetics for mammalian channels, but is still manyfold slower than Ca,1 and Ca,2 channels. The slowness of deactivation kinetics suggests that native LCa,3 currents may pass a deactivating tail current upon membrane repolarization.

Macroscopic, native Ca\textsuperscript{2+} currents are typically equal or larger than Ba\textsuperscript{2+} currents at equimolar concentrations although the unitary conductance is reported to be equal in high Ca\textsuperscript{2+} or Ba\textsuperscript{2+} (13). Macroscopic Ca\textsuperscript{2+} currents range from smaller, equal or larger than Ba\textsuperscript{2+} currents for recombinant Ca,3.2, Ca,3.3 or Ca,3.1 channels, respectively (14). Reasons for the relative differences in Ca\textsuperscript{2+} and Ba\textsuperscript{2+} permeability of different channel types are not clearly understood. LCa,3 resembles Ca,3.2 and other HVA snail Ca\textsuperscript{2+} channels, conducting larger amplitude whole cell Ba\textsuperscript{2+} currents than Ca\textsuperscript{2+} currents (fig. 7a) (7). Ba\textsuperscript{2+} as a charge carrier results in a slight hyperpolarizing shift in the current-voltage relationships compared to Ca\textsuperscript{2+} (fig. 7b), but there is still a ~50% increase in whole cell Ba\textsuperscript{2+} conductance compared to Ca\textsuperscript{2+} in the absence of driving force changes (fig. 7c). Kinetics are also faster when Ba\textsuperscript{2+} is the charge carrier, with faster time to peak (fig. 7d) associated with more rapid inactivation kinetics (fig. 7e). Ca\textsuperscript{2+}-dependent inactivation typically associated with Ca,1 channels is not a property of LCa,3 or other T-type channels (14).

Ni\textsuperscript{2+} traditionally has been considered to be a blocker that distinguishes T-types from other channels but only one of the three cloned mammalian T-channels, Ca,3.2, is strongly inhibited by Ni\textsuperscript{2+} (15). LCa,3 is approximately equally sensitive to Ni\textsuperscript{2+} as Ca,3.1 and Ca,3.3, with an IC\textsubscript{50} of 300 ±29.24 μM (fig. 8a), but all of these T-type channels are ~20 to 60 fold less sensitive than Ca,3.2 (15). Lee and colleagues (16) identified that the unusual Ni\textsuperscript{2+} sensitivity of Ca,3.2 critically involves His-191, imbedded in a helix-turn helix motif known as the S3b-S4 voltage sensor paddle in Domain I (17) (fig. 8c). Interestingly, LCa,3 does not have a corresponding His-191 residue of Ca,3.2, but neither does the sequence of the S3b–S4 voltage sensor paddle of LCa,3 compare well with any of the Ca,3 channels (fig. 8c). LCa,3 has an eight amino acid insert in this short linker region and extra positive and negative charges compared to the mammalian Ca,3 channels (fig. 8c). The Ni\textsuperscript{2+} dose response curve does not perfectly fit the data (fig. 8b), but a biphasic dose-response curve does, having a high affinity IC\textsubscript{50} of 27.25 ±2.74 μM (38%) and a lower affinity IC\textsubscript{50} of 1064.54 ±79.11 μM (62%) (fig. 8b, insert).

Mibefradil was marketed by Roche as a drug for treatment of hypertension and angina (18), before it was withdrawn in 1998 for its potential side effects. It is a non-selective antagonist, but typically has a ~ ten-fold
greater selectivity for T-type channels over L-type Ca\(^{2+}\) channels. LCa\(_3\) is in the range of sensitivity to mibefradil (680 ±0.03 nM) as mammalian T-type channels (fig. 8d). Caution must be heeded when directly comparing results from different studies since mibefradil is highly sensitive to the charge carrier, charge carrier concentration, and holding potential (19).

**DISCUSSION**

**Introduction to LCa\(_3\).** Here we describe the first in vitro expression characteristics of a snail homolog of mammalian T-type channels and, also remarkable, is that LCa\(_3\) is only one of two full length cDNA sequences determined for non-mammalian T-types to date. cDNAs are assembled from predicted exons from a number of invertebrate sequenced genomes (eg. *Drosophila melanogaster* Ca-a1T-RB, accession # NM_132068), but low homology outside of the conserved transmembrane domains indicates that the predicted transcript assemblies are likely erroneous when analysed with multiple sequence alignments of cDNAs derived from mRNA (LCa\(_3\) and *C. elegans* cca-1B, human Ca\(_3.1\) to Ca\(_3.3\)). LCa\(_3\) codes for a 322 kDa protein of 2886 amino acids, which is the largest protein of any reported four repeat ion channel expressed to date, including 1.25 times larger than mammalian T-type channels and 1.5 times larger than cca-1B from *C. elegans*, the only other reported invertebrate cDNA coding for a T-type channel. Whether T-type channels in other phylogenetic groups are this large or possibly even larger is not known.

**Permeation and the DDEE selectivity filter.** The transmembrane regions are not responsible for most of the extra mass of LCa\(_3\), and include the highly-conserved, voltage-sensor domain (S1 to S4) the outer helix (S5), the P-loop and inner helix (S6) in all four repeat domains (7). A unique “DDEE” selectivity filter (20) and a gating brake (21) are two trademarks of T-types that distinguish them from the Ca\(_1\) and Ca\(_2\) Ca\(^{2+}\) channel families. Flexible side chains of each domain harboring key glutamate residues (EEEE) contribute to the selectivity filter by extending into the permeation pathway, where they are expected to bridge Ca\(^{2+}\) ions as they pass through the pore of high voltage-activated Ca\(_1\) and Ca\(_2\) channels (B. Zhorov, personal communication). A highly conserved aspartate (D) residue upstream in the selectivity filter and adjacent to the glutamate residue in Domain II may serve to attract incoming Ca\(^{2+}\) ions to the ion selective pore of all Ca\(^{2+}\) channels, according to modeling studies by B. Zhorov (personal communication). T-type channels are reported to have a lower Ca\(^{2+}\) selectivity over monovalent cations since the estimated reversal potential is less positive than high voltage activated Ca\(_1\) and Ca\(_2\) channels (+40 mV vs. +60 mV) (22). Shortened carbon side chains in Domains I and II of T-types (DDEE instead of EEEE) may bridge Ca\(^{2+}\) ions less stringently, resulting in lower pore selectivity for Ca\(^{2+}\) ions in favor of faster kinetics that is typical for T-type Ca\(^{2+}\) channels. Interestingly, inactivation kinetic changes mirror changes in activation kinetics in T-types (12) and a modified EEDD locus alters gating properties (20).

No obvious conclusion can be drawn from differences in permeability for Ba\(^{2+}\) and Ca\(^{2+}\) ions among T-type channels. Only LCa\(_3\) and mammalian Ca\(_3.2\) channels have larger macroscopic Ba\(^{2+}\) than Ca\(^{2+}\) currents. Interestingly, greater macroscopic currents in Ba\(^{2+}\) over Ca\(^{2+}\) are a consistent feature with snail Ca\(^{2+}\) channels expressed in HEK-293T cells including LCa\(_1\) (9) and LCa\(_2\) (23).

**Gating brake.** A gating brake shared amongst T-type channels is considered to prevent channel opening at hyperpolarized potentials, since nucleotide polymorphisms in patients with childhood absence epilepsy or strategically placed deletions in this region, produce channels that open at even more negative potentials than typical T-type channels (24). The proximal I-II loop of LCa\(_3\) is predicted to contain the helix-loop-helix gating brake structure (21), and more...
distally the I-II loop has been ascribed to regulating of the surface expression of T-types (25). It may be more than coincidence that the gating brake is in the equivalent position where β subunits associate with and alter the biophysical properties of HVA Ca,1 and Ca,2 channels, as well as regulate/modulate their expression (e.g. protein folding, turnover, and membrane trafficking) (26). Indeed, invertebrate LCa,3 does not require accessory beta or alpha2delta subunits and robustly expresses in human HEK-293T cells, at an efficiency that rivals the mammalian T-type channels. Continued transfection in the presence of G418 antibiotic selection has generated a number of stable HEK-293T cell lines for LCa,3. A high constitutive expression of LCa,3 under the strong mammalian CMV promoter argues in favour of greater transcriptional controls for T-type channel expression in native cells, compared to perhaps more post-translational checkpoints regulating the expression of Ca,1 and Ca,2 channels which are known to form complex, multimeric assemblies along the secretory pathway.

**Overall shared features of T-type channels.**

Scoring of the overall amino acid conservation between invertebrate and mammalian genes can lead to an overestimates of the degree of structural divergence, since sequence not under selection will drift substantially, over the hundreds of millions of years separating their evolution. Comparing the in vitro expression characteristics between LCa,3 and mammalian T-types suggest a structural equivalency in core regions, despite the overall sequence divergence of different channels, revealing a set of quintessential properties shared by all T-types. Voltage properties are tightly regulated with fast and transient kinetics, slow deactivation, window currents produced by overlapping activation and availability curves and channel activity limited to a narrow window of sub-threshold voltages where channels are available and conducting. Also, similar drug sensitivities of LCa,3 for Ni2+ ions and mibefradil suggest conserved residues in the outer pore and the aqueous permeation pathway between the selectivity filter and the aqueous, pore-lined, inner S6 helices (inverted tepee-shape) as predicted from the three dimensional structure of crystallized K channels (27). Probing the affinity of a number of different T-type channel drugs will assist in interpreting the structural variants in the snail channel pore versus the mammalian ones.

**Primitive features in invertebrate channels.**

Invertebrate Ca2+ channels of the high-voltage variety are also highly conserved in their biophysical properties. Rat Ca,1.2 and snail LCa,1 channels, for example are so alike that there are no reliable biophysical features, outside of drug sensitivity that separate the two channels transfected in HEK-293T cells (28). Differences outside of biophysical features appear to reflect the primitiveness of the invertebrate homologue, reminiscent of a state preceding the evolution of specializations in electromechanical coupling, such as the tetradic organization in skeletal muscle where mammalian Ca,1.1 channels are directly coupled to ryanodine receptors of the sarcoplasmic reticulum (29). Invertebrate muscles lack tetrads, or an equivalent Ca,1.1 channel that mediates muscle contraction (29). More indirect coupling, with Ca2+ serving as a short range transmitter, is also a feature of invertebrate neurotransmission. Invertebrate Ca,2 channels that are responsible for transmitter release lack a II-III loop structure containing the synaptic protein binding site of Ca,2.1 and Ca,2.2 channels (11), and also exhibit a synaptic organization lacking key structural proteins present in mammalian synapses (such as Bassoon and CAST) and a synaptic substructure, such as a Drosophila T-bar that is unlike the mammalian presynaptic density (30).

**T-type channel diversity.** T-type channels are modulated through intracellular signalling cascades and coupled to other ion channels (31), but there is little to indicate that T-types serve as instruments for electromechanical coupling in cell-type specific, multisubunit
complexes in the manner of Ca,1 and Ca,2 channels (32). Structural diversity in the three mammalian T-type channels arose out of genomic duplication, perhaps creating some overlapping redundancy in function. Yet, the presence of unique biophysical properties, tissue specificity, modulation and putative protein-protein interactions sites suggests otherwise, indicating that the different genes may provide specialized functions in mammals. Examples that illustrate this functional divergence include the contribution to rebound burst firing in thalamocortical neurons by Ca,v,1, the involvement of Ca,v,2 in pain sensitivity, relaxation of coronary arteries and secretion of aldosterone, and the involvement of Ca,v,3 in long lasting bursts in the inferior olive and habenula served by its slower kinetics and a larger window current range compared to other T-type channels (7). Distinct, regional antibody staining within individual central neurons suggests that each gene may serve particular roles within somatic, dendritic and perinuclear compartments (33,34). Whether the diversity of mechanisms in mammals is contained within a single invertebrate Ca,v,3 gene and its alternative splicing has not been explored.

Wide range of functions expected for abundant T-type channel transcript. Here we show that LCa,v,3 is the most abundant Ca,2+ channel transcript in the Lymnaea nervous system, and our previous analysis indicates that this reflects a transcript profile in an individual snail neuron (11). Quantitative RT-PCR of single, identified, respiratory, VD4 neurons, measured in replicates of six neurons, indicate that LCa,v,3 is manyfold more abundant than either LCa,v,1 or LCa,v,2 channel expression (11). Their abundance in invertebrates may reflect a wide range of functions associated with T-Type channels such as a) shaping nerve action potentials and pacemaking, b) non-electrogenic role for T-types in providing Ca,2+ through window currents (2); c) roles in differentiating and proliferating cells (3) and secretion (35). Some invertebrates also appear to have additional roles that are not served by mammalian T-type channels, such as excitation contraction coupling in jellyfish muscle cells (36) or facilitating the contraction of pharyngeal muscles in nematodes (37). Interestingly, T-Type spikes can provide qualitatively different information than Na spikes in the same invertebrate axons. Weak depolarizations initiate slow swimming via T-type spikes while stronger pacemaking inputs initiate a fast escape swimming response mediated by overshooting Na spikes in the same axons, presumably operating in the availability range outside of T-type channels (38).

Drug binding — Nickel. LCa,v,3 has equal (~300 μM IC50) Ni2+ sensitivity as Ca,v,1 and Ca,v,3 channels. We report that the Ni2+ dose-response curve for LCa,v,3 is biphasic, indicative of two components of drug block. A similar biphasic Ni2+ block is apparent in dose response data for mammalian recombinant channels fig.3D,(16), fig 2B, (15) fig 7b, (39); and in native currents fig. 3A, (40) fig 6b, (41) fig 7b (39). A biphasic response might result from two Ni2+ binding sites. Jones and colleagues suggest that Cav3.1 indeed has two binding sites, one in the outer pore and another deeper site within the pore pathway that is strongly affected by the permeant ion (42).

Unusually sensitive Ni2+ block (5-10 μM IC50) is a property of Ca,v,2 channels, and critically involves a His-191 residue (16) in what has been described as the S3b–S4 voltage sensor paddle for Na channels based on the X-ray structure of K channels (43). More than His-191 may be critical in the S3b–S4 voltage sensor paddle since a similar high affinity cation block of Cav3.2 channels by extracellular Zn2+ involves the His-191 residue and two residues directly upstream of His-191, in particular, Asp-189 and Gly-190 (44). The S3b–S4 is considered to carry most of the gating charge, and likely drives the conformational changes required for pore opening and closing. It seems probable that Ni2+ associates with the S3b–S4 paddle motif of Ca,v,2 in a manner similar to how tarantula
and scorpion toxins immobilize the voltage-sensor of Na channels (17). Ca,3.2 is inhibited by Ni\(^{2+}\) independently of voltage, and is similarly blocked with Ca\(^{2+}\) or Ba\(^{2+}\) as a charge carrier, which is consistent with an inhibition by a mechanism outside the permeation pathway (42). Interestingly, LCa,3 has an eight amino acid insert in this short S3b–S4 region with extra positive and negative charges compared to Ca,3 channels. The effect of the insert on Ni\(^{2+}\) block or voltage-gating, if any, is not known. Other regions may also contribute to Ni\(^{2+}\) block. High affinity Zn\(^{2+}\) block in Cav3.2 channels also involves a neutral Ala-140 in IS2 that is negatively-charged (Asp-140) in corresponding position of less sensitive Cav3.1 and Cav3.3 channels (44). Future chimera work may be important to evaluate whether LCa,3 with a positively-charged His-140 at this position influences cation block.

**Drug binding – mibefradil.** A number of new and potent T-type channel blockers are being explored, and mibefradil serves as the first T-type channel blocker that was clinically available (18). Interestingly, mibefradil block of snail LCa,3 channel is in the range of potency of mammalian T-types. With doses spanning the mid-range of the IC\(_{50}\) (680 ±0.03 nM), we observed that the mibefradil block of LCa,3 would not readily stabilize, with accumulation of a slow, but progressive block during long periods (tens of minutes) of continuous perfusion. We assume that this reflects a use-dependence often ascribed to mibefradil block (45). A slow time course of mibefradil block may also be explained by a reported accumulation of a hydrolyzed metabolite, and more membrane-impermeant form of mibefradil (dm-mibefradil) that has an affinity for calcium channels from the cytoplasm (46). Further probing of different structures with mammalian and snail homologs will provide an opportunity for describing the high affinity drug binding in T-type channels.

**Summary and future prospects.** Expression of an invertebrate T-type channel has combined features that are reminiscent of all mammalian Ca,3.1, Ca,3.2, and Ca,3.3 channels (see Table I). LCa,3 is 25% larger than any voltage-gated ion channel expressed to date, and is the most abundantly expressed Ca\(^{2+}\) channel transcript in the snail nervous system. Window currents in invertebrate and mammalian T-type channels suggests a likely non-electrogenic role for T-types in providing Ca\(^{2+}\) for proliferating and differentiating cells, and in the developing embryo. Alternative-splicing of the single invertebrate gene may provide the structural diversity for shaping the window current and firing patterns catered for individual network requirements. We anticipate that the snail will provide unique perspectives for probing T-type channel physiology. Much can be learned from the simple molluscan preparation where only a single T-type channel gene is expressed in native cells and where there is a relative ease in probing of physiological mechanisms in single identified cultured neurons and intact networks in the brain that underly well-described behaviours (47). An invertebrate channel also provides an opportunity to reflect on evolutionary mechanisms. “Channel I” as it was first described has turned out to be the most challenging Ca\(^{2+}\) channel to analyse since it was identified by Hagiwara and colleagues (1975) more than thirty-five years ago. There is some truth in the following summary statement by Gray and MacDonald (48), reflecting on the present status of the T-type channel field: “[The] Physiologic regulation of T-type channels is simultaneously well-documented and very obscure.”
REFERENCES


FOOTNOTES

We would like to thank E. Perez-Reyes and B. Zhorov for helpful discussions, H. Vigil-Guitierrez for his assistance in generating LCa,3 antibodies, S. Lam for his support in setting up the electrophysiological experiments, and A. N. Boone for editing of the manuscript. This research was supported by NSERC of Canada, and a NSERC CGS-D graduate scholarship to A. Senatore.
FIGURE LEGENDS

Fig. 1. Full length snail LCa,3 is the largest identified voltage-gated ion channel expressed to date. It is coded by a 9,031 bp cDNA transcript that forms a 2886 aa protein with a MW of 322 kDa. (A) N-terminus closely matches with a putative start site derived from marine snail Aplysia californica EST (EB302921) and slightly resembles N- and C-termini of human Ca,3.1-3.3. (B) LCa,3 is 1.25x larger than human Ca,3 channels and 1.5x larger than nematode T-type, cca-1B, and all other four repeat ion channels. (C) LCa,3 is larger than human Ca,3 channels in the N- and C-terminus, and also the I-II and II-III cytoplasmic linkers.

Fig. 2. Singleton, snail T-type Ca2+ channel gene is distantly related to vertebrate homologs, and is the most abundant Ca2+ channel transcript in the snail brain. (A) Most parsimonious gene tree generated using multiple aligned sequences, analysed in PAUP 4.0 (D.L. Swofford) and illustrated with TreeView (R.D.M. Page). Sequences include official human sequences (http://www.iuphar-db.org); LCa,3 (AF484084) and yeast gene Cch1 from S. pombe (CAB11726) and S. cerevisiae (CAA97244). Numbers at branchpoints represent bootstrap values based on 100 replicates in heuristic search. Phylogram branches are scaled by their length and rooted with Cch1 Ca2+ channel homologs from fungi species; (C) Percent amino acid similarity scores were generated from EMBOSS NEEDLE (EMBL); (B) Southern blot indicates a single copy gene in the Lymnaea genome. T-type probe hybridized to create banding pattern (white arrows) on blot created from membrane transfer of genomic DNA digested with either a) EcoRV, b) HindIII, c) EcoRI, or d) XhoI. Probe contained an EcoRV restriction site, so the probe hybridized to two genomic DNA fragments digested with EcoRV. (D) Densinometric intensity of RT-PCR bands (illustrated in inset) generated from Lymnaea brain tissue.

Fig. 3. Running window of similarity (A) and alignments (B, C) between amino acid sequence of distant T-type channel homologs (snail LCa,3 and human Ca,3.3) reveal that the invariant structures for T-type channels are harboured in six membrane spanning segments in all four Domains (I, II, III, and IV), including an ion conducting pore (S5-P-loop-S6) and voltage sensor (S1-S4). Illustrated is the position in the I-II linker where LCa,3 polyclonal antibody (Ab) was generated in rabbits against a 200 amino acid peptide. (B) Amino acid sequence alignment of the re-entrant P-loop located between S5 and S6 of each of the four domains illustrating the signature sequence (EEDD locus) that influences Ca2+ ion permeation and selectivity. Conserved Aspartate (D) residue (1097 in LCa,3) in a position downstream of selectivity filter glutamate (E) residue is positioned to attract incoming Ca2+ ions to the pore (B. Zhorov, personal communication). LCa,3 contains a neutral isoleucine (I) in the outer pore at position 468 where mammalian T-type channels have a negatively charged residue (E or D) that influence pore blocking drugs. (C) Alignment of the cytoplasmic gating brake in proximal I-II linker. The gating brake is thought to prevent T-type channel gating at more hyperpolarized potentials.

Fig. 4. Transient transfection of HEK-293T cells harbouring the pRES2-EGFP plasmid containing invertebrate T-type channel cDNA reveal highly abundant channels and characteristic T-type channel properties. (A) Membrane delimited staining of LCa,3 (inset) is evident in eGFP-positive cells, but only with LCa,3-specific antibody and not with pre-immune serum or with LCa,1-transfected cells. (B) Box chart indicating the current density (pA/pF) of LCa,3 expression on 3 or 6 days after transfection. The box chart also illustrates mean, median +/- 1 SD, min/max current densities. (C) Sample LCa,3 currents are shown in response to 5ms voltage steps from a -110 mV holding potential. Illustrated is an ensemble of rapidly-activating and inactivating Ca2+ currents where each trace “crosses over” the previous one from rest to peak, and (D) the resulting normalized peak currents plotted as a function of voltage step, indicating low threshold of activation (~65 mV) and maximal currents generated at a step to ~35 mV. Current-voltage relationships were curve-fitted with an Ohmic-Boltzmann function. (E) The
increase in inactivation kinetics ($\tau_{\text{inact}}$) follows closely the increasing speed at which the current approaches peak ($t_{\text{peak}}$) also reflected in the faster rate of activation, curve-fitted and represented by $\tau_{\text{act}}$.

**Fig. 5.** Invertebrate LCa,3 has a large, persistent window current up to 1.8% of the total current near the resting membrane potential. (A) Sample current traces of maximal Ca,3 currents (step to -35 mV) in response to 1s inactivating prepulse. (B) Boltzmann-fitted inactivation curve generated by plotting fraction of maximal current as a function of prepulse voltage. Fraction of maximal conductance at each voltage was plotted as an activation curve, curve-fitted with a Boltzmann function. Activation curve was derived from the current-voltage relationship, minus the ohmic changes due to driving force (illustrated in fig 4D). (C) Calculation of the window currents were based on the product of the fraction of the whole cell conductance and fraction of available, non-inactivated channels at each voltage. Inset: A window current was measured at the end of a long, 1s voltage-step. At 1 s, majority of open channels will have inactivated, leaving only open channels that persist under steady-state conditions, with a maximum at the resting membrane potential (-65 mV).

**Fig. 6.** Invertebrate LCa,3 slowly deactivates similar to mammalian T-type channels. Sample tail currents and curve fitting of decay rate of tail currents ($\tau$, ms) were generated by hyperpolarizing steps between -110 and -60 mV for 450 ms from a 7 ms depolarizing step to -35 mV.

**Fig. 7.** LCa,3 currents are larger and faster when Ba$^{2+}$ is the charge carrier. (A) Sample traces and (B) current-voltage relationships of LCa,3 currents were generated from depolarizing voltage steps from a holding potential of -110 mV, while either micro-perfusing extracellular solution containing 5mM Ba$^{2+}$ or 5mM Ca$^{2+}$. (C) Whole cell Ba$^{2+}$ conductance was estimated to be ~ 50% greater than Ca$^{2+}$ conductance at all voltages. (D) Kinetics of activation (time to peak current, ms) and (E) inactivation decay (tau curve fit, ms) are faster when barium instead of calcium is the charge carrier.

**Fig. 8.** Invertebrate T-type channels have similar Ni$^{2+}$ and mibefradil sensitivity as mammalian T-types. A) Time course of Ni$^{2+}$ inhibition of normalized LCa,3 peak currents (inset, representative traces). B) Cumulative dose-response is illustrated, with an IC$_{50}$ (300 ± 29.2 μM) value that overlaps with IC$_{50}$ of Ca,3.1 (304.8 ± 6.2 μM; Kang et al., 2006). Inset: A better fit illustrated with a biphasic dose-response curve. C) T-type channel alignments in the region of the S3b-S4 paddle of Domain I illustrating the His-191 required for high Ni$^{2+}$ sensitivity of Ca,3.2 channels. LCa,3 has an eight amino acid insert with additional charged residues in the relative position of the His-191 residue in Ca,3.2. D) Cumulative dose response curve of mibefradil block of LCa,3 (inset, representative traces), indicating an IC$_{50}$ (300 ± 29.2 μM) value that is reminiscent of the IC$_{50}$ for mammalian Ca,3 channels.
Table I - Comparison of biophysical parameters for recombinant LCav3 and mammalian T-Type channels expressed in human cell lines

<table>
<thead>
<tr>
<th>Electrophysiology</th>
<th>LCav3</th>
<th>n</th>
<th>Cav3.1 Ref</th>
<th>Cav3.2 Ref</th>
<th>Cav3.3 Ref</th>
<th>Ref</th>
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<td>Activation</td>
<td>V0.5</td>
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<td>-44.6 ± 0.7*</td>
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<td>K</td>
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<td></td>
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<td>6.0 ± 0.1</td>
<td>6.3 ± 0.1</td>
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<td>Peak of IV, mV</td>
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<td>-25</td>
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<td>-74.1 ± 1.6**</td>
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<td>-78.1 ± 1.2**</td>
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<td>2.76 ± 0.13</td>
<td>5.0 ± 0.2**</td>
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<td>5.3 ± 0.2**</td>
<td></td>
<td>3,4</td>
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<td></td>
<td>5.5 ± 0.5**</td>
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<tr>
<td>Kinetics at -20mV</td>
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<td>1.6 ± 0.2*</td>
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<td>2.9 ± 0.1*</td>
<td></td>
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<td></td>
<td>Tau inact</td>
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<td>14 ± 1.0**</td>
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<td>80 ± 5**</td>
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<td>1.12 ± 0.1†</td>
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<td>250 ± 22</td>
<td>4.9 ± 2.0**</td>
<td>12 ± 2**</td>
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<tr>
<td></td>
<td>Mibefradil (IC50 uM)</td>
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<td>0.68 ± 0.03</td>
<td>1.2 ± 0.2**</td>
<td>9</td>
<td>9</td>
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<td></td>
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<td></td>
<td>1.1 ± 0.2**</td>
<td></td>
<td>1.5 ± 0.1**</td>
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1 - Baumgart et al., 2008 (25); 2 - Shcheglovitov et al., 2008 (49); 3 - Arias-Olguin et al., 2008; 4- Vitko et al., 2007 (24); 5-Gomora et al., 2002 (50); 6 - Chemin et al., 2002 (51); 7-Kang et al., 2006 (16); 8-Lee et al., 1999 (15); 9- Martin et al., 2000 (19)

* p < 0.05, ** p < 0.005 (One Way ANOVA)

§2 mM instead of 5 mM Ca2+ in extracellular solution
ψ10 mM Ba2+ in extracellular solution
Figure 1

A

N-terminus

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<th>Aplysia EST</th>
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C-terminus

<table>
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<tr>
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<th>hCav3.2</th>
<th>hCav3.3</th>
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<td>SGLSSDPADLDP</td>
<td>TPAPGGADDPV</td>
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B

C

By guest on November 9, 2016 http://www.jbc.org/ Downloaded from
Figure 2

A

S. cervisceae
S. pombe

Ca,1.1 (α₁S)
Ca,1.2 (α₁C)
Ca,1.3 (α₁D)
Ca,1.4 (α₁F)

LCa,1

Ca,2.1 (α₁A)
Ca,2.2 (α₁B)
Ca,2.3 (α₁E)

LCa,2

Ca,3.1 (α₁G)
Ca,3.2 (α₁H)
Ca,3.3 (α₁I)

LCa,3

B

bp

20 000
7 000
4 000
1 000

1000

100

a b c d

C

percent similarity

60
50
40

LCav3 vs Cav3.1
LCav3 vs Cav3.2
LCav3 vs Cav3.3
LCav3 vs Cav3.1 vs Cav3.2 vs Cav3.3

D

RT-PCR intensity from brain tissue

NALCN
LCav1
LCav2
LCav3
actin

+ -
Figure 3

A

Running window of similarity for LCav3 vs Cav3.3

Relative amino acid position

B

EEDD locus

LCav3 IP   hCav3.3
V S F D N I G A W V A I F Q V I S L E S W V I M Y H
I N F D N I G A W V A I F Q V I T E G W V E I M Y Y

hCav3.3
V Y S F D N L Q A L M A L F V L A S K D G W V Q I M Y T
I N F D N L Q A L M A L F V L A S K D G W V N I M Y N

LCav3 IIP   hCav3.3
Y N F D N L G Q A L M A L F V L A S K E G W V Q I M Y T
I N F D N L G Q A L M A L F V L A S K E G W V N I M Y N

C

end of IS6

helix 1

turns

helix 2

LCav3 GATING BRAKE

hCav3.3

18
Figure 5

A

B

C
Figure 6

![Graph showing deactivation kinetics and repolarization potential](image-url)

- Deactivation kinetics: 
  - Time constants (τ) are measured in milliseconds (ms).
  - The deactivation kinetics curve shows how the current decays over time.

- Repolarization potential (mV): 
  - Repolarization potentials range from -110 mV to -35 mV.
  - The graph compares the deactivation kinetics across different repolarization potentials.

- Currents: 
  - The graph includes a current scale of 300 pA with a 4 ms time scale.

- Data points: 
  - The data points are plotted on the graph, indicating specific values of deactivation kinetics for different repolarization potentials.
Figure 7

A

5 mM Ca

5 mM Ba

100 ms

1 nA

B

Voltage (mV)

-80 -60 -40 -20 0

1/ I / I max

-1.0

5 mM Ca

5 mM Ba

C

Voltage (mV)

-60 -40 -20 0

g Ba / g Ca

1.0

D

Voltage (mV)

-60 -40 -20 0

Time to peak (ms)

30 32 34 36 38 40 42 44

5 mM Ca

5 mM Ba

E

Voltage (mV)

-60 -40 -20 0

Tau (ms)

10 20 30 40 50 60 70 80

5 mM Ca

5 mM Ba
Figure 8

A

B

C

D

IC50 = 300 +/- 29.2 μM

IC50 = 677 +/- 30.4 nM

His-191

His-191

His-191

IC50 = 300 +/- 29.2 μM

IC50 = 677 +/- 30.4 nM
Transient and big are key features of an invertebrate T-type channel (L_{Ca,3}) from the central nervous system of *Lymnaea stagnalis*

Adriano Senatore and J. David Spafford

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