Expression and Modulation of an Invertebrate Presynaptic Calcium Channel α₁ Subunit Homolog*

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Here we report the first assessment of the expression and modulation of an invertebrate α₁ subunit homolog of mammalian presynaptic Caᵥ₂ Calcium channels (N-type and P/Q-type) in mammalian cells. Our data show that molluscan channel (LCaᵥ₂a) isolated from Lymnaea stagnalis is effectively membrane-targeted and electrophysiologically recordable in tsA-201 cells only when the first 44 amino acids of LCaᵥ₂a are substituted for the corresponding region of rat Caᵥ₂.1. When coexpressed with rat accessory subunits, the biophysical properties of LCaᵥ₂a-5'rba resemble those of mammalian N-type calcium channels with respect to activation and inactivation, lack of pronounced calcium dependent inactivation, preferential permeation of barium ions, and cadmium block. Consistent with reports of native Lymnaea calcium currents, the LCaᵥ₂a-5'rba channel is insensitive to micromolar concentrations of ω-conotoxin GIVA and is not affected by nifedipine, thus confirming that it is not of the L-type. Interestingly, the LCaᵥ₂a-5'rba channel is almost completely and irreversibly inhibited by guanosine 5'-3-O-(thio)triphosphate but not regulated by syntaxin1, suggesting that invertebrate presynaptic calcium channels are differently modulated from their vertebrate counterparts.

Calcium entry through voltage-gated calcium channels mediates a plethora of cytoplasmic responses, including the activation of enzymes, the initiation of gene transcription, neurite outgrowth and neurotransmitter release (1–3). Based on their functional and pharmacological profiles, voltage-gated calcium channels have been classified into T-, N-, L-, P/Q-, and R-types, each with unique physiological roles (1). With the exception of the T-types, voltage-gated calcium channels are heteromultimers comprised of a principal α₁ subunit that defines the calcium channel subtype plus the accessory β (4), α₁-δ (5), and possibly γ (6) subunits which modulate the functional and pharmacological properties of the α₁ subunit (1, 7). Molecular cloning has identified 4 genes, each encoding β and α₁-δ subunits, 8 genes encoding γ subunits, and 10 genes representing different types of calcium channel α₁ subunits (1). The various α₁ subunits fall into three distinct classes. The Caᵥ₁ class encodes L-type calcium channels, Caᵥ₂.1, Caᵥ₂.2, and Caᵥ₂.3, respectively, represent P/Q-type, N-type, and R-type channels, and Caᵥ₃ encodes the family of T-type calcium channels (1).

Caᵥ₂.1 (P/Q-type) and Caᵥ₂.2 (N-type) channels are densely localized at presynaptic zones of vertebrate neurons (8–10) where they are physically coupled to proteins of the synaptic vesicle release proteins such as syntaxin1, SNAP-25, and synaptotagmin (1, 2, 7, 11). This possibly serves to optimize the efficiency of synaptic transmission and as a negative feedback mechanism allowing the regulation of calcium channel activity during various steps of exocytosis (7, 12). Mammalian N-type and P/Q-type calcium channels are differentially inhibited upon activation of G protein-coupled receptors (7, 13–15). This effect is mediated by G protein βγ subunits and may serve to fine-tune synaptic activity.

Invertebrate species do not appear to contain the same structural diversity of vertebrate calcium channel genes in their genomes and, with one possible exception (16), possess only single homologs of the three major calcium channel families, Caᵥ₁, Caᵥ₂, and Caᵥ₃ (12, 17). Invertebrate Caᵥ₂ representatives are considered functional as well as structural correlates of both mammalian N- (Caᵥ₂.2) and P/Q-type (Caᵥ₂.1) calcium channels (12, 17). Caᵥ₂ homologs from Drosophila (DmCaᵥ₂A/ cac) (18, 19), Caenorhabditis elegans (unc-2) (20), and more recently, the freshwater mollusk Lymnaea stagnalis (LCaᵥ₂a) (21), are required for invertebrate synaptic transmission, reminiscent of the roles of N- and P/Q-type channels in mammalian synapses (3, 22). The invertebrate Caᵥ₂ calcium channel α₁ subunits, unlike mammalian N-type and P/Q-type calcium channels, do not display an elongated domain II-III linker region, which in mammalian synaptic channels characteristically contain interaction sites for syntaxin, SNAP-25, and synaptotagmin (19, 21). However, although full-length sequences of invertebrate presynaptic calcium channels have been in the public domain for years, there has not been a report describing the functional expression of any of the cloned invertebrate Caᵥ₂ synaptic calcium channels. An exception to this is a single report of expression of a squid Caᵥ₂ homolog in Xenopus oocytes (23), a system known to endogenously express calcium channels as well as low levels of synaptic proteins. This has precluded a detailed functional analysis of these channels and has been an obstacle to taking full advantage of invertebrate synaptic preparations to address fundamental aspects of synaptic transmission at the molecular level.

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Here we report the expression and characterization of a L. stagnalis Cav2 calcium channel α1 subunit homolog (LCa2a).

We show that LCa2a channels are ineffectively targeted to the plasma membrane but that this can be overcome by the replacement of a short stretch of amino acids at the N-terminal region of LCa2a (44 amino acids) with its counterpart from rat Ca2.1. When coexpressed with rat β3 and α2δ, the functional properties of modified LCa2a-5 rBa channel are indistinguishable in many ways those of mammalian N-type calcium channels, showing similar activation and inactivation behavior, preferential permeation of barium over calcium, a lack of pronounced calcium-dependent inactivation, and complete block by cadmium ions. Consistent with what has been reported for native Lymnaea calcium currents in neurons (24, 25), the LCa2a-5 rBa channel is insensitive to micromolar concentrations of ω-conotoxin GVIA and does not have characteristics of L-type channels with regard to nifedipine sensitivity. As expected from the lack of an identifiable syntaxin binding site (21), the coexpression of the channel with Lymnaea syntaxin1 did not affect channel function. Interestingly, the channel was irreversibly inhibited in the presence of GTP·S51 but not functionally affected by syntaxin1, suggesting that invertebrate calcium channels may display distinct regulation from their vertebrate counterparts.

**EXPERIMENTAL PROCEDURES**

*Preparation of Full-length LCa2a and Lsytx1 cDNAs for Expression—* Full-length cDNA homologs of Lymnaea Cav2.2 (LCa2a; GenBank™ accession number AF484082) and syntaxin1 (Lsytx1; GenBank™ accession number AF484088) were constructed from fresh Lymnaea brain cDNA by PCR using proofreading Turbo Pfu (Stratagene) polymerase and primers flanking the identified start and stop codons of the open reading frame (molecular cloning of LCa2a and Lsytx1, described in Spafford et al. (21)). The 2141- and 290-amino acid coding region of LCa2a and Lsytx1 were inserted using primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS. A consensus Kozak sequence was constructed in the 5′ NoI site immediately upstream from the start (ATG) codon, CGGCCGCCACC(ATG). When functional expression of LCa2a failed, the construct LCa2a-5′ rBa was designed. For LCa2a-5′ rBa, a silent XhoI restriction site was created at arginine, position 45, and the 5′ end of rat Ca2.1 homologue (gi:203110) was inserted into primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS. A consensus Kozak sequence was constructed in the 5′ NoI site immediately upstream from the start (ATG) codon, CGGCCGCCACC(ATG). When functional expression of LCa2a failed, the construct LCa2a-5′ rBa was designed. For LCa2a-5′ rBa, a silent XhoI restriction site was created at arginine, position 45, and the 5′ end of rat Ca2.1 homologue (gi:203110) was inserted into primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS. A consensus Kozak sequence was constructed in the 5′ NoI site immediately upstream from the start (ATG) codon, CGGCCGCCACC(ATG). When functional expression of LCa2a failed, the construct LCa2a-5′ rBa was designed. For LCa2a-5′ rBa, a silent XhoI restriction site was created at arginine, position 45, and the 5′ end of rat Ca2.1 homologue (gi:203110) was inserted into primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS. A consensus Kozak sequence was constructed in the 5′ NoI site immediately upstream from the start (ATG) codon, CGGCCGCCACC(ATG). When functional expression of LCa2a failed, the construct LCa2a-5′ rBa was designed. For LCa2a-5′ rBa, a silent XhoI restriction site was created at arginine, position 45, and the 5′ end of rat Ca2.1 homologue (gi:203110) was inserted into primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS. A consensus Kozak sequence was constructed in the 5′ NoI site immediately upstream from the start (ATG) codon, CGGCCGCCACC(ATG). When functional expression of LCa2a failed, the construct LCa2a-5′ rBa was designed. For LCa2a-5′ rBa, a silent XhoI restriction site was created at arginine, position 45, and the 5′ end of rat Ca2.1 homologue (gi:203110) was inserted into primer-incorporated 5′ NoI and 3′ XhoI restriction sites into the polylinker of the mammalian expression vector PMT2XS.

**Sequence Comparisons—** Lymnaea genes and rat homologs were aligned using a progressive pairwise, multiple alignment in PILEUP (UNIX-based, GCG Wisconsin Package 2002, Accelrys, Madison, WI). Aligned rat genes included Ca2.1 (P/q type) (GI:1705706), Ca2.2 (N-type) (GI:254553410), syntaxin1A (GI:417843), and syntaxin1B (GI:631888).

*Preparation of LCa2a Polyclonal Antibody—* The antigen to make the calcium channel polyclonal antibody was derived from a 15-mer peptide, KAEDNENDSEQNDND (Henk Hilkman, Netherlands Cancer Institute—Amsterdam), corresponding to amino acids 418-432 of LCa2a cytoplasmic I-H linker. The purity of the peptide was confirmed by matrix-assisted laser desorption ionization mass spectrometry and alkylation high performance liquid chromatography. Rabbits were immunized for a 4-week period with adjuvants and antigen conjugated to the carrier protein KLH (Washington Biotechnology Inc., Baltimore, MD). IgG rabbit antiserum was tested for immunoreactivity with the antigen by dot blot.

*Transient Transfection of Mammalian Cells—* Human embryonic kidney tsA201 cells were grown and transfected using standard calcium phosphate protocol (26). cDNAs transfected included a CDNAs construct encoding for green fluorescent protein (Clontech), calcium channel subunits rat α2δ, and rat β3 and either LCa2a or LCa2a-5′ rBa or rat Ca2.2, plus when stated, Lsytx1, rat syntaxin1A, or C-terminal fragment of the β-adrenergic receptor kinase (β-ARKct). Cells slatted for immunohistochemistry were left to incubate at 37 °C on poly-L-lysine-coated dishes whereas those slated for electrophysiological experiments were incubated at 28 °C for 2–4 days before analysis.

*LCa2a Immunostaining in Mammalian Cells—* Mock-transfected tsA-201 cells or tsA-201 cells transfected with rat α2δ, β3 and either LCa2a or LCa2a-5′ rBa were incubated at 37 °C for 4 days, fixed with 1% paraformaldehyde for 12 h at 4 °C, then permeabilized in 1% Nonidet-P40 (4-(4-phenoxyxethanol polyethylene glycol)-isoctyl phenyl ether) for 1 h at room temperature. Afterward, cells were washed in TBS-BSA-Triton blocking agent (50 mM Tris, 150 mM NaCl, 1 g/liter bovine serum albumin; Sigma-Aldrich, A9647), 0.5% Triton-X100, pH 7.4, and then incubated with 1:1000 dilution, LCa2a anti-rabbit polyclonal antibody in TBS-BSA-Triton overnight at 4 °C. The next day, cells were washed 10× in TBS (50 mM Tris, 150 mM NaCl, pH 7.4), wet-dried, and mounted in fluorescence anti-fading media (ProLong AntiFade, Molecular Probes). Images were visualized and analyzed on an Olympus confocal microscope.

**Immunoblot Analysis of LCa2a and Lsytx1 Protein Expression in tsA-201 Cells—**Transiently transfected or untransfected tsA-201 cells incubated for 4 days at 37 °C were harvested for Western blot analysis using a standard procedure (26). Proteins were detected with primary antibodies at 1:2000 dilution, raised in rabbits against LCa2a or Lsytx1 (JAN 002, Alomone Labs, Jerusalem, Israel). Color detection of gel blots were processed using previously described methods (21).

*Functional Analysis and Analysis of LCa2a CDNAs Expression—* Calcium channel activity in transiently transfected tsA-201 cells was characterized via whole cell patch clamp using an Axopatch 200B amplifier (Axon Instruments, Union City, CA); pCLAMP 9.0 software, and previously described recording procedures and solutions (26). Data analysis was carried out using Clampfit (pClamp 9, Axon Instruments) and SigmaPlot 2000 (Jandel Scientific, SPSS Science, Chicago, IL.). Steady state inactivation curves were fit with standard Boltzmann relations I = I(1 + exp(Va - V))/S), where I is the normalized peak current amplitude, V is the holding potential, Vi is the half-inactivation potential, and S is a slope factor. Whole cell current-voltage relations were fitted with the equation I = GV - Erev))/(1 + exp(V - Vrev)/S), where G is the maximum slope conductance, I is the peak current amplitude, V is the test potential, Erev is the reversal potential, Vi is the half-activation potential, and S is a slope factor inversely proportional the effective gating charge. Time constants for inactivation, v, were determined from monoeponential fits to the raw data (Fig. 3A). Error bars reflect S.E., and numbers in parentheses reflect numbers of experiments. Statistical analysis was carried out using Sigmasstat (Jandel Scientific). Differences between mean values from each experimental group were tested using paired and unpaired Student’s t tests and were considered significant if p < 0.05.

**RESULTS AND DISCUSSION**

**The N Terminus Region of Cav2 Calcium Channels Regulates Membrane Trafficking—** We have recently reported the effects of RNA-mediated interference knockdown of a Cav2 calcium channel homolog on Lymnaea synaptic transmission (21). Our data revealed that synaptic transmission in identified Lymnaea neurons was dependent on a Cav2 calcium channel homolog (LCa2a) that is capable of associating with the scaffolding proteins Mint-1 and CASK but which curiously lacks the synaptic protein interaction site common to mammalian presynaptic calcium channels (21) (see Fig. 1, A and B). To functionally characterize the LCa2a2a channels, we generated a full-length cDNA inserted in a mammalian expression vector and coexpressed the channel with rat β1b and α2δ accessory subunits and an enhanced green fluorescent protein expression marker in tsA-201 cells. Repeated attempts (n = 24) to record current activity via whole cell patch clamp recordings failed, consistent with the lack of any reports of expression of synaptic invertebrate calcium channels in cell lines. To determine whether our inability to record from LCa2a calcium channels was due to inefficient protein synthesis or membrane targeting, we generated an antibody based upon a 15-mer peptide sequence of LCa2a in a region of low similarity among calcium channels and, thus, a low probability of interaction with other

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1 The abbreviations used are: GTP·S, guanosine 5′-3-O-(thiotriphospho- phate); BSA, bovine serum albumin.
proteins (see Fig. 1A). Using this antibody, we determined the expression pattern of LCav2.2 in transfected tsA-201 cells via confocal fluorescence microscopy. The antibody produced almost no background staining in mock-transfected cells (Fig. 2A). When cells were transfected with LCav2.2 together with appropriate ancillary subunits (Fig. 2B), staining could be observed in cytoplasmic compartments, but much less membrane staining was apparent, suggesting that LCav2.2 protein is synthesized but not efficiently targeted to the plasma membrane, thus accounting for the lack of barium currents in our electrophysiological analysis.

We then attempted a strategy used previously to obtain expression of a mammalian Ca\textsubscript{2,2} calcium channel variant in Xenopus oocytes (27–29) by replacing part of the N-terminal region of the LCav2.2 channel (44 amino acids) with the corresponding sequence from rat Ca\textsubscript{2,1} (creating LCav2.2-5\textsuperscript{rbA}). As shown in Fig. 2C, LCav2.2-5\textsuperscript{rbA} is detected in the plasma membrane as rings of fluorescence of differing intensities around the cell edges, apparent in approximately one-third of all transfected cells, similar to the fraction of green fluorescent protein-positive cells in typical transfections. Single bands of predicted size (240 kDa) for LCav2.2 were detected in an immunoblot of LCav2.2 and LCav2.2-5\textsuperscript{rbA}-transfected tsA-201 cells but not in mock-transfected cells (Fig. 2D). Together with the relatively low abundance of membrane staining and absence of observed barium currents in LCav2.2-transfected cells, this indicates that the native N terminus of LCav2.2 either directly antagonizes plasma membrane trafficking in mammalian cells or that it might be missing an N-terminal domain required for efficient trafficking or stabilization of channels in mammalian cell membranes (see Fig. 2E for sequence alignment of the rat Ca\textsubscript{2,2} and LCav2.2 N-terminal regions). It is interesting to note that among rat Ca\textsubscript{2,2} channels, there is a common, almost identical 28-amino acid N-terminal sequence just upstream of where LCav2.2 differs with rat channels (see boxed amino-acids, Fig. 2E), raising the possibility that this motif may perhaps be involved in membrane targeting. Further construction of chimeras will be needed to substantiate this possibility.

**The Biophysical Properties of LCav2.2-5’rbA Are Similar to Those of Mammalian N-type Channels**—When analyzed electrophysiologically, the LCav2.2-5’rbA channel construct resulted in robust barium currents in 46 of 82 green fluorescent protein-positive cells when transiently expressed in tsA-201 cells. This clearly contrasts with the lack of detectable expression of the wild type LCav2.2 channel. We therefore carried out a detailed analysis of biophysical properties of the LCav2.2-5’rbA calcium channel. A shown in Fig. 3A, the LCav2.2-5’rbA channel supports barium currents with moderate inactivation kinetics. As seen from the ensemble current-voltage relations (Fig. 3B), currents first activate at about 20 mV and peak near +20 mV. The half-activation potential determined from the fit of the IV curve was +8.5 mV, consistent with LCav2.2-5’rbA acting as a high voltage-activated calcium channel. A similar value was obtained from fits to individual IV relations (10.8 ± 1.9 mV, n = 26). The peak of the IV curve of LCav2.2-5’rbA is shifted about 20 mV positive to the human Ca\textsubscript{2,2} (N-type) calcium channel (30) and about 10 mV positive to the

**Fig. 1.** A, schematic representation of the \( \alpha _{2} \) subunit of LCav2.2 calcium channel with highlighted regions of significance described in the text (indicated by bold segments). Note that the II-III linker of LCav2.2 has a dramatically shortened intracellular loop compared with rat Ca\textsubscript{2,2} homologs. B, sequence alignment of the shorter II-III linker region of LCav2.2 with the equivalent region in rat Ca\textsubscript{2,2}, which is longer and contains a synaptic protein interaction (synprint) site common to vertebrate presynaptic calcium channels (dotted line above sequence). Indicated are arrows delimiting the homologous region in rat Ca\textsubscript{2,2} (\( \alpha _{II} \)) that is absent in a human \( \alpha _{II} \Delta 1 \) and \( \Delta 2 \) splice variants. \( \alpha _{II} \Delta 2 \) eliminates most of the synprint site, and as a consequence, there is a loss of syntaxin1A binding capacity in the human II-III linker (46). (A) and (B) refer to small insertions of 4 and 13 amino acids, respectively, found within C-terminally truncated, b variant of LCav2.2.
replaced barium as the external charge carrier (see the inset). This effect may be due to a reduction in single channel conductance, an effect on maximum open probability, or a combination thereof and is seen with most types of mammalian high voltage-activated calcium channels with the exception of Ca_{2.3} (33). These data are consistent with the observation that half-activation potential shifts of this magnitude are more typical to L-type calcium channels, with N-type and P/Q-type calcium channels but much less pronounced than the degree of speeding is comparable with that reported for rat Cav2.1 and Cav2.2 calcium channels. It is interesting to note that half-activation potential shifts of this magnitude are more typical to L-type calcium channels, with N-type and P/Q-type channels showing a smaller (~10 mV) effect (33).

We also examined the time course of inactivation in both barium and calcium. As shown in Fig. 4C, when the current records of Fig. 4A were superimposed to overlap at peak, a small degree of kinetic speeding could be observed in calcium, perhaps indicative of a small degree of calcium-sensitive inactivation. Examination of this effect at a number of different test potentials shows that calcium significantly speeds the rate of inactivation at more depolarized voltages (Fig. 4D). This small degree of speeding is comparable with that reported for rat P/Q-type calcium channels but much less pronounced than the calcium-dependent inactivation of L-type calcium channels. It is, however, important to note that the calcium-induced ~20-mV shift in half-activation potential could potentially re-
sult in a similar shift in the voltage dependence of the time constant for voltage-dependent inactivation, thus attributing the observed effects purely to voltage-dependent rather than calcium-dependent inactivation (i.e. compare the inactivation time constants in barium and calcium at +40 and +60 mV, respectively). Taken together, our data thus indicate that LCa,2a-5’rbA calcium channels undergo only little if any calcium-dependent inactivation.

LCa,2a-5’rbA Channels Are ω-Conotoxin GVIA-resistant—It has been shown previously that native Lymnaea neuronal whole cell calcium currents are insensitive to classical blockers such as ω-conotoxin GVIA and nifedipine (24, 25). Indeed, to date no selective blocker of these native currents has been identified. As shown in Fig. 5A, the LCa,2a-5’rbA channel shows the predicted lack of nifedipine and ω-conotoxin GVIA block. At concentrations (i.e. 3 μM GVIA and 5 μM nifedipine) at which mammalian N-type and L-type channels would, respectively, be completely blocked (35, 36), the LCa,2a-5’rbA channel showed only about a 10% reduction in peak current amplitude in response to ω-conotoxin GVIA and no detectable inhibition by nifedipine. In contrast, application of 100 μM cadmium completely abolished current activity within 10 s of application, as expected from a high voltage-activated calcium channel. The lack of conotoxin inhibition is interesting given the relatively high degree of sequence homology within the putative ω-conotoxin GVIA binding region of the Ca,2.2 calcium channel domain III S5-S6 region (Fig 5B, see Refs. 27 and 36). By contrast, the inhibition by cadmium ions is consistent with the sequence conservation of the narrow region of the pore (Fig. 4E, see Refs. 37 and 38).

LCa,2a-5’rbA Is Uniquely Regulated by G Proteins—Both rat N-type and P-type calcium channels are inhibited by direct interactions with G protein βγ subunits (see Refs. 39–41). A hallmark of G protein inhibition of these channels is that it can be relieved after the application of a strong depolarizing prepulse (see 42, 43). Under control conditions, the application of such a prepulse resulted in a slight decrease in peak current amplitude by 13 ± 2.9% (n = 16), presumably due to a small degree of inactivation occurring during the prepulse and indicating a lack of tonic (background) G protein inhibition. To elicit a putative G protein inhibition of LCa,2a-5’rbA channel activity, we added 100 μM GTPγS to the patch pipette but consistently failed to observe current activity upon cell rupture in seven experiments. In one additional experiment, however, current activity could be observed but was completely eliminated within 2 min of cell rupture and could not be recovered with application of pre pulses. These data suggest the possibility that rapid dialysis of the cell with GTPγS may have resulted in an irreversible inhibition of LCa,2a-5’rbA activity. To examine this possibility, the very tip of the recording pipette was filled with intracellular recording solution, and the rest of the pipette was back-filled with GTPγS-containing solution, thus allowing us to slow the dialysis of the cell with GTPγS. As shown in Fig. 6A, this resulted in the appearance of inward barium currents. However, unlike under control conditions, where currents remained stable over a time course of 10–15 min (not shown), channel activity decayed rapidly, presumably due to activation of G proteins in response to dialysis with GTPγS. The application of pre pulses had only little effect on current amplitude, indicating that membrane depolarization was ineffective in reversing the GTPγS-mediated inhibition. For comparison, rat N-type calcium channels would under these conditions display a −200–300% increase in peak current amplitude in response to application of the prepulse (44).

This leaves two possibilities. First, as in mammalian Ca,2.2 calcium channels, Gβγ subunits liberated by GTPγS-induced dissociation of the heterotrimeric G protein complex could perhaps directly bind to the channel to inhibit channel opening. If so, then binding would have to be unusually tight since pre pulses were ineffective in relieving the GTPγS-mediated inhibition. As seen in Fig. 6B, the LCa,2a-5’rbA channel and the rat Ca,2.2 channel share a high degree of sequence homology in the first putative G protein binding motif in the domain I-II linker (which overlaps with the calcium channel β subunit interaction site, alpha interaction domain sequence), whereas in the second putative G protein region identified in rat Ca,2.1 and Ca,2.2 channels (7, 15), there is little sequence similarity to the LCa,2a channel. It is, thus, conceivable that this results in a more stable G protein-channel interaction that is resistant to membrane depolarization.

Alternatively, it is possible that Gβγ subunits might be incapable of interacting with the LCa,2a-5’rbA channel. Instead,
GTPγS might trigger an intracellular signaling cascade that could secondarily inhibit channel activity. A number of electrophysiological studies in preparations of identified molluscan neurons (including *Lymnaea*) could be consistent with the existence of such a mechanism (24). In *Helisoma*, Phe-Met-Arg-Phe-NH₂ triggers a PTX sensitive, Gₐ-mediated inhibition of calcium currents, which results in a depression of neurotransmitter release (45) and inhibition of growth cone motility (46). This effect could be mimicked by GTPγS and was shown to be irreversible (45, 46). Similar observations with Phe-Met-Arg-Phe-NH₂ inhibition of calcium currents have been reported for neurons of *Helix aspera* (47), *Aplysia californica* (48), and *Lymnaea* (49). However, although there are some apparent parallels to our observations with transiently expressed LCav2a-5/rbA channels, it remains to be determined whether GTPγS similarly affects native *Lymnaea* synaptic calcium currents. Nonetheless, it seems clear that the regulation of transiently expressed LCav2a-5/rbA calcium channels appears to be fundamentally different from those of their mammalian counterparts, but additional studies will need to be conducted to pinpoint the precise signaling mechanism underlying the unique regulation of this Cav2 calcium channel homolog.

**Syntaxin1 Regulation of the LCav2a-5/rbA Channel**—We have shown previously that unlike the rat N-type calcium channel (1, 2, 26, 28, 32, 50), the LCav2a calcium channel is incapable of directly interacting with syntaxin1 (21). This is due primarily to the absence of the synaptic protein interaction site in the LCav2a II-III linker region (Fig. 1B). Hence, one would predict that the coexpression of syntaxin1, which in rat N-type calcium channels results in a pronounced hyperpolarizing shift in half-inactivation potential (28, 32, 50), should not affect LCav2a-5/rbA channel gating. Rather than using rat syntaxin1A or 1B, which we have characterized previously, we used *Lymnaea* syntaxin1 (Lsytx₁) for coexpression studies. To demonstrate expression of Lsytx₁ in tsA-201 cells, we first generated a Western blot of tissue homogenated from mock- and Lsytx₁-transfected cells probed with an antibody to rat syntaxin1 (Fig. 7A). We then coexpressed Lsytx₁ with the rat
FIG. 5. A, representative time course of block of LCa,2a-5 rbA calcium channels by 3 μM ω-conotoxin GVIA, 5 μM nifedipine, and 100 μM cadmium. Note that only cadmium appears to mediate significant inhibition. Inset, summary of the blocking effects of GVIA, nifedipine, and cadmium in the form of bar graphs. Cadmium always completely inhibited current activity, and hence, no error bars are evident. B, sequence alignment of the region from domain III S5 into the pore linking III5 to III6 of rat Ca,2.1, rat Ca,2.2, and the LCa,2a calcium channels, which is considered especially critical for ω-conotoxin binding in Ca,2.2 channels.

FIG. 6. A, time course of current amplitude in response to intracellular dialysis with 100 μM GTPγS (n = 7). Currents were elicited by stepping from −100 mV to +20 mV, and every second test pulse was preceded by a depolarizing prepulse (PP) to +150 mV for 50 ms (open symbols). For each experiment, all current amplitudes were normalized to the peak current value seen with the first test pulse. Note that current rapidly decays in response to GTPγS dialysis, but that this inhibition cannot be relieved by application of prepulses. B, sequence alignment of the linker region between domains I and II of rat Ca,2.1, rat Ca,2.2, and LCa,2a calcium channels. Note the high degree of conservation in the first putative Gβγ/calcium channel β subunit binding site (alpha interaction domain), which contrasts with the sequence divergence shown in the second Gβγ interaction site.
N-type calcium channel to determine whether Lsytx1 mediated similar functional effects to those described for rat syntaxin1A, that is, a hyperpolarizing shift in the midpoint of the steady state inactivation curve, and a tonic G protein inhibition of the channel triggered by syntaxin binding to the channel, which can be assessed by application of strong depolarizing prepulse (26, 32, 50). Interestingly, unlike rat syntaxin1A and 1B, Lsytx1 did not affect the position of the steady state inactivation curve of the rat N-type channel (control, \( V_h = -44.8 \pm 1.7 \) mV, \( n = 7 \); Lsytx1, \( V_h = -47 \pm 2.4 \) mV, \( n = 10 \), see Fig. 7B) despite being able to bind to its domain II-III linker region as we showed recently (21). The lack of Lsytx1 expression on rat N-type channel inactivation is, however, somewhat surprising since Lsytx1 is highly homologous to both rat syntaxin1A and 1B (see Fig. 7C), both of which affect N-type channel gating (32). This result may, thus, provide novel clues about the syntaxin structural determinants that are required to affect N-type channel gating. In the presence of Lsytx1, we did, however, detect tonic G protein inhibition of the rat N-type channel (32 ± 7% current enhancement by the prepulse, \( n = 10 \), not shown), which differed significantly from background. Although this effect was not nearly as large as that described by us previously for rat syntaxin1A (80% enhancement after the prepulse; see Ref. 32), this observation together with the Western blot shown in Fig. 7A suggests that Lsytx1 was functionally expressed in tsA-201 cells and is active on the N-type channel.

**Fig. 7.** A, Western blot stained with a syntaxin1 antibody illustrating *Lymnaea* syntaxin1 (Lsytx1) expression in Lsytx1-transfected tsA-201 cells (~35-kDa band) and in *Lymnaea* brain homogenate. Note the absence of signal in the control lane of the mock-transfected cells. B, comparison of half-inactivation potentials obtained with LCa\(_{2.6}\)/5\rbA or rat Ca\(_{2.2}\) (each coexpressed with rat \( \beta_{1b} \) and \( \alpha_{2-3} \) subunits) with or without coexpression of Lsytx1, and as indicated, the C terminus of the \( \beta \)-adrenergic receptor kinase, \( \beta \)-ARKct, which serves as a G\(_{\text{q/11}} \) sink; see Ref. 39). Note that neither the rat N-type channel nor the LCa\(_{2.6}\)/5\rbA channel undergoes a negative shift half-inactivation potential. Numbers in parentheses indicate the numbers of experiments. C, sequence alignment of rat syntaxin1A, rat syntaxin1B and *Lymnaea* syntaxin1. Note the high degree of homology among the three syntaxin isoforms.
We next examined the action of Lsytx1 on the functional properties of the LCa2a-2a-5-rbA calcium channel. To eliminate any syntaxin-mediated G protein inhibition that might interfere with our ability to record from the channel, we also coexpressed the C-terminal fragment of the β-adrenergic receptor kinase (β-ARKct), a known inhibitor of Gβγ-mediated signaling events (26). Under these circumstances, the half-inactivation potential of the channel was not significantly affected by the presence of Lsytx1 (Fig. 7C), consistent with our previous report showing that Lsytx1A is incapable of binding to the channel in vitro (21). These data suggest that the modulation of presynaptic calcium channel gating by syntaxin1 is likely a vertebrate specialization.

**Potential Limitations of Our Study**—In this study, the 5′ end of rat Cav2.1 was necessary to promote the expression of this channel in mammalian cells. Without this modification, the functional description of the invertebrate channel would not have been possible, thus making use of a chimeric channel was a necessary evil. Based on previous structure function studies on mammalian calcium channels, many of the functional properties that we examined here (permeation, P loops (37, 38); voltage-dependent inactivation, II-1 linker and S6 segments (51–56); calcium-sensitive inactivation, C terminus (57–60); dihydropyridine sensitivity, IIIS5, IIIS6, and IVS6 segments (61, 62); conotoxin block, IIIS5-S6 region (27, 36); syntaxin regulation, II-III linker (1, 2, 7, 11, 26, 28, 32, 50)) are unlikely to be affected by the presence of the mammalian Cav2.1 N-terminal sequence. However, this may not be so with regard to G protein regulation of the channel, if it were to be mediated by a direct action of Gβγ subunits on the channel. Although the major Gβγ interaction sites on the mammalian Cav2 channels are found in the domain II-1 linker and the C-terminal regions (15, 40, 63), the N terminus has been implicated in regulating the functional effects of G protein βγ subunits on N-type channel activity (64). Thus, whereas mammalian Cav2.1 channels are typically only weakly regulated by direct action of Gβγ (13, 39, 41), we cannot rule out the possibility that the presence of the Cav2.1 N terminus could enhance a putative direct Gβγ modulation of LCA2a. However, as we discussed above, in light of previous literature, it appears more likely that the GTPγS-mediated inhibition is due to an indirect regulation by one or more second messengers. Future experiments will attempt to delineate the exact messenger pathway involved and whether the observed effects are affected by the presence of the Cav2.1 N terminus.

**Novel Perspectives from Our Studies**—Here we provide for the first time a functional description of an expressed invertebrate synapatic Cav2 calcium channel in a mammalian cell line. First, we show here that properties of mammalian Cav2 channels cannot be universally applied to invertebrates, since toxin sensitivity and regulation of the channels by syntaxin1 appear to be unique to vertebrate channels. At the molecular level, such differences may in part reflect modifications or adaptations in the calcium-dependent neurotransmitter release process in either invertebrates or vertebrates.

Second, despite structural divergences, many of the characteristic biophysical features of mammalian P/Q- and N-type channels are found in the LCa2a-2a-5-rbA channels. Selective permeability to ions (barium and calcium) and responsiveness to changes in membrane voltage, including the rate and threshold of activation and inactivation, are remarkably similar, suggesting that the fundamental biophysical characters responsible for calcium- and voltage-dependent synaptic transmission are conserved at the level of the calcium channel. This similarity is shared at a biophysical level of invertebrate synaptic transmission where kinetic features of calcium-dependent neurotransmitter release are comparable with mammalian ones.

Third, as we reported previously, the invertebrate channel LCa2a lacks an equivalent for the mammalian synaptic protein interaction (synprint) site in the II-III linker, enabling it to bind synaptic proteins such as Lymnaea syntaxin1 or even mammalian syntaxin1A. Conversely, there is a reverse compatibility from mammals to invertebrates, where mammalian synprint binds with high affinity to invertebrate synaptic proteins, including syntaxin1, SNAP-25, and synaptotagmin1 (21). A functional consequence is that application of a synprint peptide to an invertebrate synapse blocks synaptic transmission as has been reported in mammals (21). To our surprise however, the invertebrate syntaxin (Lsytx1) does not appear to be functionally compatible with mammalian Cav2 channels. Even though Lsytx1 is strikingly homologous to both mammalian syntaxin1A and syntaxin1B (see Fig. 7C) and capable of physically binding to Cav2.2 (21), we did not observe an Lsytx1-mediated reduction in Cav2.2 channel availability. This brings a unique perspective to the relationship between synaptic calcium channels and synaptic proteins, suggesting that Cav2 channels and syntaxin1 may have coevolved to allow for syntaxin-mediated regulation of Cav2 channel activity. Indeed, minor residue changes in syntaxin1 and more profound adaptations such as the insertion of a –250–300-amino acid synprint domain into vertebrate Cav2 calcium channels appear required to allow a functional regulation of mammalian Cav2 channels by syntaxin1. The lack of this interaction in invertebrates may perhaps provide an opportunity to reconstitute functional interactions between synaptic calcium channels and synaptic proteins and investigate their consequences on synaptic release, thus providing novel insights into the intricacies of synaptic transmission.

Finally, identified synapses between Lymnaea neurons are highly amenable for analysis using molecular and electrophysiological approaches and optical imaging (21, 65–67). A combined approach using in vitro expression and hypothesis testing in native invertebrate neurons may, thus, permit us to dissect the interplay between presynaptic calcium channels, second messenger systems, and synaptic proteins.

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