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G-proteins modulate invertebrate synaptic calcium channel (LCa_v2) differently from the classical voltage-dependent regulation of mammalian $Ca_v2.1$ and $Ca_v2.2$ channels

Xuan Huang, Adriano Senatore, Taylor F. Dawson, Quyen Quan and John David Spafford*

Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

*Author for correspondence (spafford@uwaterloo.ca)

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SUMMARY

Voltage-gated calcium channels in the Ca_v2 channel class are regulators of synaptic transmission and are highly modified by transmitter inputs that activate synaptic G-protein-coupled receptors (GPCRs). A ubiquitous form of G-protein modulation involves an inhibition of mammalian $Ca_v2.1$ and $Ca_v2.2$ channels by Gβγ dimers that can be relieved by high-frequency trains of action potentials. Here, we address whether the ubiquitous and versatile form of G-protein regulation in mammals is also found in simpler invertebrate nervous systems. Remarkably, the invertebrate LCa_v2 channel from the pond snail, *Lymnaea stagnalis*, does not bear any of the hallmarks of mammalian, voltage-dependent G-protein inhibition of $Ca_v2.2$. Swapping either the I-II linker or N-terminus of $Ca_v2.2$, which serve as key binding domains for G-protein inhibition, does not endow invertebrate LCa_v2 channels with voltage-dependent G-protein modulatory capacity. Instead, *in vitro* expressed LCa_v2 channels are inhibited slowly by the activation of cAMP, in a manner that depends on G-proteins but does not depend on Gβγ subunits. A similar G-protein and cAMP-dependent inhibition of nifedipine-insensitive LCa_v2 currents is also consistent in native and identified *Lymnaea* VD4 neurons. The slower inhibition using a cellular messenger such as cAMP may meet the modulatory needs in invertebrates while an activity-dependent regulation, evolving in vertebrates, provides a more dynamic, fine-tuning of neurosecretion by regulating the influence of neurotransmitter inputs through presynaptic GPCRs.

Key words: calcium channel, patch clamp electrophysiology, G-protein, *Lymnaea stagnalis.*

INTRODUCTION

Voltage-gated calcium channels in the Ca_v2 channel class are regulators of synaptic transmission and are highly modified by neurotransmitter inputs that activate synaptic G-protein-coupled receptors (GPCRs). This was first described by Dunlap and Fischbach, who showed that activating GABAB, serotonin or adrenergic receptors shortened the action potential duration by inhibiting N-type calcium channels in chick sensory neurons (Dunlap and Fischbach, 1978; Dunlap and Fischbach, 1981). Since this seminal work was published over 25 years ago, much more is now known of the molecular mechanisms of the neurotransmitter-mediated regulation of GPCRs (Tedford and Zamponi, 2006). GPCRs are membrane-associated and complexed with the Ca_v2 calcium channels [N-type ($Ca_v2.2$) or P/Q-type ($Ca_v2.1$)] at synapses (Tedford and Zamponi, 2006). Upon receptor activation, G-protein βγ subunits dissociate from Gα-GTP subunits and directly bind and inhibit calcium channels at the synaptic membrane in a membrane-delimited manner (Herlitze et al., 1996). This inhibition is pertussis-toxin-sensitive (Tedford and Zamponi, 2006) and thus involves Gαi/o βγ subunits and is more pronounced for $Ca_v2.2$ than for $Ca_v2.1$ channels (Currie and Fox, 1997). The G-protein inhibition can be artificially relieved using electrophysiological protocols such as pre-pulse facilitation (PPF) (Bean, 1989). A strong conditioning, depolarizing pre-pulse promotes facilitating or enhancing calcium currents by temporarily disassociating the inhibitory Gβγ subunits from calcium channels (Tedford and Zamponi, 2006). Relief of the G-protein inhibition by the arrival of high-frequency action potential trains in the presynaptic terminal causes a temporary enhancement of intracellular calcium levels and transmitter secretion mediated by Ca_v2 channels (Park and Dunlap, 1998). This activity-dependent G-protein regulation in mammals serves as a critical form of short-term synaptic plasticity. A major question is whether this ubiquitous and versatile form of regulation is also found in simpler nervous systems, such as invertebrates.

We have previously cloned and expressed an invertebrate calcium channel homolog, LCa_v2, from the pond snail, *Lymnaea stagnalis*, which is a singleton homolog in invertebrates to the mammalian $Ca_v2.1$ and $Ca_v2.2$ channels (Spafford et al., 2003a). We previously showed that, like its mammalian counterpart, LCa_v2 mediates neurotransmitter secretion at nerve synapses (Spafford et al., 2003b) and robustly expresses in HEK-293T human cell lines in the presence of accessory α2δ and β subunits (Spafford et al., 2003a). Here, we report for the first time an evaluation of mammalian G-protein regulation in an invertebrate. We show that the invertebrate LCa_v2 channel does not exhibit any of the hallmarks of mammalian voltage-dependent G-protein inhibition. Instead, *in vitro* expressed LCa_v2 channels are inhibited slowly by the activation of cAMP, in a manner that does not depend on Gβγ subunits. A similar G-protein and cAMP-dependent inhibition of nifedipine-insensitive LCa_v2 currents is also consistent in native and identified *Lymnaea* VD4 neurons. While a form of inhibition of calcium channels through activation of G-protein subunits is present in invertebrate LCa_v2 channels, it is independent of voltage, slower to develop and requires enzymatic amplification steps mediated through generating a cellular second messenger, cAMP. The slower inhibition using a cellular messenger such as cAMP may meet the modulatory needs in invertebrates while an activity- dependent regulation, evolving in vertebrates, provides a more dynamic, fine-tuning of neurosecretion

by regulating the influence of neurotransmitter inputs through presynaptic GPCRs.

MATERIALS AND METHODS

Cloning of LCa$_v$2 and construction of chimeras

To study the characteristics of the LCa$_v$2 calcium channel, the wild-type *LCa$_v$2* gene was cloned into pIRES2-EGFP vector from a previously modified LCa$_v$2 construct, dubbed 5′*RatCa$_v$2.1-LCa$_v$2*, in pMT2 plasmid, which bears an N-terminus from mammalian Ca$_v$2.1 (Spafford et al., 2003a; Spafford et al., 2004; Spafford et al., 2006). The wild-type channel was reconstructed with the N-terminus of wild-type LCa$_v$2 (GenBank Accession No. AF484082) using *Xho*I and *Mlu*I restriction enzyme sites. The *Mlu*I restriction site was previously created by silent mutation of CGC to CGT, an arginine residue at position 232 using QuikChange mutagenesis (Spafford et al., 2003a). Similar silent mutagenesis was used to introduce silent restriction enzyme sites to facilitate the generation of chimeras. *Bam*HI (TCG to TCC, serine residue at position 1096) and *Spe*I (CTT to CTA, leucine residue at position 1513) sites were created, which flanked 5′ and 3′ ends of the I–II linker, respectively. A *Bst*bI site (GAG to GAA, glutamate residue at position 307) was created to delimit the 3′ end of the N-terminal sequence, while an *Xho*I site flanked the 5′ position upstream of the Kozak and start codon sequence. The corresponding I–II linker and N-terminus from rat Ca$_v$2.2 with compatible restriction site ends were used to replace the original LCa$_v$2 sequences, resulting in the *LCa$_v$2* ($_L1_R2_L3_L4$) chimera and the *LCa$_v$2* ($_R1_L2_L3_L4$) chimera, respectively.

Transfections

All culture reagents as well as GenElute HP Endotoxin-Free Plasmid Maxiprep Kit for purification of plasmid DNA for transfections were purchased from Sigma-Aldrich Canada (Oakville, Ontario). For electrophysiology, 10 μg of calcium channel subunits [Ca$_v$2.2 (GenBank Accession No. NP_671482) or LCa$_v$2 α1 subunit, accessory subunits: α2δ1 (NP_037051), β1b (NP_059042)] and sometimes G-protein subunits Gβ1 (AAD00650) and γ2 (AAB82554) harbored in mammalian expression vectors were heterologously expressed by transfection using either calcium-phosphate or Lipofectamine (Invitrogen Canada, Burlington, Ontario) into human embryonic kidney cell line (HEK293T, M. Calos, Stanford University, USA) at 40–50% confluency. HEK-293T cells were cultured in DMEM with 10% FBS and supplemented with 0.5% (v/v) penicillin–streptomycin solution. At least 3–4 h before transfection, cells were re-plated in 60 mm (diameter) sterile Petri dishes containing 3–6 pre-sterilized poly-lysine coated glass cover slips (Circles No. 1; 0.13–0.17 mm thick; size, 12 mm; Fisher Scientific Canada, Ottawa, Ontario) used for recording. 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. Cells were allowed to recover from washing at 37°C for 2 h and were then left at 28°C for at least 48 h before patching.

Whole-cell recording

HEK-293T cells positively transfected with calcium channel subunits were identified by green fluorescence, emitted by EGFP on the bicistronic pIRES2–EGFP plasmid (Clonetech, Mountain View, CA, USA) containing LCa$_v$2 calcium channels. Electrophysiological recordings were carried out in voltage-clamp mode at room temperature, with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), while monitored by the epifluorescence microscope (Axiovert 40 CFL; Zeiss Canada, Toronto, Ontario). Cells were bathed in external solution containing barium as the charge carrier (20 mmol l^{-1} $BaCl_2$, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes, 40 mmol l^{-1} TEA-Cl, 65 mmol l^{-1} CsCl, 10 mmol l^{-1} glucose, pH adjusted to 7.2 with TEA-OH, filtered through a 0.22 μm filter). Patch pipettes (25 MΩ; World Precision Instruments, Sarasota, FL, USA) were filled with internal solution (108 mmol l^{-1} Cs-methanesulfonate, 4 mmol l^{-1} $MgCl_2$, 9 mmol l^{-1} EGTA, 9 mmol l^{-1} Hepes, pH adjusted to 7.2 with CsOH, filtered through a 0.22 μm filter). Voltage commands were generated and data were acquired using a PC computer equipped with a Digidata 1440A interface in conjunction with pClamp10.1 software (Molecular Devices, Sunnyvale, CA, USA). Recorded currents were filtered at 10 kHz using a low-pass Bessel filter and digitized at a sampling frequency of 2 kHz. Only recordings with minimal leak (<10%) 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). Electrophysiology figures were illustrated in Origin 8 (OriginLab Corporation, Northampton, MA, USA).

Current–voltage relationships were obtained by holding cells at –100 mV before stepping to test potentials ranging from –50 to +60 mV for 150 ms. Ca^{2+} current activation curves were constructed by converting the peak current values from each current–voltage relationship data set to conductance using the equation $G_{Ca}=I_{peak}/(V_{command}-E_{Ca})$, where I_{peak} is the peak current, $V_{command}$ is the command pulse potential, and E_{Ca} is the Ca^{2+} 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: $G/G_{max}=\{1+[\exp(-V_{command}-V_{1/2})/k]\}-1$, where G is the peak conductance, G_{max} is the maximal peak Ca^{2+} conductance, $V_{command}$ is the conditioning potential, $V_{1/2}$ is the half-maximal activation, and k is the activation slope factor.

The voltage dependence of inactivation was measured as the fraction of peak currents at a test depolarization step to +20 mV from a –100 mV holding potential, after steady-state voltage conditions, prepared with a long 4 s pre-pulse holding potential ranging from –90 to +30 mV. Normalized data were averaged and curve fit with a Boltzman equation $I/I_{max}=\{1+\exp[(V_{inact}-V_{1/2}/k)]\}-1$, 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.

To study the voltage-dependent G-protein facilitation, a paired pulse protocol was used to observe the pre-pulse facilitation. Facilitation was recorded by providing a +150 mV strong depolarization lasting for 50 ms, 25 ms before the 40 ms +20 mV testing potential, while cells were held at –100 mV. Facilitation was calculated by dividing the value of the peak current with a pre-pulse to the value of the peak current without a pre-pulse.

Isolation and recording of snail neurons

The great pond snail, *Lymnaea stagnalis* (Eukaryota; Metazoa; Mollusca; Gastropoda; Pulmonata; Basommatophora; Lymnaeoidea; Lymnaeidae; Lymnaea) were raised in 38 liter tanks linked through an 85% recirculating system with artificial freshwater at room temperature, and fed growth-chamber-raised romaine lettuce *ad libitum*, supplemented with spirulina pellets. *Lymnaea* neurons for whole-cell patch clamp recording were prepared from

2–3-month-old, juvenile snails. Outer and inner sheaths of *Lymnaea* brains were removed using fine forceps after trypsin exposure (2 mg ml^{-1}) and trypsin inhibitor (2 mg ml^{-1}) treatment. Isolated VD4 neurons were isolated from pinned brains in high osmolarity defined media (DMEM supplemented with 20 mmol l^{-1} D-glucose) using a fire-polished glass pipette (50 µm). Neurons were cultured onto poly-L-lysine-pretreated cover slips and recorded 18–24 h after incubation in brain conditioned media. VD4 neurons were recorded in 2 mmol l^{-1} $BaCl_2$ plus (in mmol l^{-1}) 47.5 TEA-Cl, 1 $MgCl_2$, 10 Hepes and 2 4-aminopyridine (4-AP), pH 7.9 (adjusted with TEA-OH). VD4 neurons were recorded with 1.5–2 MΩ pipettes in calcium plus ATP/GTP-containing solution (in mmol l^{-1}): 29 CsCl, 2.3 $CaCl_2$, 10 ethylene glycol tetraacetic acid (EGTA), 10 Hepes, 2 ATP-Mg and 0.1 GTP-Tris, with pH 7.4 (adjusted with CsOH).

Immunolabeling

Standard HEK-293T cells tend to wash away during repeated antibody application and washing steps during an immunolabeling protocol. So, for immunolabeling work, a GripTite™ 293 MSR cell line (Invitrogen Canada Inc., Burlington, ON, Canada) was used instead, which is a genetically engineered cell line expressing a human macrophage scavenger receptor that promotes strong adherence of HEK-293T cells to culture plates. GripTite™ 293 MSR cells were transfected with calcium channel subunits and adhered onto cover slips coated with 1 µg ml^{-1} poly-D-lysine (Sigma-Aldrich Canada) and incubated at 28°C for 5 days and fixed with 1% paraformaldehyde in PBS (preheated to 37°C) at room temperature for 2 h then at 4°C overnight. After washing, blocking was carried out by application of PBS-T 3% BSA and incubation at room temperature for 2 h. Rabbit polyclonal anti-LCa_v2 antibodies (Spafford et al., 2003a) were diluted 1:200 in PBS-T 3% BSA and applied to cells overnight at 4°C. Cells were then washed three times and then blocked with PBS-T 5% BSA for 45 min prior to application of secondary antibody. Cells were then incubated with Alexa Fluor 633 goat anti-rabbit IgG (H+L) (Molecular Probes™, Invitrogen, Eugene, OR, USA) secondary antibody diluted 1:5000 in PBS-T 3% BSA for 45 min at room temperature, then washed. Cover slips were dried at 37°C for 30 min and mounted onto glass slides using FluorSave™ Reagent (Calbiochem® Biochemicals, EMD Chemicals, Inc., San Diego, CA, USA). Images were captured using a Zeiss LSM 510 META confocal microscope.

RESULTS

Introduction to the LCa_v2 calcium channel

Invertebrates, including *Lymnaea*, usually possess only one gene homolog to mammalian calcium channels in each of the three families. Shown in Fig. 1A is a gene tree illustrating the relationships of human homologs to an identified set of calcium channel subunit genes from *Lymnaea stagnalis*, all previously expressed in human cell lines: *LCa_v1* (Spafford et al., 2006), *LCa_v2* (Spafford et al., 2003a) and *LCa_v3* (Senatore and Spafford, 2010). Ca_v3 channels such as LCa_v3 bear transient, T-Type currents that are low voltage-activated (LVA) and open at resting membrane potentials (Senatore and Spafford, 2010). Ca_v1 and Ca_v2 channels are structurally more similar to each other and cluster together (see Fig. 1A). Opening of Ca_v1 and Ca_v2 channel gates requires strong depolarizations to a threshold significantly above the resting membrane potential, hence the term high voltage-activated (HVA). Ca_v1 channels produce L-type currents and mediate functions such as skeletal muscle and heart contraction, gene transcription and the endocrine release of hormones (Snutch et al., 2005). Ca_v2 channels, in particular $Ca_v2.1$ and $Ca_v2.2$ in mammals and invertebrate LCa_v2, are highly specialized non-L-type channels, which mediate neurotransmitter release at nerve synapses (Fig. 1A) (Spafford and Zamponi, 2003). Modulation of the activity of synaptic $Ca_v2.1$/$Ca_v2.2$ calcium channels by G-proteins is ubiquitously featured in mammals as a key mechanism of channel regulation (Tedford and Zamponi, 2006). Comparison with invertebrates provides insights into the structural and functional evolution of this mode of regulation.

In vitro expression of LCa_v2 in HEK-293T cell lines

To functionally characterize the G-protein regulation of the synaptic LCa_v2 channel *in vitro*, the full-length 6426 bp sequence coding for the 2144 amino acid LCa_v2 channel was placed in bicistronic vector

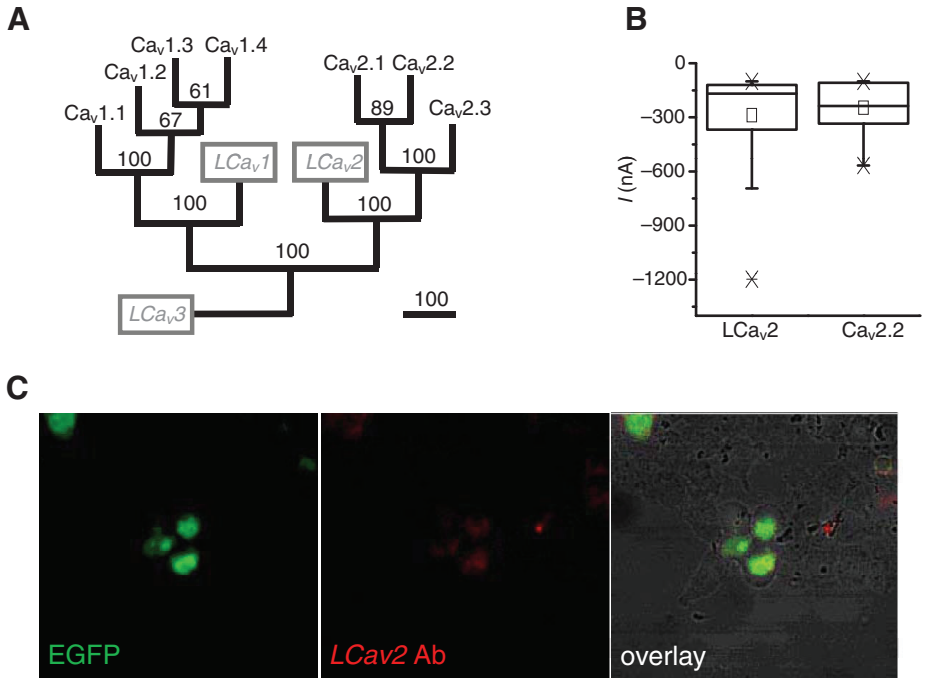

Fig. 1. Introduction to *Lymnaea* synaptic Ca_v2 channel and its *in vitro* expression in HEK-293T human embryonic kidney cell line. (A) Most parsimonious gene tree generated using multiple aligned sequences, analysed in PAUP4.0 (D. L. Swofford) and illustrated with TreeView (R. D. M. Page). Sequences include official human sequences (http://www.iuphardb.org) and *Lymnaea* homologs *LCa_v1* (AAO83838), *LCa_v2* (AAO83841) and *LCa_v3* (AAO83843). Numbers at branchpoints represent bootstrap values based on 100 replicates in heuristic search. (B) Box chart indicating the sizes of LCa_v2 currents recorded 4 or 8 days after transfection of LCa_v2 in pIRES2 vector with accessory α2δ and Ca_vβ subunits. The box chart also illustrates mean, median ± 1 s.d., min/max current densities. (C) EGFP fluorescent cells corresponded with cells bearing LCa_v2 polyclonal antibody staining (illuminated with Alexa Fluor 633 goat anti-rabbit secondary antibody) in transfected GripTite™ 293 MSR cell line.

pIRES2–EGFP, where enhanced GFP (EGFP) and LCa_v2 channel are generated from the same mRNA *via* transcription from a CMV promoter. Using EGFP fluorescence on the pIRES2-EGFP plasmid as a marker, cells containing adequately sized LCa_v2 currents could be readily identified for whole-cell patch clamp recording (see Fig. 1C). A minimum threshold of 100 nA amplitude was chosen for all electrophysiological recordings used in this study, which bore a mean ± s.e.m. for mammalian $Ca_v2.2$ of 287±38.91 nA (range=100.5–1197.44 nA, N=34) and a mean ± s.e.m. for *Lymnaea* LCa_v2 of 246±28.34 nA (range=100.2–567.7 nA, N=42) (Fig. 1B). Our first experiments were to measure parameters that are modulated by G-proteins such as voltage sensitivities (Fig. 2) and channel kinetics (Fig. 3).

Similar voltage sensitivities of LCa_v2 and $Ca_v2.2$

LCa_v2 and mammalian $Ca_v2.2$ ($Ca_v2.2$) calcium currents (Fig. 2A) were generated in 10 mV voltage steps from a holding potential of –100 mV in whole-cell voltage clamp and were plotted as a peak current *versus* test voltage (Fig. 2B). Both LCa_v2 and $Ca_v2.2$ channels respond to depolarization as expected for HVA channels, with a threshold of current generation above resting potentials at approximately –20 mV and rising to a peak at 20 mV in the presence of 20 mmol l^{-1} extracellular Ba^{2+} as the charge carrier (see Fig. 2B). The half-activation potential determined from the Boltzmann transformations of individual activation curves was nearly identical for LCa_v2 channels (8.4±0.708 mV, N=7) and $Ca_v2.2$ channels (8.3±0.573 mV, N=6) (Fig. 2C). Steady-state availability curves were generated by measuring the fraction of maximal current during a test pulse after a long 4 s pre-pulse at different voltages. The half-inactivation potential generated from Boltzmann curve fits of individual availability curves corresponds to voltages at which there were 50% available and non-inactivated channels during steady-state conditions. Both LCa_v2 and $Ca_v2.2$ have voltage sensitivities of inactivation in the range expected for HVA channels, although the half-inactivation potential for LCa_v2 (–39.3±0.453 mV, N=9) was ~10 mV more positive than for $Ca_v2.2$ (–49.2±0.422 mV, N=10) (Fig. 2D).

Faster kinetics of LCa_v2 *versus* $Ca_v2.2$

Dramatic differences are apparent for the gating kinetics between LCa_v2 and $Ca_v2.2$ channels. This is illustrated in Fig. 3A, plotted as averaged, normalized current ± s.e.m. of N=6 current traces for LCa_v2 and $Ca_v2.2$ channels elicited by a single voltage step to +40 mV for 150 ms from a holding potential of –100 mV. Tau values extrapolated from the single exponential curve fits are illustrated for kinetics of activation (Fig. 3B) and kinetics of inactivation decay (Fig. 3C). At every potential, LCa_v2 had significantly faster activation kinetics than $Ca_v2.2$ and also faster

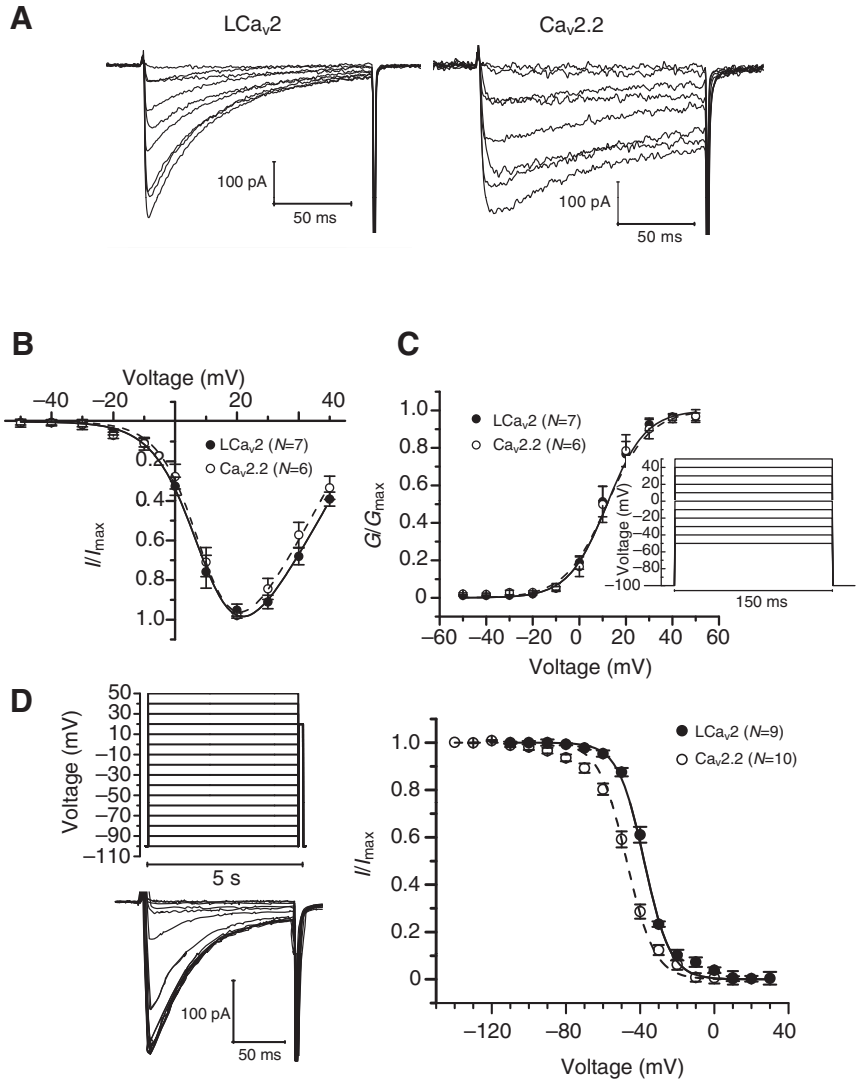

Fig. 2. Similarities in the voltage sensitivities of invertebrate LCa_v2 and mammalian $Ca_v2.2$ synaptic calcium channels expressed in HEK-293T cell lines. (A) Representative current traces of LCa_v2 and $Ca_v2.2$ channels in response to depolarizing voltage steps from –50 mV to +40 mV in 10 mV steps from a holding potential of –100 mV. (B) Plot of the peak current *versus* step voltage for LCa_v2 and $Ca_v2.2$ curve fitted with Ohmic–Boltzmann or (C) illustrated as activation curves fitted with a Boltzmann function. (D) The steady-state availability of LCa_v2 and $Ca_v2.2$ in response to a –100 mV to +20 mV test pulse, after channel inactivating pre-pulses of 5 s duration at voltages ranging from –130 mV to +30 mV. Stimulation protocol and sample LCa_v2 traces (left) and Boltzmann-fitted steady-state availability curves for LCa_v2 and $Ca_v2.2$ (right).

kinetics of inactivation decay at potentials above +20 mV. Evaluation of the effects of G-proteins on biophysical parameters such as activation kinetics for LCa_v2 and $Ca_v2.2$ channels is illustrated in Fig. 4.

Assessment of G-protein modulation of LCa_v2

Pre-pulse relief of G-protein inhibition was generated with a strong depolarizing pre-pulse (150 mV for 50 ms duration), 25 ms preceding the current generating test pulse (+PP), and the degree of facilitation was measured as the ratio of current change in the presence (+PP) and absence (–PP) of a G-protein relieving pre-pulse. $Ca_v2.2$ currents were facilitated dramatically, almost doubling the maximal peak current size (1.95±0.11 +PP/–PP ratio, *N*=8; see Fig. 4B), in response to a strong depolarizing pre-pulse when $Ca_v2.2$ channels were co-expressed with G-protein β1and γ2 subunits. Comparing sample traces, the voltage-dependent relief of G-protein inhibition for $Ca_v2.2$ (Fig. 4A; sample trace, right) was not evident for LCa_v2 (see Fig. 4A; sample trace, left). Background expression of endogenous G-protein subunits in HEK-293T cells provides a reduced (1.37±0.07+PP/–PP ratio, *N*=9), but still statistically significant, pre-pulse facilitation of $Ca_v2.2$, while there was no enhancement of current with a depolarizing pre-pulse for LCa_v2 in the absence (1.01±0.03 +PP/–PP ratio, *N*=8) or presence (0.95±0.03 +PP/–PP ratio, *N*=7) of exogenous G-protein subunits. The LCa_v2 +PP/–PP ratio near 1.0 reflects a lack of voltage-dependent G-protein modulation for LCa_v2 channels.

Hallmark features of G-protein modulation, such as changes in activation kinetics and voltage sensitivity (Tedford and Zamponi, 2006), are also absent for LCa_v2. Gβγ bound to $Ca_v2.2$ channels characteristically slows the onset of channel opening, leading to a delayed rate of rise to the generation of peak current (–PP; see Fig. 4A, right), while activation kinetics becomes faster after pre-pulse relief of the Gβγ inhibition (+PP; see Fig. 4A, right). The faster activation kinetics for $Ca_v2.2$ in the presence of pre-pulse relief of G-protein inhibition is reflected in the shorter tau constants derived from exponential fits of the rate of activation [2.600±0.161 (+PP) *versus* 2.951±0.268 (–PP), *N*=9]. Faster activation kinetics and shorter tau constants during pre-pulse relief of G-protein inhibition were even greater when exogenous Gβγ was cotransfected with $Ca_v2.2$ [2.165±0.187 (+PP) *versus* 2.860±0.302 (–PP), *N*=8] (Fig. 4C).

As expected for channels that are not regulated by G-proteins in a voltage-dependent manner, LCa_v2 did not bear any kinetic differences in activation (+PP or –PP; see Fig. 4A, left), which was reflected in unchanging activation tau constants in the absence [0.845±0.0902 (+PP) *versus* 0.853±0.0631 (–PP), *N*=7] or presence of exogenous G-protein βγ subunits [0.686±0.125 (+PP) *versus* 0.630±0.118 (–PP), *N*=8]. Normally, G-proteins also shift the activation voltage to more depolarizing potentials but this was also not apparent for LCa_v2 (Fig. 4D).

Poorly conserved G-protein modulation sites in LCa_v2

The lack of G-protein modulation of LCa_v2 channels under the identical conditions for $Ca_v2.2$ channels, such as the presence of accessory α2δ1 and Ca_vβ1 subunits and G-protein β1 and γ2, suggests that there are structural elements lacking for mammalian G-protein modulation in the LCa_v2 channel. Voltage-dependent G-protein modulation requires pre-association of a Ca_vβ subunit to the Ca_v2 α1 subunit, so the determinants for Ca_vβ subunit binding are also critical for understanding voltage-dependent G-protein modulation (Zhang et al., 2008). Key structures in the calcium channel for Ca_vβ subunit association include the N-terminal end of the cytopasmic I–II linker of the Ca_v2 channel, which can be divided into a rigid α-helical IS6-AID linker sequence (20 amino acids) (Vitko et al., 2008) followed by an α-helical, alpha-interacting domain (AID) (18 amino acids) (Opatowsky et al., 2004) (see Fig. 5A,C). Primary sequences for G-protein βγ binding with Ca_v2–Cavβ complexes are reported to primarily include sequences in the I–II linker at a Gβγ-1 site overlapping with Ca_vβ and a downstream Gβγ-2 site in the I–II linker (Fig. 5C) (Tedford and Zamponi, 2006). The N-terminus

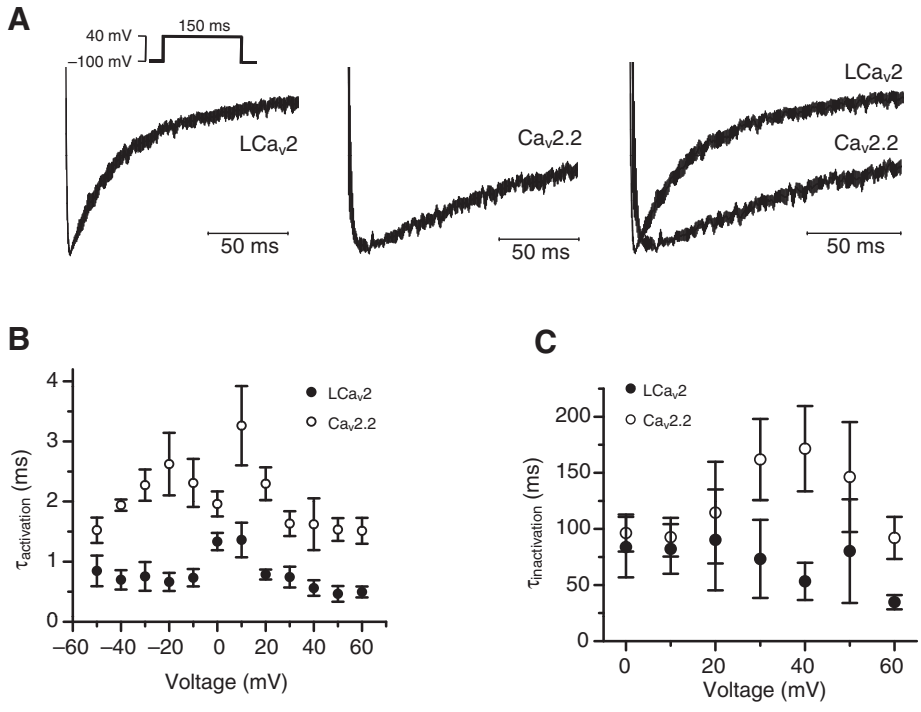

Fig. 3. Dramatic differences in the kinetics of activation and inactivation of invertebrate LCa_v2 and mammalian $Ca_v2.2$ synaptic calcium channels expressed in HEK-293T cell lines. (A) Mean and s.e.m. (*N*=6) of overlapping traces of LCa_v2 and $Ca_v2.2$ channel currents elicited by a voltage step to +40 mV for 150 ms from a holding potential of –100 mV. Tau values acquired from exponential curve fits of kinetics of (B) activation and (C) inactivation decay for LCa_v2 and $Ca_v2.2$ channels.

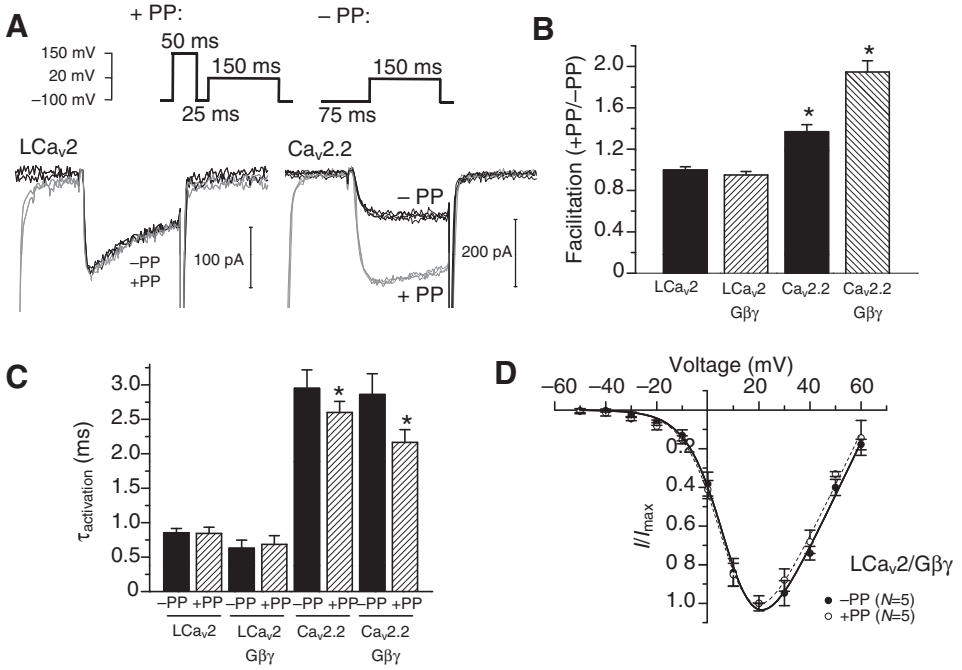

Fig. 4. Lack of evidence for the voltage-dependent G-protein modulation of $Ca_v2.2$ channels in invertebrate LCa_v2 synaptic calcium channels expressed in HEK-293T cell lines. (A) Comparison of pre-pulse facilitation of LCa_v2 and $Ca_v2.2$ channels cotransfected with G-protein β1 and γ2 subunits. Stimulation protocol (above) and sample traces (below). Facilitation is only evident in $Ca_v2.2$ channels, reflected in an increase in peak current size in response to a 150 mV depolarizing pre-pulse of 50 ms duration (+PP) in comparison to the same test pulse without the G-protein relieving pre-pulse (–PP). (B) Histogram mean ± s.e.m. of facilitation data illustrated as a ratio of the peak facilitated current (+PP) over the unfacilitated current (–PP). $Ca_v2.2$ channels facilitated with an even greater response in the presence of exogenous G-protein β1 and γ2 subunits (*P<0.05), while LCa_v2 lacked any facilitation. (C) Tau constants (ms) derived from the exponential fits of activation in the absence (–PP) or presence (+PP) of facilitating, depolarizing pre-pulses. Activation kinetics of $Ca_v2.2$ channels was faster for facilitated currents, with even faster kinetics in the presence of exogenous G-protein β1 and γ2 subunits (*P<0.05), while LCa_v2 lacked changes in activation kinetics. (D) Peak current sizes of LCa_v2 channels expressed with G-protein β1 and γ2 subunits elicited from a holding potential of –100 mV to a range of –50 mV to +60 mV in 10 mV steps. Facilitation was assessed as the relative increase in current during a depolarizing pre-pulse (+PP) normalized to current size without a depolarizing pre-pulse (–PP). No differences in the current–voltage curves +PP *versus* –PP suggest an absence of G-protein regulation of voltage sensitivity of LCa_v2.

of $Ca_v2.2$, labeled NTB (Fig. 5B), has also been identified as significant in G-protein βγ binding (Agler et al., 2005).

Mammalian sequences do not endow LCa_v2 with voltage-dependent G-protein modulation

The I–II linker or the N-terminus of $Ca_v2.2$ was swapped into LCa_v2 to test whether either of these regions would endow LCa_v2 channels with mammalian voltage-dependent G-protein modulation. Unique restriction sites spanning the N-terminus (*Xho*I–*Bst*BI) and I–II linker (*Bam*HI–*Spe*I) were created by site-directed mutagenesis for insertion of PCR-based synthetic DNA fragments of the $Ca_v2.2$ sequence with appropriate restriction site ends. G-protein modulation was evaluated with the pre-pulse facilitation protocol. Absence of enhancement of current resulting from the depolarizing pre-pulse suggests that neither LCa_v2 harboring the I–II linker (L1R2L3L4LCa_v2, N=5) nor the N-terminus (R1L2L3L4-LCa_v2, N=6) of $Ca_v2.2$ create voltage-dependent G-protein modulation in LCa_v2, even in the presence of exogenous G-protein βγ subunits (Fig. 6).

LCa_v2 is inhibited by cAMP *via* a Gβγ subunit independent mechanism

Preliminary investigation suggests that invertebrate synaptic Ca_v2 channels are inhibited by G-proteins but that it does not involve Gβγ subunits (Spafford et al., 2003a). Treatment of LCa_v2 channels with a non-hydrolysable GTP analog, GTP-γS, leads to a constitutive activation of G-proteins and a voltage-independent modulation, where peak currents run down over time without relief of the inhibition by depolarization pre-pulses (Spafford et al., 2003a). A possible downstream target for this G-protein activation is a cytoplasmic second messenger, which may mediate the inhibition of LCa_v2 channels. Micro perfusion of a cell-permeable cAMP analog, 8-bromo-cAMP (8Br-cAMP), caused a slowly developing inhibition of LCa_v2 channel activity *in vitro* that required 1–3 min to develop (see Fig. 7A, sample time course) and reached an average peak of inhibition of 33.7±9.4% (N=6) for LCa_v2 currents (Fig. 7B). The possible involvement of G-protein βγ subunits was specifically addressed by co-transfection of βark-ct, the C-terminus of the beta adrenergic receptor kinase. βark-ct binds G-protein βγ subunits with high affinity and thus serves as a scavenger of G-protein βγ subunits. 8Br-cAMP did inhibit LCa_v2 channels in the presence of βark-ct, suggesting that GPCR activation elevates cAMP levels through activation of G-protein α but not βγ subunits.

The inhibition of LCa_v2 channels *via* a cAMP pathway was confirmed in *Lymnaea* VD4 neurons. VD4 neurons bear two HVA currents, including an L-type (or LCa_v1) current and an LCa_v2 current, and they operate in the same voltage range. It was previously shown that LCa_v2 channels can be separated from LCa_v1 channels with 10 μmol l^{-1} nifedipine, which completely blocks LCa_v1 channels without affecting LCa_v2 channels *in vitro* (Spafford et al., 2006). LCa_v1 current generally comprises ~20% of the total calcium current in VD4 neurons, but this value may

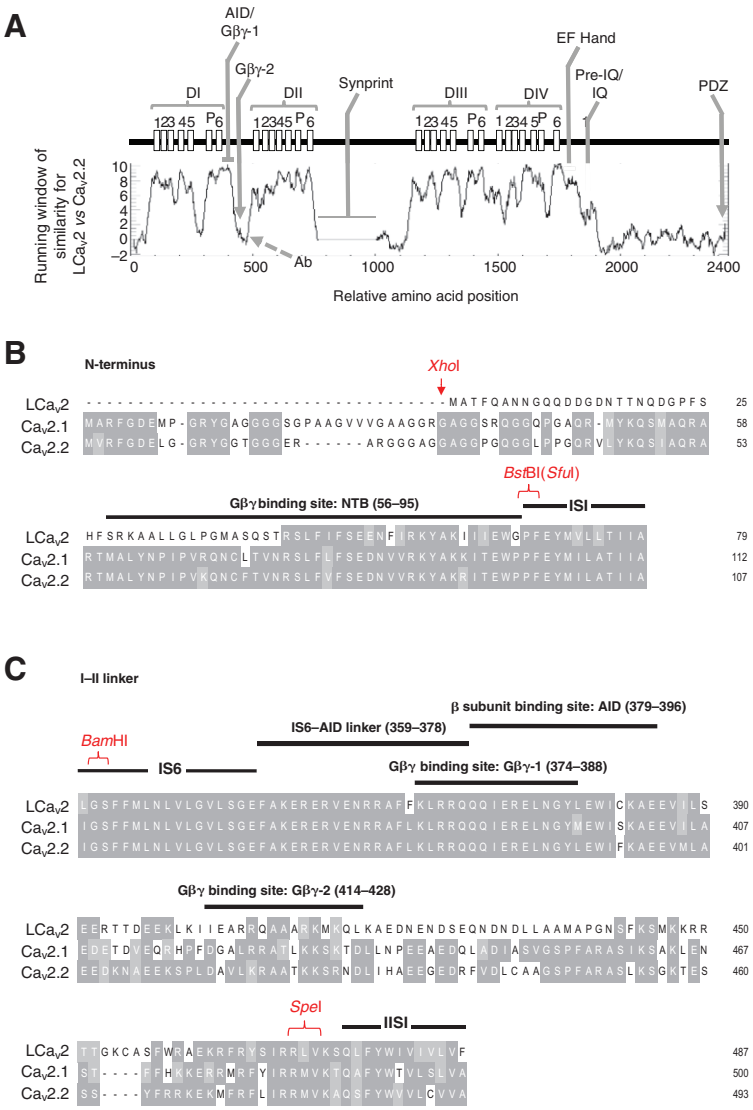

Fig. 5. Poorly conserved sequences of the I–II linker and N-terminus of invertebrate LCa$_v$2 calcium channels, which are known to harbor key elements for mammalian voltage-dependent G-protein modulation. (A) Running window of similarity between aligned amino acid sequence of LCa$_v$2 and Ca$_v$2.2 reveals invariant structures in six membrane-spanning segments in all four domains (I, II, III and IV), including an ion-conducting pore (S5-P-loopS6) and voltage sensor (S1–S4). Illustrated is the highly conserved AID sequence involved in high-affinity binding to Ca$_v$β subunits, a putative EF Hand region, a Pre-IQ/IQ motif for calmodulin binding and a C-terminal PDZ binding domain. Shown is the position in the I–II linker where LCa$_v$2 polyclonal antibody (Ab) was generated in rabbits. Absence of conservation of LCa$_v$2 sequences in the putative G-protein binding sites in (C) the I–II linker (βγ-2) and (B) the N-terminus (NTB) of Ca$_v$2.1 and Ca$_v$2.2 channels. Conserved and similar sequences in alignments (B,C) are colored dark and light grey boxes, respectively. Highly conserved helical structures in AID and IS6–AID linker are required for voltage-dependent G-protein modulation. Site-directed mutagenesis was carried out in LCa$_v$2 to swap N-termini (*Xho*I–*Bst*BI) and I–II linker sequences (*Bam*HI–*Spe*I) of Ca$_v$2.2 into LCa$_v$2.

vary between 10% and 35% depending on the cell (Spafford et al., 2006). The nifedipine-insensitive calcium current is likely to be conducted by LCa$_v$2 channels, since it can be blocked with 100 μmol l^{-1} cadmium, which completely blocks LCa$_v$2 *in vitro* (Spafford et al., 2006). Perfusion of 8Br-cAMP onto the nifedipine-insensitive currents in VD4 neurons caused a slowly developing inhibition (2–5 min) (Fig. 7C) that peaked at 26±11% inhibition (N=4; Fig. 7D). This response to cAMP corresponds to the *in vitro* results of a cAMP-mediated inhibition of LCa$_v$2 *via* a voltage-independent activation of G-proteins, through Gα and not Gβγ subunits.

DISCUSSION

Introduction to G-protein regulation of Ca$_v$2 channels

Calcium ions passed through voltage-gated calcium channels service a diverse array of functions, including the activation of highly sensitive intracellular signaling pathways that respond to slight changes in cytosolic Ca^{2+} levels. For this reason, even slight changes in channel gating characteristics can cause dramatic responses such as at nerve synapses, where the secretory capacity is strongly dependent on the gating behavior of Ca$_v$2 channels. G-protein βγ subunits participate in a form of short-term synaptic plasticity because of the voltage-sensitive nature of the G-protein binding to Ca$_v$2 channels (Tedford and Zamponi, 2006). Relief of the G-protein βγ inhibition by action potential trains provides temporary rises in intracellular calcium and corresponding increases in neurosecretion (Park and Dunlap, 1998). The inhibition and disinhibition of Ca$_v$2 channels is highly responsive to changes in the pool of activated Gβγ subunits from neurotransmitter inputs that secrete onto GPCRs of the presynaptic membrane (Tedford and Zamponi, 2006). Variety in types of GPCRs and G-proteins and distinctly different regulation between Ca$_v$2.1 and Ca$_v$2.2 channels provide enormous variety in neurocomputational possibilities (Tedford and Zamponi, 2006). It is particularly important to evaluate whether this ubiquitous and versatile mechanism in mammals is also present in Ca$_v$2 channels of the invertebrate nervous system.

Evolutionary relationship of Ca$_v$2 channels

The relationship between Ca$_v$2 and other calcium channels is reflected in the gene tree of homologs in Fig. 1. A likely genomic duplication event led to speciation of genes within the three calcium channel classes (Ca$_v$1, Ca$_v$2 and Ca$_v$3), whilst the invertebrates mostly retained single gene homologs in the three classes. Ca$_v$3 channels operate at the resting membrane potential

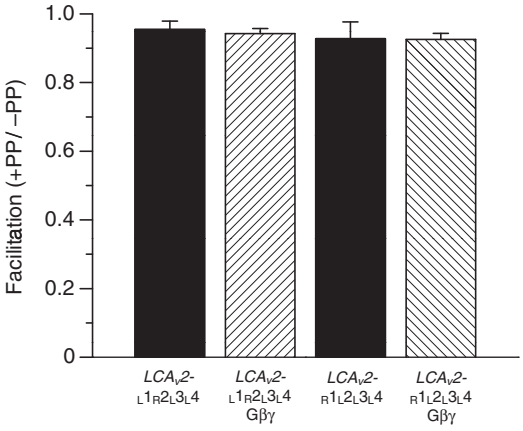

Fig. 6. Replacement of the *Lymnaea* I–II linker ($_L1_R2_L3_L4$) or the N-terminus ($_R1_L2_L3_L4$) with mammalian Ca$_v$2.2 sequence does not endow *LCa$_v$2* with the capacity for mammalian voltage-dependent G-protein modulation. Facilitation was assessed with chimeric channels expressed in HEK-293T cells in the presence or absence of coexpressed G-protein βγ subunits. Since the ratio of the tested current size in response to a depolarizing pre-pulse (+PP) *versus* its absence (–PP) was not greater than 1, it suggests a lack of voltage-dependent G-protein modulation in the chimeric LCa$_v$2 channels.

and thus are more associated with shaping the firing behavior of action potentials and providing calcium at resting membrane potentials (Senatore and Spafford, 2010). Ca$_v$1 and Ca$_v$2 channels require a strong depolarization to a firing threshold and thus are recruited by nerve activity (Snutch et al., 2005). Ca$_v$1 and Ca$_v$2 channels serve as electromechanical response units transducing action potentials to functions. Examples for Ca$_v$1 channels include skeletal muscle contraction (Ca$_v$1.1) and heart contraction (Ca$_v$1.2), gene transcription (Ca$_v$1.2), endocrine release of hormones (Ca$_v$1.3) and vision (Ca$_v$1.4) (Snutch et al., 2005). Mammalian Ca$_v$2.1 and Ca$_v$2.2 channels and invertebrate LCa$_v$2 are specialized channels for synaptic junctions, where they are closely embedded with the release machinery associated with the release of transmitter at the presynaptic membrane (Spafford and Zamponi, 2003).

Differences in biophysical characteristics between Ca$_v$2 channels

Side-by-side comparison of invertebrate LCa$_v$2 and mammalian Ca$_v$2.2 channels *in vitro* indicates that the voltage sensitivity of both activation and inactivation is similar, although LCa$_v$2 bears a ~10 mV shift in the steady-state inactivation compared with Ca$_v$2.2 (Fig. 2).

Similar voltage sensitivities between vertebrate and invertebrate orthologs are indicative of similar responsiveness to calcium channel gating during action potential volleys served to the presynaptic terminal. Constraints on the voltage range of channel activity reflect the shared responsibility of LCa$_v$2 and Ca$_v$2.2 in gating the precise rises in intracellular calcium for mediating transmitter release at nerve synapses (Dodge et al., 1967).

More variable between calcium channels is the rate of change of gating modes, reflected in activation and inactivation kinetics and interpreted as tau curve fits of the descending and rising slopes, respectively, of the whole-cell currents. LCa$_v$2 has dramatically faster activation and inactivation kinetics and shorter tau constants for activation and for inactivation in the voltage range greater than +20 mV (Fig. 3). Activation and inactivation rates are highly variable amongst Ca$_v$1 and Ca$_v$2 calcium channels and can be modified to a tremendous extent by accessory subunits, especially the cytoplasmic beta subunit that it is paired with (Snutch et al., 2005). Mammals have four different beta subunit genes (*β1*, *β2*, *β3* and *β4*), each possessing tremendous variability in alternative splicing, which serves to fine-tune biophysical parameters such as gating kinetics of the Ca$_v$1 and Ca$_v$2 channels (Snutch et al., 2005). Although invertebrates, such as *Lymnaea*, have only a single β subunit gene (Spafford et al., 2004), we have observed parallel alternatively spliced patterns of similar exons in the N-terminus and

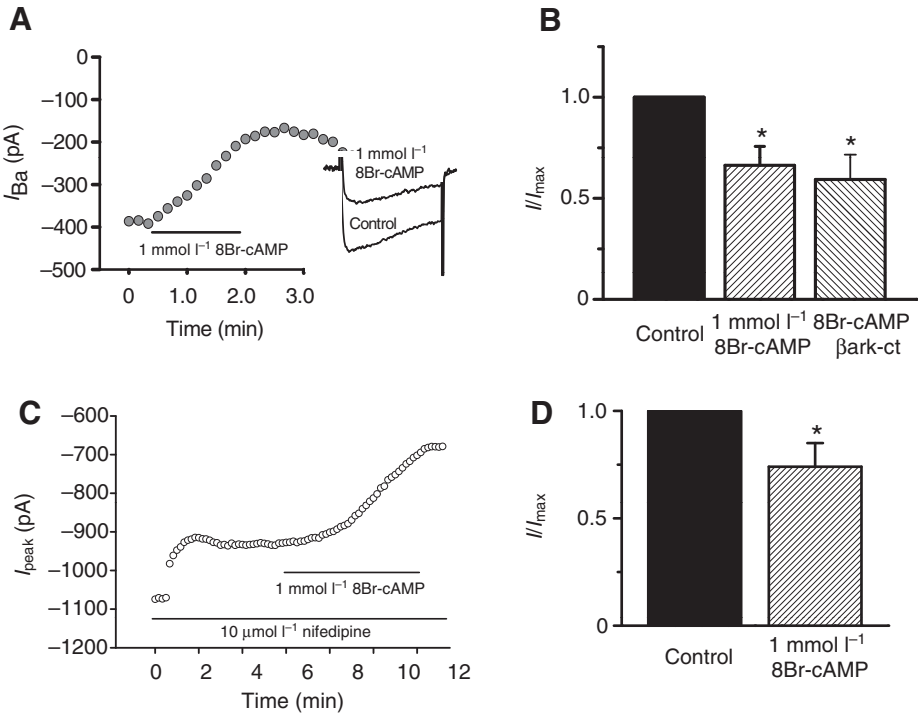

Fig. 7. Invertebrate LCa$_v$2 calcium channels *in vitro* are slowly inhibited by cAMP through a G-protein pathway that does not involve Gβγ subunits. (A) Membrane-permeant 8-bromo-cAMP (1 mmol l^{-1}) perfused onto LCa$_v$2 channels expressed in HEK-293T cells causes a slowly progressing inhibition of the calcium current. (B) Histogram illustrating the mean (+ s.e.m.) cAMP-mediated inhibition (26±11% inhibition, *N*=4, **P*<0.05) and the absence of effect of coexpression of β-adrenergic receptor kinase c-terminus (βark-ct) that operates as an effective scavenger of free G-protein βγ subunits. (C) A similar slowly progressing inhibition of nifedipine-insensitive LCa$_v$2 currents in VD4 neurons in response to cAMP application. (D) Histogram (mean + s.e.m.) illustrating a similar inhibition of current in VD4 neurons (33.7±9.4%, *N*=6, **P*<0.05) as with transfected channels in HEK-293T cells (compare with B).

HOOK region, which can create diversity in gating kinetics of invertebrate Ca_v1 or Ca_v2 channels (T.F.D., S. Harel, A.S., A. Boone and J.D.S., unpublished observations).

Lack of voltage-dependent G-protein modulation in LCa_v2

A voltage-dependent G-protein modulation is ubiquitously featured in mammalian $Ca_v2.1$ and $Ca_v2.2$ channels (Tedford and Zamponi, 2006). Gβγ subunits are considered to stabilize a closed state conformation of channels in a 'reluctant' gating mode (Bean, 1989). The transition from a reluctant to 'willing' gating mode can be simulated artificially using stimulation protocols such as pre-pulse facilitation (PPF) (Tedford and Zamponi, 2006). As observed for $Ca_v2.2$ currents, G-protein inhibition was associated with a delay in activation kinetics (measured as longer activation time constants) (Bean, 1989). Strong depolarizing pre-pulses were facilitating as reluctant calcium channels unbind from Gβγ subunits and transition to an opening willing mode (Fig. 4) (Bean, 1989). LCa_v2 completely lacked any of the features of voltage-dependent G-protein modulation such as kinetic slowing of inactivation, pre-pulse facilitation or change in voltage dependence of activation (Fig. 4). The lack of voltage-dependent G-protein modulation suggests that the mammalian $Ca_v2.2$ sequence that renders the channels sensitive to voltage-dependent G-protein inhibition is missing in LCa_v2.

Structural features involved in G-protein modulation

A key feature of mammalian G-protein modulation is a necessary pre-associated accessory beta subunit to $Ca_v2.2$ channels, but the key binding surfaces between invertebrate LCa_v2–$Ca_v\beta$ subunits are highly conserved (Spafford et al., 2004). Thus, it is not likely that invertebrate calcium channel beta subunit interactions are responsible for the absence of mammalian G-protein modulation. Ca_v1/Ca_v2 channels associate *via* a highly conserved AID peptide (alpha-interacting domain) sequence in the cytoplasmic I–II linker, which forms an alpha helix that is deeply embedded in the guanylate kinase (GK) domain of $Ca_v\beta$ (Fig. 5B) (Opatowsky et al., 2004). Also important upstream of the AID sequence is a highly conserved IS6–AID sequence, which also contributes to a rigid secondary structure for beta subunit binding and is considered to be important for voltage-dependent charge movements of IS6 (Vitko et al., 2008) and to drive the G-protein βγ unbinding (Fig. 5B) (Zhang et al., 2008). Disruption of the rigid α-helical structure in the IS6–AID sequence or prevention of $Ca_v\beta$ subunits from binding to the AID sequence completely eliminates voltage dependence of G-protein regulation (Zhang et al., 2008).

G-proteins closely associate with the region of the I–II linker that associates with β subunits, and indeed one putative Gβγ binding site (Gβγ-1), with a signature QQIER motif, is not likely to be a key determinant because the sequence is buried when $Ca_v\beta$ is complexed with Ca_v2 channels (Fig. 5) (Opatowsky et al., 2004). Gβγ-2 serves as a second identified Gβγ binding site downstream of Gβγ-1 in the I–II linker (Tedford and Zamponi, 2006), and its sequence is not conserved between LCa_v2 and $Ca_v2.1/Ca_v2.2$ channels (Fig. 5B). Gβγ subunits also associate with an NTB sequence in the N-terminus of $Ca_v2.2$ (Fig. 5B) (Agler et al., 2005) that is not well conserved in LCa_v2 channels. However, neither the replacement of the I–II linker nor the N-terminus of $Ca_v2.2$ into LCa_v2 was sufficient (Fig. 6) to endow LCa_v2 with mammalian G-protein modulation. One possibility reported by Yue and colleagues is that the N-terminus and I–II linker unite together and form a platform for G-protein modulation (Agler et al., 2005), but testing this option has not been possible since double replacement of the N-terminus and I–II linker of $Ca_v2.2$ into LCa_v2 did not produce channels with sufficiently resolvable currents expressed *in vitro*.

Voltage-independent G-protein regulation

Despite lacking determinants for voltage-dependent regulation, LCa_v2 is modulated by G-proteins in a voltage-independent manner that does not involve Gβγ subunits. In particular, activation of G-proteins inhibits calcium channel activity through a cytosolic messenger cascade involving cAMP that progresses over a slow time course (minutes). It is likely that the downstream target of cAMP is the LCa_v2 channel, since the degree of cAMP inhibition of LCa_v2 alone, transfected in HEK-293T cells (Fig. 7B, 26±11% inhibition, N=4), was similar to the degree of cAMP inhibition (Fig. 7D; 33.7±9.4%, N=6) of the HVA, nifedipine-insensitive current in VD4 neurons. Ca_v2 channel inhibition through a cAMP-mediated pathway provides a means of regulating transmitter release by activation of dopamine (Barnes et al., 1994) or serotonin (McCamphill et al., 2008) GPCRs on presynaptic *Lymnaea* VD4 neurons. Voltage-independent regulation of $Ca_v2.2$ channels has also been reported in select mammalian neurons, utilizing a number of different G-protein-dependent second messenger pathways (Tedford and Zamponi, 2006).

Importance of both forms of G-protein regulation

All $Ca_v2.2$ channels in mammalian neurons are inhibited by means of the voltage-dependent pathway mediated by direct interaction of βγ subunit heterodimers (Tedford and Zamponi, 2006). While the only form of G-protein regulation for LCa_v2 is voltage independent, this form of regulation is only observed in select neuron types in mammals (Tedford and Zamponi, 2006). Interestingly, just the selective inclusion of a short C-terminal exon (exon 37a) in $Ca_v2.2$ channels creates a tyrosine kinase phosphorylation site for voltage-independent G-protein modulation in mammalian nociceptive neurons (Raingo et al., 2007).

A question arises as to why some mammalian neurons selectively maintain voltage-independent forms of G-protein regulation while simultaneously maintaining a ubiquitous, voltage-dependent form (Tedford and Zamponi, 2006). Voltage-dependent G-protein modulation exerts an inhibition and relief of inhibition on $Ca_v2.1/Ca_v2.2$ channels in a manner proportional to a changing pool of activated Gβγ subunits. This allows for rapid and dynamic regulation of intracellular calcium and neurosecretion by transmitters that secrete onto appropriate presynaptic GPCRs in the presynaptic terminal. Changes in calcium channel activity occur rapidly and with little amplification and take place in conditions where the ligands for GPCRs are in abundance. Also, the voltage-dependent unbinding of Gβγ subunits operates like a high-pass or low-cut filter. Brief stimuli do not dislodge Gβγ subunit complexed to Ca_v2 channels, but stronger stimuli overcome the inhibition, with a relief of Gβγ subunit inhibition that is in proportion to the firing of action potential trains. Gβγ subunit inhibition thus serves as a critical form of short-term synaptic plasticity that causes a temporary enhancement of neurotransmitter release with the arrival of high-frequency action potential trains in the presynaptic terminal.

Voltage-independent inhibition lacks the dynamic aspects of the Gβγ inhibition but uniquely provides a persistent inhibition independent of cellular activity through activation of Gα subunits. The inhibition has a slower onset and recovery and requires amplification through cell signaling cascades. Many different intracellular pathways may converge into the cell signaling cascade, providing a highly modifiable response. Another advantage is a consistency in the inhibition even if the GPCR ligand is not in great

abundance. Both forms of G-protein inhibition thus provide qualitatively different responses. The combination of both pathways simultaneously utilizes both Gα and Gβγ of the heterotrimeric G-protein in the inhibition of calcium channels to maximize the fine-tuning of calcium influx in mammals.

Lack of voltage-dependent regulation of invertebrate Ca$_v$2 channels reflects a less modifiable invertebrate synapse

Lack of the voltage-dependent G-protein modulation in LCa$_v$2 suggests that unique determinants, such as in the N- and C-terminus in mammalian synaptic calcium channels, evolved as a vertebrate specialization for G-protein modulation. While it is not possible to discount other factors that could account for the lack of voltage-dependent modulation of LCa$_v$2, such as structural differences in the invertebrate Ca$_v$β subunits or the G-proteins themselves, as being incompatible with mammalian G-protein modulation, these are not likely to be of importance due to the high conservation of Ca$_v$β subunits and G-protein subunits in invertebrates. *Lymnaea* G-protein β1 (GenBank Accession # CAA80652) is 89% similar and 84% identical to mammalian G-protein β1 subunit, and all the critical determinants circumscribed for G-protein modulation (Tedford et al., 2006) are also conserved in *Lymnaea* G-protein β1. Each of the four Ca$_v$β subunit types (β1, β2, β3, β4) influences the degree of G-protein modulation, but only a minimal and highly conserved core GK domain invariant in invertebrate beta subunits is required for mammalian voltage-dependent G-protein modulation (Zhang et al., 2008).

Other modulatory structures, such as the synprint region, are also lacking in invertebrate Ca$_v$2 channels, suggestive of a primitive condition for invertebrate synaptic calcium channels (Spafford et al., 2003b). Synprint is a large 245 amino acid platform in the cytoplasmic II–III linker of Ca$_v$2 channels for binding of synaptic proteins such as syntaxin1, SNAP-25, cysteine string protein (CSP) and synaptotagmin (see Fig. 5B) (Spafford and Zamponi, 2003) and serves as a highly integrated center for modulation. For example, syntaxin1A binding to the synprint region promotes voltage-dependent G-protein inhibition of Ca$_v$2.2 channels (Jarvis et al., 2002), while PKC-dependent phosphorylation of the channel antagonizes the G-protein inhibition *via* βγ subunit dimers (Viard et al., 2004). Mammalian sequences have likely been adapted to embed Ca$_v$2 channels into the synaptic vesicle fusion apparatus and couple with GPCRs. These form part of a presynaptic complex in mammals that contains a web of regulatory and scaffolding proteins in the active zone (Schoch and Gundelfinger, 2006). Invertebrates exhibit a synaptic organization lacking key structural proteins such as Bassoon and CAST present in mammalian synapses and bear a synaptic substructure, like the *Drosophila* T-bar, which is unlike the mammalian presynaptic density (Atwood, 2006). The lack of activity-dependent G-protein regulation is one of these features lacking in invertebrates. A slower inhibition using a cellular messenger like cAMP may meet the modulatory needs in invertebrates while an activity-dependent regulation, evolving in vertebrates, provides a more dynamic, fine-tuning of neurosecretion.

LIST OF SYMBOLS AND ABBREVIATIONS

8Br-cAMP	8-bromo cyclic AMP
CMV	cauliflower mosaic virus
EGFP	enhanced green fluorescent protein
G	conductance
GPCR	G-protein-coupled receptor
HEK-293T	human embryonic kidney cells 293T
HVA	high voltage-activated channel
I	current
LVA	low voltage-activated channel
PP	pre-pulse
$V_{command}$	conditioning potential
βark-ct	the C-terminus of the beta adrenergic receptor kinase

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