In Vitro Characterization of L-Type Calcium Channels and Their Contribution to Firing Behavior in Invertebrate Respiratory Neurons

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INTRODUCTION

Voltage-gated calcium channels are key mediators of a wide range of cellular responses, ranging from regulating neuronal firing patterns to the initiation of neurosecretory events.

The mammalian CNS expresses multiple types of voltage-gated calcium channels with distinct cellular and subcellular distributions and highly specific cellular functions (for review: Catterall 2000; Snutch et al. 2004). For example, T-type calcium channels are abundantly expressed in dendrites where they contribute to the initiation of burst firing (Perez-Reyes 2003). N- and P/Q-type calcium channels are localized to presynaptic nerve termini where their opening is intimately linked to the release of neurotransmitters (Spafford and Zamponi 2002). L-type calcium channels are typically expressed on cell bodies, where they are thought to initiate the activation of calcium-dependent enzymes and gene transcription (Dolmetsch et al. 2001; Weick et al. 2003). However, it remains unknown to what extent L-type calcium channels contribute directly to the electrical properties of the CNS. Understanding the exact role of L-type channels in this process has been hampered by the fact that the mammalian CNS expresses both Cav1.2 and Cav1.3 L-type calcium channel isoforms (Hell et al. 1993; Westenbroek et al. 1998). These two channel types exhibit distinct sensitivities to dihydropyridine (DHP) antagonists with complete inhibition of the Cav1.3 subtype only at high DHP concentrations at which specificity for L-type channels is no longer assured (Koschak et al. 2001; Xu and Lipscombe 2001). For example, DHPs block T-type calcium channels (Shecheglovitov et al. 2005), N-type currents, and sodium and potassium currents in the micromolar range (Triggle 2003), and nifedipine has been shown to act as a sequestragen in some type of neurons (Hirasawa and Pittman 2003). Knockout mouse studies have provided only a marginal advantage, as knockout of Cav1.2 is embryonic lethal (Seisenberger et al. 2000) and that of Cav1.3 does not display neurological deficits outside of hearing loss, suggesting that compensation from other calcium channel subtypes is likely to occur such that these mice have a relatively normal phenotype (Clark et al. 2003).

In contrast with the vertebrate nervous system, invertebrates express a singleton homologue representing the three major calcium channel families (Spafford et al. 2003b). As a result, invertebrate model systems such as Drosophila (Kawasaki et al. 2002), Caenorhabditis elegans (Jospin et al. 2002), or Aplysia (White and Kaczmarek 1997) have been successfully used to address fundamental aspects of calcium channel physiology. One such model organism, the pulmonate freshwater pond snail Lymnaea stagnalis, provides the added advantage of a readily identifiable and well-characterized neuronal network that controls respiratory function. We have recently reported the cloning, expression, and characterization of the Lymnaea Cav2 calcium channel gene that is responsible for neurotransmitter release (Spafford et al. 2003a,b). Here, we report the cloning, functional and physiological characterization of LCav1, a homologue to vertebrate L-type calcium channels. We show that LCav1 encodes a channel with typical L-type calcium channel properties such as slow activation and DHP sensitivity. Using information obtained from pharmacological studies in the tsA-201 cell expression system, we show that LCav1 is essential for maintaining the efficacy of repolarization during a long spike train. The channel thereby contributes to the synchronization of neuronal firing patterns in the respiratory network that underlies the rhythmic breathing in L. stagnalis.

METHODS

Preparation of full-length LCav1a cDNA for in vitro expression

Full-length LCav1a was created by PCR on cDNA reverse transcribed from RNA isolated from the CNS of L. stagnalis, using
prooftreading Turbo Pfu (Stratagene) polymerase and primers flanking the start and stop codons of the putative open reading frame. Molecular identification and cloning of LCav1a,b,c variants were described previously in (Stafford et al. 2003b) and deposited (as GenBank Accession Nos. AF484079-81). Two putative, in frame start sites were present in the amino terminus of the full-length mRNA for LCav1, creating variants of 2,078 amino acids (~237 kDA) or 2,190 amino acids (~247 kDA). The longer variant ~247 amino acid was cloned, using primer incorporated 5’ and 3’ restriction sites, into the polycliner of bicystronic vector pRRES2-EGFP (BD Biosciences).

Sequence comparisons and phylogenetic analysis

LCav1a was aligned with invertebrate orthologs and human Cav1 channels by modified progressive pairwise, multiple alignment in PILEUP (UNIX-based, GCG Wisconsin Package 2002; Accelrys, Madison, WI) and visually displayed in PLOTSIMILARITY (Accelrys). Gene tree was generated from PILEUP alignment of Lymnaea and human Cav channels imported into PAUP 4.0. Consensus gene tree generated from the Branch-and-Bound algorithm was tested for robustness in 100 bootstraps and displayed in TREEVIEW 1.6.6 (Rod Page, Glasgow).

Transient transfection of mammalian cells

LCav1a (6 µg) in a pRRES2 bi-cystronic EGFP construct or LCav2a and EGFP (BD Biosciences) was transfected in tsA-201 human embryonic kidney cells together with 6 µg accessory rat α2-δ1 and rat β1b subunits using a standard calcium phosphate protocol (Stafford et al. 2003a). For this study, a mammalian α2-δ subunit was used in lieu of an comparable, as yet unidentified, Lymnaea homologue. We have also coexpressed a rat β1b subunit with LCav1a, which is consistent with the approach taken in a previous characterization of LCav2a in mammalian cells (Stafford et al. 2003a). Cells for transient transfection were plated on glass coverslips at 10% confluence and maintained in a humidified environment of 5% CO2 in standard DMEM supplemented with 10% FBS and 50 U/ml penicillin-streptomycin. Twelve hours after transfection, cells were washed in fresh media and allowed to recover at 37°C for another 12 h. Cells were then incubated at 28°C for 3–6 days before electrophysiologically recording.

Whole cell recording of mammalian cells and Lymnaea VD4 neurons in vitro

Calcium channel activities of Lymnaea neurons and transfected mammalian tsA-201 cells were measured using whole cell (membrane ruptured) recording technique. In brief, transfected tsA-201 cells were bathed and recorded with barium as the charge carrier (20 mM BaCl2) or calcium (20 mM CaCl2) in extracellular solution containing (in mM) 40 tetraethylammonium chloride (TEA-Cl), 1 MgCl2, 10 HEPES, 10 glucose, and 65 CsCl, pH 7.2 (adjusted with TEA-OH). For transfected cells, patch pipettes (3–4 MΩ) were filled with intracellular solution containing (in mM) 108 cesium methane sulfonate, 4 MgCl2, 10 EGTA, and 9 HEPES, pH 7.2 (adjusted with CsOH). The average current size for LCav1a was ~156.1 ± 28 pA (n = 17), and 285.5 ± 39 pA (n = 6; means ± SE) for LCav2a. For analysis of calcium-dependent inactivation in low intracellular EGTA, intracellular solutions contained (in mM) 118 cesium methane sulfonate, 4 MgCl2, 0.1 EGTA, and 9 HEPES, pH 7.4 (adjusted with CsOH). VD4 neurons were recorded in BaCl2 or CaCl2 (2 mM) plus (in mM) 47.5 TEA-Cl, 1 MgCl2, 10 HEPES, and 2 4-aminoypyridine (4-AP), pH 7.9 (adjusted with TEA-OH). VD4 neurons were recorded with larger bore pipettes (1.5–2 MΩ) and calcium plus ATP/GTP-containing solution (in mM): 2 CaCl2, 2.3 CaCl2, 10 ethylene glycol-β-aminoethyl ether)N,N,N’,N”-tetraacetic acid (EGTA), 10 HEPES, 2 ATP-Mg, and 0.1 GTP-Tris, with pH 7.4 (adjusted with CsOH).

Whole cell recordings were made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). The voltage command generation and data acquisition were carried out using a PC computer equipped with a Digitida 1322A interface in conjunction with pClamp 9.1 software (Axon Instruments). Recorded currents were filtered at 1 kHz using a 4-pole Bessel filter and digitized at a sampling frequency of 2 kHz. Series resistance was compensated by 80%. Current-voltage relationships were obtained by holding cells at −80 mV before stepping to test potentials ranging from −50 to +60 mV. The voltage dependence of inactivation was measured at a test depolarization of +10 mV, after prepulse holding potentials ranging from −90 to +30 mV over 10 s. All solutions were purchased from Sigma-Aldrich, (St. Louis, MO). Nifedipine and BayK 8644 were used from 10 mM stock concentration dissolved in dimethylsulfoxide (DMSO). There were no visible changes associated with the highest DMSO concentration (0.1%) used in working solutions.

Data analysis was carried out using Clampfit (pClamp 9, Axon Instruments) and SigmaPlot 2000 (Jandel Scientific, SPSS science, Chicago, IL). A standard Boltzmann equation \( I = I_\text{rev} \frac{1}{1 + \exp(V - V_\text{rev})/G} \) was used to curve fit steady-state inactivation where \( I \) is the normalized peak current amplitude, \( V \) is the holding potential, \( V_0 \) is the half-inactivation potential, and \( S \) is a slope factor. Whole cell current voltage relations were fitted with the equation \( I = G(V - E_{rev})[1 + \exp(V - V)/G] \) where \( G \) is the maximum slope conductance, \( E_{rev} \) is the peak current amplitude, \( V \) is the test potential, \( E_{rev} \) is the reversal potential, \( V_0 \) is the half activation potential, and \( S \) is a slope factor inversely proportional the effective gating charge. Time constants for inactivation and recovery from inactivation were gathered from a monoeponential fit of the raw data. Data statistics were illustrated in figures as means ± SE, with numbers of trials in parentheses. Significant differences between mean values were tested using paired and unpaired Student’s t-test and considered significant if \( P < 0.05 \). Data analysis was carried out using Clampfit (pClamp 9, Axon Instruments) and SigmaPlot 2000 (Jandel Scientific).

Microelectrode recording of Lymnaea identified neurons and cultured synapses

Lymnaea neurons for recording were prepared from ~2- to 3-mo old snails raised in laboratory conditions at room temperature, using previously described methods (Syed et al. 1990, 1999). Isolated brains, washed three times in normal saline with gentamicin, 50 µg/ml, were exposed to trypsin (2 mg/ml) followed by trypsin inhibitor (2 mg/ml) or protease (2 mg/ml). Outer and inner sheaths surrounding ganglia were mechanically removed by fine forceps in high osmolarity defined media (20 mM n-glucose). Rhythmic pattern activity from the intact ganglia was recorded as described previously (Syed et al. 1990, 1999). For neuronal culture, cells were isolated and extracted from Lymnaea brains by applying gentle suction via a syringe attached to fire-polished glass pipettes (50–90 µm tip diameter). Singlet neurons and synaptic pairs formed between somata of identified Lymnaea VD4 and LpED1 neurons were plated on poly-L-lysine-precoated coverslips and recorded 18–24 h after incubation in brain conditioned medium. For sharp electrode recordings, electrical signals were amplified (NeuroData Instrument) and recorded in standard Lymnaea saline (51.3 NaCl, 1.7 KCl, 4.0 CaCl2, and 1.5 MgCl2, buffered in HEPES to pH 7.9) with glass microelectrodes filled with a saturated solution of potassium sulfate (K2SO4; electrode resistance, 20–40 MΩ). CaCl2 (10 mM) replaced external calcium and magnesium ions in experiments with 2.5 times normal calcium saline. Resting membrane potentials varied considerably with an average of ~75.5 ± 19 mV. Spike threshold was relatively constant at ~40 to ~45 mV, requiring an average of 24 pA to reach threshold. Bursting in VD4 was evoked by injecting 40-pA current above the threshold level (i.e., 64 pA) required to generate single spikes. Drugs were applied by bath exchange (2 ml/min; sharp electrode recordings) or local perfusion by pipette driven by gravity.
flow (whole cell recording). All drug effects were considered if there was substantial (>80%) recovery after washout.

RESULTS

LCav1 encodes an ancestral L-type calcium channel gene

We isolated a full-length cDNA encoding the LCa1a calcium channel gene via PCR from cDNA generated from brain enriched tissue of L. stagnalis. The isolated gene is a homologue to members of the mammalian Cav1 calcium channel family (Fig. 1A). Only a singleton Ca1,1 homologue has been identified in C. elegans and Drosophila and a similar feature is likely in Lymnaea but cannot be demonstrated in the absence of detailed genomic analysis. A comparison with mammalian L-type calcium channel sequences indicates a high degree of conservation in the four transmembrane domains, in the calcium channel β subunit interaction site (AID), as well as in the EF hand and IQ motif regions that are thought to be critical for calcium dependent inactivation (CDI) (Catterall 2000; Snutch et al. 2004) (Fig. 1, B and C). The LCav1 gene includes a potential alternate splice site in the C-terminal region (Fig. 1C) as well as two putative start sites within the amino terminal region. A full-length cDNA construct encoding the splice variant bearing the longer amino and carboxyl-termini (LCav1a) was assembled and used for functional expression studies.

Functional expression of LCav1a and LCav2a in tsA-201 cells yields whole cell currents (Fig. 2A) in barium that activate at −20 mV and peak near +20 mV (Fig. 2B) and are remarkably similar to what has been described for native barium currents in Lymnaea neurons (Byerly et al. 1985). While its voltage dependence of activation is similar to that observed with the synaptic Lymnaea Cav2 (LCav2a) channel, LCav1a inactivates and activates with significantly slower kinetics (Fig. 2, C and D), it shows a 20-mV more-depolarized half-inactivation potential (Fig. 2E), and it is significantly slower to recover from inactivation (Fig. 2F). These differences in gating kinetics between LCav1a and LCav2a channels are reminiscent of what is observed with mammalian calcium channel subtypes (Yasuda et al. 2004) and suggest that these two calcium channel subtypes are likely to contribute differentially to electrical activity of Lymnaea neurons (see following text).

We then examined calcium-dependent inactivation (CDI) properties of LCav1 (Fig. 3). LCav1a displayed a significant degree of CDI observed in standard buffer containing 10 mM EGTA similar to that observed with classical mammalian L-type calcium channels (Fig. 3B). Correspondingly, LCav1a has a high sequence similarity across the region contributing to CDI (see sequence alignment in Fig. 3A). In contrast LCav2 (Spafford et al. 2003a) and known mammalian non-L-type channels do not bear significant CDI in standard 10 mM EGTA buffer (Liang et al. 2003). Figure 3C examines CDI of whole cell currents recorded from Lymnaea VD4 (visceral dorsal 4) neurons, a cholinergic neuron that forms part of a respiratory pattern generator in the Lymnaea brain (Syed et al. 1996, 1999). Consistent with the data obtained in tsA-201 cells, only the nifedipine-sensitive component of VD4 neurons had observable CDI (Fig. 3C). In tsA 201-cells, replacement of barium with calcium reduced peak current amplitude by ~50%, consistent with what is observed with mammalian L-type channels (Fig. 3D).

We then examined the pharmacological properties of transiently expressed LCav1a channels (Fig. 4). LCav1a was sensitive to nifedipine with half-maximal block occurring at 450 nM when cells were held at −80 mV (Fig. 4B). The nifedipine sensitivity of LCav1a is in the same range as that reported for rat L-type calcium channels. Nifedipine block of LCav1a was state dependent as reflected by a −15-mV shift in half-inactivation potential in the presence of 100 nM nifedipine (Fig. 4B, inset). At a concentration of 10 μM, nifedipine completely blocked LCav1a channels, while leaving LCav2a activity un-
affected (Fig. 4C). Similarly, the DHP agonist BAYK 8644 selectively enhanced LCa1a currents. In contrast, cadmium similarly inhibited both channel types almost completely at 30 and 100 μM with LCa2 channels being slightly more sensitive to 10 μM at lower concentrations compared with LCa1a. There are two important implications of these data. First, the current enhancement by agonist BAYK 8644 is somewhat unexpected considering that the LCa1a sequence contains asparagine and isoleucine residues in positions 981 and 1394, respectively (Fig. 4A). In the corresponding positions, mammalian Cav1.2 and Cav1.3 channels contain glutamine and methionine (Fig. 4A) in these positions that have been shown to be essential for DHP agonist activity (Schuster et al. 1996; Wappl et al. 2001; Mitterdorfer et al. 1996). More importantly, our findings obtained with transiently expressed channels allow us to use nifedipine as an effective tool to isolate LCa1 from LCa2 channels in neurons.

**LCAv contributes only a small fraction of total barium current in VD4 neurons**

To ascertain the function of LCa1 calcium channels in Lymnaea neurons, we recorded whole cell calcium currents from VD4 neurons in the presence and absence of 10 μM nifedipine. As shown in Fig. 5A, nifedipine reduced peak current amplitude of VD4 whole cell barium currents by ~20%, unmasking a current with rapid inactivation kinetics that is presumably carried by LCa2. The nifedipine-sensitive current component obtained by subtraction of the current traces recorded before and after nifedipine application exhibits a slowly inactivating waveform, consis-
tent with our observations with transiently expressed $LCav1a$ (Fig. 2C). Interestingly, previous RNAi knockdown of $LCav2$ in *Lymnaea* VD4 neurons unveiled a much faster inactivating current (Spafford et al. 2003b). This kinetic difference could have resulted from the compensatory turning on or off of genes during the 4-day exposure to RNAi, such as overexpression of a different splice variant of $LCav1$, upregulation of a faster T-Type channel ($LCav3$), or potentially regulation of genes that might alter $LCav1$ kinetics.

In 17 VD4 neurons examined, nifedipine reduced peak current amplitude by 15.77 ± 1.8%, whereas cadmium virtually eliminated all calcium current activity (Fig. 5B). Hence, almost all the calcium current is carried by high-voltage-activated ($LCav1 + LCav2$) channels, whereas T-type current that is expected to be cadmium insensitive is negligible. Figure 5C illustrates in form of averaged data that the nifedipine-sensitive current in VD4 neurons. D: effect of replacing permeant ion species on peak current amplitude with transiently expressed $LCav1a$ channels. Data in barium and calcium were obtained for the same cell in each case, and current amplitudes were normalized to that observed in barium.

Fig. 3. Effects of permeant ion species on channel properties. A: sequence alignment of the highly conserved, C-terminal region delimited by the C-terminal EF hand region and IQ motif, encompassing the calmodulin binding site of human L-type homologue (Cav1,1.2) and the region corresponding to $LCav1a$. B: time constants of inactivation obtained for transiently expressed $LCav1a$ channels bathed in recording solution containing either 20 mM barium or 20 mM calcium and with standard, 10 mM EGTA containing, internal recording solution in the patch pipette. The concentration of permeant ion was 2 mM in each case. Note that calcium-dependent inactivation is readily apparent for both the $LCav1a$ channel and the nifedipine-sensitive current in VD4 neurons. D: effect of replacing permeant ion species on peak current amplitude with transiently expressed $LCav1a$ channels. Data in barium and calcium were obtained for the same cell in each case, and current amplitudes were normalized to that observed in barium.

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previously described. Spafford et al. 2003b). This treatment significantly reduced, but did not completely eliminate the nifedipine-sensitive current (\(n/H11005\)) compared with mismatch controls (not shown). Although this is consistent with \(\text{LCav1}\) corresponding to the nifedipine-sensitive current component, the incomplete action of this oligonucleotides prevented their further use in the ensuing physiological experiments.

**LCav1 channels are required for high-frequency burst firing**

To ascertain the contribution of \(\text{LCav1}\) channels to synaptic activity, we isolated VD4 and left pedal dorsal 1 (LPeD1) neurons, paired them to allow the formation of soma-soma synapses, and then carried out dual microelectrode recordings in the absence and the presence of nifedipine. As we described in detail previously, these synapses are functionally equivalent to neurite-neurite synapses and are thus a convenient model to examine aspects of synaptic transmission (Spafford et al. 2003b; Syed et al. 1999). As shown in Fig. 6A, current injections into the presynaptic VD4 neuron evoke, depending on the amount of current injected, either single spikes or a rapid burst of action potentials that terminates on spontaneous repolarization. The postsynaptic neuron responds with single postsynaptic potentials to each single spike and with compound excitatory postsynaptic potentials (EPSPs) in response to a presynaptic burst discharge. Block of \(\text{LCav1}\) channels with nifedipine does not affect the generation of single spikes but often results in spike broadening and a loss of afterhyperpolarization (see Fig. 6B, inset), suggesting that block of \(\text{LCav1}\)
channels prevents adequate repolarization during a burst. More strikingly, nifedipine prevents high-frequency burst activity in the presynaptic neuron, thus leading to loss of synchronous release (Fig. 6A). Normal bursting activity of the presynaptic VD4 neuron is restored on wash. In the presence of nifedipine, the repolarization amplitude within the burst spike becomes progressively reduced with increasing spike number (Fig. 6B). In addition, the interspike interval is decreased (Fig. 6C), and spike broadening is observed early with the burst (Fig. 6D). It is important to note that partial block of LCav1 channels with \( \cong 3 \) \( \mu \text{M} \) nifedipine did not produce a consistent effect on bursting behavior, suggesting that complete or nearly complete block of LCav1 was necessary for the manifestation of the physiological effects. Collectively, our data indicate that the ability of the presynaptic VD4 neuron to undergo high-frequency burst firing is critically dependent on the presence of L-type calcium channels. In contrast, LCav1 does not appear to be required for synaptic transmission per se (Fig. 6E).

One possible mechanism that could account for the LCav1-mediated effect on bursting activity is a reduction in the activity of calcium-activated potassium [K(Ca)] channels due to reduced calcium entry via LCav1. If so, then application of TEA, a known blocker of K(Ca) channels (Mathie et al. 1998) should mimic the effect of nifedipine on burst firing behavior, suggesting that complete or nearly complete block of LCav1 was necessary for the manifestation of the physiological effects. Collectively, our data indicate that the ability of the presynaptic VD4 neuron to undergo high-frequency burst firing is critically dependent on the presence of L-type calcium channels. In contrast, LCav1 does not appear to be required for synaptic transmission per se (Fig. 6E).

Effects of nifedipine were indeed attributable to its L-type calcium channel blocking activity rather than perhaps a non-specific action of nifedipine on K(Ca). Indeed, we also could not detect any effect of 10 \( \mu \text{M} \) nifedipine on whole cell potassium conductance in VD4 neurons (not shown), again suggesting that nifedipine did not act directly on K(Ca) channels. If calcium entry and subsequent activation of K(Ca) channels is indeed essential for maintaining burst activity, then it should be possible to at least partially overcome the effects of nifedipine by boosting calcium entry through LCav2 channels. To test this hypothesis, we blocked LCav1 channels via nifedipine and then raised the extracellular calcium concentration from 4 to 10 \( \text{mM} \), which is expected to raise peak calcium influx by 10–15\% in Lymnaea neurons (Byerly et al. 1985). As shown in Fig. 7, C and D, in the presence of elevated external calcium, the nifedipine induced inhibition of burst firing activity became slightly, albeit significantly attenuated. Although the additional calcium influx mediated by LCav2 could not anywhere completely compensate for the loss of calcium entry via LCav1, these data are consistent with the need for a global rise in calcium and subsequent activation of K(Ca) to sustain burst activity.

**LCav1 channels synchronize neuronal activity in a respiratory neuronal network**

VD4 neurons from part of a neuronal network that controls breathing behavior of *L. stagnalis* (Syed et al. 1990, 1999). The functional connectivity of the individual neurons that form this pattern generator has been well characterized and involves a critical interplay among VD4, RPcD1, IP3I neurons (see Fig. 8A). The synaptic connections between conditionally bursting neurons IP3I (expiration) and VD4 (inspiration) are mutually
inhibitory and comprise the “half center” of the central pattern generator (CPG). The activities of these two neurons are in turn regulated by RPeD1, which makes biphasic (excitation followed by inhibition) synaptic connections with IP3I, whereas its connectivity with VD4 is mutually inhibitory. Considering the key role of LCav1 channels in high-frequency bursting behavior of VD4 neurons, one might predict that coordination of firing behavior among these neurons should be drastically altered in the presence of nifedipine. To examine this possibility, we carried out simultaneous microelectrode recordings from VD4 and RPeD1 neurons in an intact Lymnaea brain preparation in the presence and the absence of nifedipine. In this case, a higher concentration of nifedipine (30 μM) needed to be applied due to access restrictions. Typically, the isolated ganglionic preparations exhibit “fictive” breathing patterns with well-characterized, alternating bursting activity in IP3I and VD4 neurons. Because IP3I is located ventrally (RPeD1 and VD4 are situated dorsally), indirect evidence for its activity is generally obtained through its excitatory effect on RPeD1 (Fig. 8A). In a semi intact animal, this alternating bursting in IP3I and VD4 controls pneumostome opening (expiration) and closing (inspiration). As illustrated in Fig. 8B (bottom), 30 μM nifedipine prevented spontaneously occurring respiratory rhythm in the CPG neurons by perhaps exclusively preventing high-frequency burst generation in VD4 (Fig. 8B). It is important to note that neither was RPeD1’s spontaneous activity blocked nor did VD4 cease to burst. However, the coordinated respiratory rhythm was completely perturbed (n = 8). These data indicate that L-type calcium channels contribute significantly to rhythmicity in the intact respiratory network of L. stagnalis.

Discussion

We have recently reported the cloning, expression, and functional characterization of LCav2, the synaptic calcium channel of L. stagnalis (Spafford et al. 2003a). Here, we report the cloning and functional characterization of a Lymnaea Cav1 channel homologue and applied this knowledge to address the fundamental role of L-type channels within the context of the function of a respiratory pattern generator. The ability to express and characterize, in isolation, the properties of Cav1 and Cav2 calcium channels is unique to the Lymnaea system and allowed us to identify nifedipine as an experimental tool to eliminate all L-type calcium channel activity in the Lymnaea nervous system. Our data show that elimination of L-type calcium channels by nifedipine inhibits repolarization in VD4 burst firing neurons, thus affecting synchronous electrical activity within the respiratory neuronal network. The physiological effects of nifedipine occurred acutely and were reversible on washout, indicating that they were direct on the calcium channels.
The biophysical characteristics of LCav1 calcium channels are consistent with those expected from an L-type channel in that the channel exhibits relatively slow gating kinetics, depolarized activation and inactivation range, DHP sensitivity, and CDI in internal recoding solutions buffered with 10 mM EGTA. It is important to note that DHP sensitivity occurred despite the fact that the Lymnaea sequences differ from mammalian channels in two key positions that have been linked to DHP agonist sensitivity in mammalian L-type channels (Mitterdorfer et al. 1996; Schuster et al. 1996; Wappl et al. 2001).

At this point, it is unclear how DHP binding is coordinated in LCav1, but it is possible that other amino acid residues mediate DHP binding. Ultimately, detailed analyses of the consequence of replacing rat Cav1.2 with corresponding LCav1 sequence may shed further light on this issue.

There appears to be a clear separation in the roles of LCav1 and LCav2 channels. LCav2 channels are essential for the release of neurotransmitter (Spafford et al. 2003b) but do not affect the ability of VD4 neurons to discharge action potential bursts (Spafford et al. 2003b). In contrast, as we show here, blockade of LCav1 does not contribute to synaptic transmission in agreement with previous studies in Aplysia (Edmonds et al. 1990) but instead appears to play an essential role in maintaining rapid spike discharges. As our data indicate, spike repolarization during a burst appears to require the concerted action of L-type calcium channels and K(Ca) channels. Functional signaling between BK K(Ca) and voltage-gated calcium channels has been described in the hippocampus (Sun et al. 2003; Tavalin et al. 2004), cerebellum (Womack et al. 2004), neocortex (Sun et al. 2003), and hair cells (Samaranayake et al. 2004). Moreover, biochemical interactions between BK and both Cav1.2 and Cav1.3 calcium channels have been reported, consistent with colocalization of calcium channels and K(Ca) channels (Grunnet and Kaufmann 2004; Liu et al. 2004). Finally, a close association of calcium channels and K(Ca) channels has been described in Helix snails where K(Ca) channel mediated repolarization of U cells does not occur when voltage-gated calcium channels are blocked by cadmium (Crest and Gola 1993).

L-type calcium channels have been linked to a number of important cellular functions, such as the triggering of calcium-dependent gene transcription (Dolmetsch et al. 2001; Weick et al. 2003), hormone secretion (Mears 2004), hearing transduction (Hudspeth 2005), and heart and smooth muscle contraction (Kamishima and Quayle 2003). Our data support a unique role of L-type calcium channels in sustaining burst firing activity in neurons within the respiratory CPG of Lymnaea. Hence, L-type calcium channels are likely to be a key factor in controlling the breathing behavior of L. stagnalis and may well play a similar role in other CPGs, including in the mammalian CNS. Indeed, there are reports that L-type calcium channel blockers block ictal activity in pilocarpine seizure models (Hadar et al. 2002) and in human epileptic tissue (Straub et al. 2000). In the spinal cords of P7 mice, nifedipine antagonizes rhythmic bursting (Jiang et al. 1999). In dopaminergic neurons, block of L-type calcium channels has been shown to inhibit apamin-induced bursting activity (Shepard and Stump 1999) and calcium-dependent spontaneous oscillations (Durante et al. 2004). The importance of K(Ca) channels in regulating bursting is supported by data obtained from BK null mice. These animals show severely reduced spontaneous tonic and bursting discharges from Purkinje cells and, as a consequence, cerebel-
lar dysfunction such as abnormal locomotion and motor coordination (Sausbier et al. 2004). Together with the data presented here, these considerations suggest that the interplay between L-type calcium channels and calcium-activated potassium conductances may directly contribute in a significant, and so far unrecognized fashion, to the electrical activities of neurons in both invertebrates and mammals.

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FIG. 8. Role of LCav1 channels in the synchronization of oscillations in a central pattern generator of the intact brain. A: diagram illustrating the organization of the 3-cell central pattern generator circuit responsible for opening (expiration) and closing (inspiration) of the respiratory orifice (pneumostome). B: alternating bursting activity in IP3I (evidenced as an excitatory drive to RPeD1) and VD4 control the activities of the expiratory and inspiratory motor neurons, respectively. The box presents expanded time scale. Nifedipine (30 μM) prevents synchronized oscillations of the central pattern generator by significantly reducing the intensity of high-frequency bursts in VD4 (see box), and as a consequence, the patterned rhythmical bursting is completely abolished. It is important to note that the tonic activity in RPeD1 remains unperturbed by nifedipine. The data shown are representative of 8 experiments.


