Graded Inhibitory Synaptic Transmission Between Leech Interneurons: Assessing the Roles of Two Kinetically Distinct Low-Threshold Ca Currents

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Submitted 17 October 2005; accepted in final form 29 March 2006

Ivanov, Andrei I. and Ronald L. Calabrese. Graded inhibitory synaptic transmission between leech interneurons: assessing the roles of two kinetically distinct low-threshold Ca currents. J Neurophysiol 96: 218–234, 2006. First published April 26, 2006; doi:10.1152/jn.01093.2005. In leeches, two pairs of reciprocally inhibitory heart interneurons that form the core oscillators of the pattern-generating network for heartbeat possess both high- and low-threshold (HVA and LVA) Ca channels. LVA Ca current has two kinetically distinct components (one rapidly activating/inactivating, \(I_{\text{CaF}}\), and another slowly activating/inactivating, \(I_{\text{CaS}}\)) that mediate graded transmission, generate plateau potentials driving burst formation, and modulate spike-mediated transmission between heart interneurons. Here we used different stimulating protocols and inorganic Ca channel blockers to separate the effects of \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\) on graded synaptic transmission and determine their interaction and relative efficacy. \(Ca^{2+}\) entry by \(I_{\text{CaF}}\) channels is more efficacious in mediating release than that by \(I_{\text{CaS}}\) channels. The rate of \(Ca^{2+}\) entry by LVA Ca channels appears to be as critical as the amount of delivered \(Ca^{2+}\) for synaptic transmission. LVA Ca currents and associated graded transmission were selectively blocked by 1 mM Ni\(^{2+}\), leaving spike-mediated transmission unaffected. Nevertheless, 1 mM Ni\(^{2+}\) affected homosynaptic enhancement of spike-mediated transmission that depends on background \(Ca^{2+}\) provided by LVA Ca channels. \(Ca^{2+}\) provided by both \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\) depletes a common pool of readily releasable synaptic vesicles. The balance between availability of vesicles and \(Ca^{2+}\) concentration and its time course determine the strength of inhibitory transmission between heart interneurons. We argue that \(Ca^{2+}\) from multichannel domains arising from \(I_{\text{CaF}}\) channels, clustered near but not directly associated with the release trigger, and \(Ca^{2+}\) radially diffusing from generally distributed \(I_{\text{CaS}}\) channels interact at common release sites to mediate graded transmission.

INTRODUCTION

Since low-voltage–activated (LVA) Ca channels were first discovered (Hagiwara et al. 1975), their properties, distribution, molecular structure, and functions of the channels have been exhaustively investigated. A wide variety of kinetically distinct LVA Ca channels has been reported in different neurons, and the cellular localization of the three identified members of the LVA Ca channel family varies, with different neurons possessing from one to all three identified LVA Ca channels (Huguenard 1996; Huguenard and Prince 1992; Lee et al. 1999b; Pan 2000, 2001; Perez-Reyes 2003; Talley et al. 1999; Tarasenko et al. 1997). Typically, LVA Ca channels are localized mainly to the dendritic tree and serve to support spiking or the pacemaker activity underlying bursting (Erickson et al. 1993; Fisher and Bourque 2001; Huguenard 1996; McCormick and Bal 1997; Yunker 2003a). In some systems, LVA Ca channels are involved in synaptic transmission (Calabrese 1998; Pan et al. 2001; Uhrenholt and Nedergaard 2005).

In leeches, two bilateral pairs of reciprocally inhibitory heart interneurons in segmental ganglia 3 (G3) and 4 (G4) form the core oscillators of the motor pattern-generating network for heartbeat. These neurons possess, in addition to HVA Ca currents, two types of LVA Ca currents, one rapidly activating/inactivating, \(I_{\text{CaF}}\), and another slowly activating/inactivating, \(I_{\text{CaS}}\). The LVA channels corresponding to these LVA currents appear to be widely distributed throughout the neuritic tree (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000; Lu et al. 1997; Olsen and Calabrese 1996). Oscillator heart interneurons are active in alternating bursts and inhibit one another by both graded and spike-mediated transmission. Although HVA Ca currents underlie spike-mediated transmission (Lu et al. 1997), LVA Ca currents serve to: 1) generate the plateau potential that drives bursts of action potential (Arbas and Calabrese 1987; Olsen and Calabrese 1996); 2) mediate graded transmission (Angstadt and Calabrese 1991); and 3) modulate spike-mediated transmission through homosynaptic enhancement (Ivanov and Calabrese 2003). Although the properties of \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\) in heart interneurons and their involvement in synaptic transmission have been extensively characterized in detailed voltage-clamp and Ca imaging studies (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000; Lu et al. 1997; Olsen and Calabrese 1996), the structural-functional organization of synaptic release sites and a precise definition of the role of the individual LVA Ca currents in graded transmission and the modulation of spike-mediated synaptic strength have not been thoroughly investigated.

Such an investigation requires developing usable agents and tools to separate the different LVA Ca channels and the HVA Ca channels, and their effects in heart interneurons. Even in vertebrates, despite the availability of a wide spectrum of selective activators and blockers of Ca channels, study of LVA channels has been challenging (Huguenard 1996; Yunker 2003b) and there is an almost complete lack of such agents for invertebrates (Jezierski et al. 2000; Kleinhaus and Angstadt 1995; Lu et al. 1997; Staras et al. 2002; Wicher and Penzlin 1997).

To address these issues, in this study, we monitored simultaneously pre- and postsynaptic Ca currents and, in some cases, changes of intracellular Ca fluorescence in leech heart interneurons of isolated G3 or G4. Using different stimulating paradigms and inorganic Ca channels blockers, we were able to
evaluate the effects of $I_{\text{CaL}}$ and $I_{\text{CaS}}$ on the amplitude and time course of inhibitory synaptic transmission. We found that 1 mM Ni$^{2+}$ selectively blocks LVA Ca currents and associated graded synaptic release, but not HVA Ca current and associated high-threshold/spike-mediated release. However, 1 mM Ni$^{2+}$ does affect homosynaptic enhancement of spike-mediated transmission similar to intracellular Ca$^{2+}$ chelators, corroborating that this plasticity is mediated by Ca$^{2+}$ entering by LVA Ca channels. We show that fast and slow low-threshold (LVA) Ca channels evoke transmitter release from the same release sites but have different degrees of efficacy in promoting release. We argue that Ca$^{2+}$ from multichannel domains arising from $I_{\text{CaL}}$ channels that are clustered near but not directly associated with the release trigger, and Ca$^{2+}$ radially diffusing from generally distributed $I_{\text{CaS}}$ channels interact at common release sites to mediate graded transmission.

**METHODS**

**Animals**

Adult leeches (H. medicinalis) were obtained from Leeches USA and Biopharm and maintained in artificial pond water (Leeches USA) at about 15°C.

**Preparation**

Leeches were anesthetized in cold saline, after which individual ganglia (midbody ganglion 3 or 4) were dissected and pinned in clear, Sylgard-coated open bath recording/imaging chamber (RC-26, Warner Instrument) with a working volume of 150 µl. The sheath on the ventral surface of the ganglion was removed with microscalpels. Ganglia were superfused continually with normal leech saline (Nicholls and Baylor 1968) containing (in mM) 115 NaCl, 4 KCl, 1.8 CaCl$_2$, 10 glucose, and 10 N$^2$-2-hydroxyethylpiperazine-N$^2$-2-ethanesulfonic (HEPES) acid buffer, adjusted to pH 7.4 with NaOH or HCl. The preparation was mounted ventral side up on the stage of an Olympus BX50WI fluorescent microscope with an Olympus 40×/0.80W water immersion objective.

**Electrophysiology**

Heart interneurons were penetrated with thin-walled (1 mm OD, 0.75 mm ID) borosilicate microelectrodes (A-M Systems), and identified by the posterolateral position of their somata on the ventral surface of the ganglion and by their characteristic pattern of rhythmic bursting. In all experiments, the recording microelectrode, inserted into a postsynaptic cell, was filled with 4 M K-acetate, 20 mM KCl (unbuffered, pH 8.4). For presynaptic current-clamp experiments, the recording microelectrode inserted into a presynaptic cell was filled with the same solution as the postsynaptic cell, and for presynaptic voltage-clamp experiments, the “presynaptic” microelectrode was filled with 1 M K-acetate, 1.5 M tetraethyl ammonium acetate (TEA-acetate), and 1.5 Cs-acetate (unbuffered, pH 7.9) to block outward currents. Microelectrodes were coated along their shanks with Sylgard 186 (Dow-Corning) and had resistances of 20–45 MΩ and time constants of 0.5–1.5 ms when capacity compensated.

Once the cells were penetrated with recording microelectrodes, for all experiments except those in Fig. 11 (see following text), the superfusate was immediately switched to Na$^{+}$-free/5 mM Ca$^{2+}$ saline containing (in mM): 110.0 N-methyl-d-glucamine (NMDG), 4.0 KCl, 5.0 CaCl$_2$, 10.0 glucose, 10.0 HEPES acid buffer, adjusted to pH 7.4 with KOH or HCl. In a few of these experiments, Ca$^{2+}$ in the saline was reduced to 2 mM with suitable osmotic adjustment of NMDG to 115.0 mM. In some cases, 150 µM Cd$^{2+}$, 1 mM Ni$^{2+}$, or both were added to the saline.

In all experiments, the activity of the postsynaptic cell was recorded in voltage-clamp mode, whereas the activity of the presynaptic cell was recorded either in current-clamp mode or in voltage-clamp mode. Voltage-clamp recordings were made with an Axoclamp-2A amplifier (Axon Instruments) in single-electrode voltage-clamp (SEVC) mode with a sampling rate of 5 kHz. Current-clamp recordings were made with an Axoclamp-2A amplifier used in discontinuous current-clamp (DCC) mode with a sampling rate of 5 kHz. In each case, the electrode potential was monitored on an oscilloscope to ensure that the potential settled between current injection cycles. All recordings were referenced to a chloridized silver wire used to ground the bath. All electrophysiological data were acquired, digitized, and stored on a Pentium IV (Intel) computer using pCLAMP 7.0/8.0 software with Digidata 1200 or 1320A interface from Axon Instruments.

All stimulus protocols were generated using the pCLAMP program CLAMPEX. The usual voltage-clamp protocol consisted of voltage pulses from a holding potential of −70 mV to various depolarizing voltages, or from different holding potential to a fixed depolarizing potential. Various approaches were used, from single voltage pulses/steps to combined pulses/steps. Software-controlled leak subtraction was implemented as previously described (Ivanov and Calabrese 2003). For the experiments of Fig. 11, the presynaptic current-clamp protocol used to study spike-mediated transmission was built to simulate normal burst activity as described previously (Ivanov and Calabrese 2003). These experiments were carried out in 5 mM Ca$^{2+}$, 20 mM Mg$^{2+}$ saline that contained (in mM) 80.5 NaCl, 4.0 KCl, 5.0 CaCl$_2$, 20 MgCl$_2$, 10.0 glucose, 10.0 HEPES acid buffer, adjusted to pH 7.4 with KOH or HCl. This elevated divalent ion solution effectively suppresses spontaneous spike activity in heart interneurons but does not appreciably affect their synaptic transmission (Nichols and Wallace 1978a). More details on all stimulus protocols used are provided in the RESULTS section.

In all experiments, the postsynaptic cell was in voltage clamp, typically held at −40 mV.

**Ca imaging**

In some experiments, we monitored changes in intracellular Ca$^{2+}$ with the fluorescent indicator Calcium Orange (Molecular Probes). In these experiments, one cell (presynaptic) was iontophoretically filled with Calcium Orange [see Ivanov and Calabrese (2000, 2003) for details of methods and indicator properties] and then repenetrated after 5–15 min with a recording microelectrode. Changes of Calcium Orange fluorescence were continuously monitored and recorded with an ICCD-350f CCD camera (Video Scope International), connected to the fluorescent microscope mentioned above, equipped with an Olympus U-MNG (exciter filter BP 530–550 nm, dichroic mirror DM 570 nm, barrier filter BA 590 nm) filter set, 10% neutral density filter, and Olympus 40×/0.80W water immersion objective and Axon Imaging Workbench 4.0 (AIW 4.0) software with a Digidata 2000 interface (Axon Instruments) on a Pentium III (Intel) computer. Intensifier gain and black (baseline) levels were adjusted to achieve minimal background fluorescence, convenient visualization of the filled neurons, and sufficient dynamic range for monitoring fluorescence changes.

Our setup permits the acquisition of full-frame images of 640×480 pixels size at a resolution of 0.379 µm$^2$ for 1 pixel (395×295 µm for full frame) with the Olympus 40×/0.80W water immersion objective. Changes of fluorescence were recorded from the approximate synaptic contact region of a heart interneuron (600–1,200 pixels, 235–470 µm$^2$), as described by Ivanov and Calabrese (2003). In all experiments, the maximal available acquisition rate (video rate, 30 Hz) was used, yielding a time resolution of 33 ms. Video signals were accumulated for 33 ms per image, without any kind of gating, using the DC mode of the camera.

To synchronize the acquisition of electrophysiological data and Ca fluorescence recording, the Digidata 2000 and Digidata 1200/1320A were connected using a DIO-3 cable interface (Axon Instruments) that
results are presented in Fig. 1C, whereas in Fig. 1B we show I_{CaF} and gIPSC_F, recorded during 100-ms depolarizing steps for comparison. The I_{CaS} obtained by this subtraction became maximal (peaked) at 277 (SD 11.21) ms (n = 6) and the corresponding maximal gIPSC_S at 405.2 (SD 14.89) ms (n = 6) during the 300-ms depolarizing step; these maximal values did not change with longer depolarizing steps. To verify the repeatability of this method for estimating fast and slow I_Ca and the corresponding gIPSCs, we averaged pre- and postsynaptic currents recorded in seven different preparations during 100- and 350-ms depolarizing steps and obtained I_{CaS} and gIPSC_S, as described above (Fig. 1D).

To evaluate further the postsynaptic effects of I_{CaF} and I_{CaS}, we integrated over time normalized I_{CaF} and I_{CaS}, and their corresponding gIPSCs evoked by depolarizing steps of different durations (Fig. 1E). The integrated postsynaptic response corresponding to I_{CaF} (gIPSC_F) evoked by 100-ms depolarizing steps was significantly greater than the integrated postsynaptic response corresponding to I_{CaS} (gIPSC_S), when evoked by depolarizing steps at 350 ms [25.116 (SD 3.576) vs. 15.908 (SD 6.510), P = 0.004231, Bonferroni test], whereas the corresponding integrated I_{CaF} and I_{CaS} were not different [6.535 (SD 1.286) vs. 6.763 (SD 1.868), P = 0.785442, Bonferroni test]. After this time, an increase in depolarization duration (≥850 ms) led to only a very small increase in integrated gIPSC, whereas the integrated I_{CaS} increased monotonically. Moreover, the time course of changes in the integrated I_{CaS} and the corresponding gIPSC_S in response to increasing depolarization were significantly different (P = 0.000001, repeated-measures ANOVA).

Thus the rapid initial transfer of smaller amount of charge by I_{CaF} (i.e., smaller Ca^{2+} entry) evoked a larger postsynaptic response than the delayed transfer of a larger amount of charge by I_{CaS} (i.e., larger Ca^{2+} entry). Such differences in effects of I_{CaF} and I_{CaS} on synaptic transmission could result from: 1) the initial I_{CaF}-evoked neurotransmitter release may severely deplete the readily releasable pool of synaptic vesicles that is common for Ca^{2+} entering by both LVA Ca currents, without sufficient replenishment by vesicles from reserve pools; 2) I_{CaF} and I_{CaS} release neurotransmitter at spatially segregated release sites with different release probabilities; 3) I_{CaF} channels are localized in closer effective proximity to the secretory trigger than I_{CaS} channels at common or segregated release sites; and/or 4) because of the cooperative binding of Ca^{2+} to the release trigger (Dodge and Rahamimoff 1967) (power-law dependency of release on intracellular Ca), postsynaptic responses evoked by I_{CaF} (rapid brief charge transfer) are much larger and faster than those evoked by I_{CaS} (slow prolonged charge transfer). Thus the amount and the rate of increase of intracellular Ca^{2+} are critical for neurotransmitter release.

**What determines the amplitude and time course of graded transmission under presynaptic voltage clamp: Ca^{2+} influx by LVA Ca channels or the availability of readily releasable synaptic vesicles?**

To begin to sort out the possibilities outlined above, we used presynaptic holding potential to set the inactivation levels of I_{CaS} and I_{CaF} and thus vary the amounts of Ca^{2+} they provide at a given test step potential. This procedure allowed us to begin to dissect how the availability of Ca^{2+} and/or of readily

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**Data analysis**

All stored data were analyzed on the same computer using pCLAMP program CLAMPFIT and Origin 7.5 (OriginLab) software. Calcium fluorescence data are presented mainly as changes in fluorescence (ΔF/F), but in some cases as fluorescence (F); in this latter case, the data are presented in units of absolute fluorescence on a scale from 0 to 255 fu (fluorescence units). Statistical analyses used one-way ANOVA, factorial ANOVA, repeated-measures ANOVA, and ANCOVA with post hoc comparisons made by Student’s t-test with Bonferroni correction for multiple comparisons (Bonferroni test), linear regression with 95% confidence interval, and linear correlation analysis, all performed with Statistica 6 and Origin 7.5 software. Results are presented/plotted as means ± SD.

**Results**

Dissection of the dynamics and synaptic effects of fast and slow low-threshold Ca currents in leech heart interneurons

To parse the relative contributions of fast and slow LVA Ca currents in leech heart interneurons to graded synaptic transmission and to determine whether they share common release sites, it was first necessary to develop tools to separate fast (I_{CaF}) and slow (I_{CaS}) LVA Ca currents. Subtraction techniques illustrated in Fig. 1 proved useful to effect this separation. Presynaptic heart interneurons were voltage clamped at −70 mV and postsynaptic heart interneurons at −40 mV, whereas depolarizing steps to −40 mV, progressively increasing in duration from 50 to 450 ms with 50-ms increment were applied presynaptically and pre- and postsynaptic currents were recorded (Fig. 1A). This type of experiment was performed in some preparations with maximal duration of depolarization =850 ms and 100-ms time increment. Although the 50-ms depolarization at −40 mV was insufficient to evoke the full time course of I_{CaF} (rapidly inactivating component of LVA Ca current) and thus evoke a fully developed gIPSC_F (fast component of graded inhibitory postsynaptic current), the 100-ms depolarization evoked a fully developed I_{CaF} with mean time-to-peak of 53.3 (SD 4.47) ms (n = 12), and gIPSC_F with mean time-to-peak of 98.2 (SD 9.63) ms (n = 12); these characteristics did not change with increase in the duration of depolarization. Depolarizing steps with duration over 100 ms led to the appearance of distinguishable I_{CaS} (slowly inactivating component of LVA Ca current) that manifested as a secondary peak in the Ca current recording and a corresponding gIPSC_S (slow component of graded inhibitory postsynaptic current) that manifested as a shoulder or plateau in the gIPSC (Fig. 1A). To minimize the effects of variability among individual traces that occurred during these experiments, we normalized pre- and postsynaptic currents (I_{Ca} and gIPSC) obtained in each individual trace by their own individual maximal (peak) values [I_{CaF} (P) and gIPSC_F (P)] that corresponded to peak I_{CaF} and IPSC_F elicited by 100-ms depolarization. To evaluate I_{CaS} and corresponding gIPSC_S, we subtracted normalized recordings of I_{CaF} and corresponding gIPSC_F obtained with a 100-ms presynaptic depolarization from normalized recordings obtained with depolarizing steps of longer duration. These subtracted

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permits one program to trigger the other. In our experiments, we used pCLAMP 7.0/8.0 protocols to trigger data acquisition by Axon Imaging Workbench, which, in turn, controlled the shutter for the imaging lamp.

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**Results**

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releasable synaptic vesicles limits the amplitude and time course both the fast and slow components of graded transmission (gIPSC\textsubscript{F} and gIPSC\textsubscript{S}). We held the postsynaptic cell at \(-35\) mV and applied depolarizing steps to \(-35\) mV (2 s) to the presynaptic cell from progressively more depolarized holding potentials (from \(-70\) to \(-45\) mV at 5-mV increments) (Fig. 2). With increasing depolarization of the presynaptic holding potential from \(-70\) to \(-45\) mV, the peak amplitude of \(I_{\text{CaF}}\) and the magnitude of \(g_{\text{IPSCF}}\), measured at 1 s \([I_{\text{CaF}}(P)]\) and at 2 s \([I_{\text{CaS}}(2\text{s})]\) as well as the corresponding gIPSCs that were evoked by the presynaptic depolarizing steps all progressively declined (Fig. 2A). Averaged data normalized by the values
obtained from presynaptic holding potential of −70 mV (∫ICaS and corresponding gIPSCs; n = 8 preparations) are shown in Fig. 2B.

Normalized ∫ICaS (1 s) and ∫ICaS (2 s) decreased steadily and to a similar extent with increasing presynaptic holding potential (no significant difference P = 0.368178; repeated-measures ANOVA). ∫ICaF (P) also decreased with increasing presynaptic holding potential but this was more gradual at the more negative holding potentials and was significantly different from the course of decrease in both ∫ICaS (1 s) and ∫ICaS (2 s).
The decrease in gIPSC values [gIPSCF (P)] and the decrease of gIPSCs values, measured at 1 s of depolarization [gIPSCS (1 s)] with increasing presynaptic holding potential were similar (no significant difference \( P = 0.113507 \), repeated-measures ANOVA); their amplitudes remained relatively constant at a holding potential of \(-60\) mV and below. The decrease in gIPSCS values measured at 2 s of depolarization [gIPSCS (2 s)] with holding potential was more steady and significantly differed from that of both the gIPSCF (P) and the gIPSCS (1 s) \( (P = 0.000076 \) and \( P = 0.0000001 \), respectively; repeated-measures ANOVA) (Fig. 2B2).

The decrease in graded transmission with increasing depolarization of presynaptic holding potential is not surprising: depolarization of the presynaptic holding potential leads to inactivation of LVA Ca currents (Angstadt and Calabrese 1991) and thus to a significant decrease in the corresponding postsynaptic responses. Figure 3, LVA Ca currents (Angstadt and Calabrese 1991) and thus to a significant decrease in the corresponding postsynaptic responses. Figure 3, LVA Ca currents (Angstadt and Calabrese 1991) and thus to a significant decrease in the corresponding postsynaptic responses. Figure 3, A–C illustrates how the changes in the components of LVA Ca current \([I_{CaF}(P), I_{Cas}(1 s), I_{Cas}(2 s)]\) and their corresponding postsynaptic responses \([gIPSCF(P), gIPSCS(1 s), \text{and } gIPSCS(2 s)]\) co- vary. Data were binned according to holding potential, which increases right to left. The gIPSCF (P) and gIPSCS (1 s) show very little change at the most negative presynaptic holding potentials \((-70, -65, -60\) mV; three right-handmost points), whereas LVA Ca currents progressively decrease (albeit \( I_{Cas} \) less) but are nevertheless at high values. Such a saturation in the postsynaptic responses mediated by \( I_{CaF}(P) \) and \( I_{Cas}(1 s) \) (Fig. 3, A and B) suggests that it is not \( \text{Ca}^{2+} \) influx, but rather the availability of readily releasable synaptic vesicles that limit amplitude of postsynaptic responses at the early stage of the test depolarization (to \( \geq 1 \) s of depolarization) at the lowest presynaptic holding potentials used. Saturation is not evident in the relation between \( I_{Cas} \) (2 s) and gIPSCS (2 s) (Fig. 3C), indicating that by 2 s graded transmission is no longer limited by the availability of readily releasable synaptic vesicles but by \( \text{Ca}^{2+} \) influx as \( I_{Cas} \) inactivated during the test step. At more depolarized holding potentials, resulting from inactivation of LVA Ca currents, Ca influx becomes limiting and gIPSCs and \( I_{Cs} \) decline in step (see Olsen and Calabrese 1996).

The time-to-peak of \( I_{CaF} \) and gIPSCF increased progressively with increasing presynaptic holding potential (Fig. 2C), but the increase in time-to-peak of gIPSCF was larger and steeper than the increase in time-to-peak of \( I_{CaF} \) \( (P = 0.0000001 \), repeated-measures ANOVA). The binned (according to holding potential) nonnormalized gIPSCF (P) correlated with binned \( I_{CaF}(P) \) time-to-peak (Fig. 3D) \( (R = 0.993775, \text{adjusted } R^2 = 0.984487, P < 0.000058) \). Such a strong correlation of the amplitudes of the postsynaptic responses (gIPSCF) with \( I_{CaF}(P) \) time-to-peak is consistent with the observations of Felmy et al. (2003) and Bollmann and Sakmann (2005), who pointed out that the amount and timing of neurotransmitter release depend on both amplitude and time course of the intracellular “Ca signal” at release sites (see also Lin and Faber 2002; Simmons 2002).

Do \( I_{Cas} \) and \( I_{CaF} \) evoke release from the same readily releasable pool of synaptic vesicles?

The analysis of the previous section suggests that \( I_{Cas} \) and \( I_{CaF} \) evoke release from the same readily releasable pool of synaptic vesicles.
synaptic vesicles. To test this hypothesis we used conditioning low-threshold presynaptic depolarizations of various durations to both deplete the readily releasable pool and inactivate LVA Ca currents and followed them by brief test pulses at various intervals to evoke \( I_{\text{CaF}} \) and the associated gIPSC. We then monitored the recovery of \( I_{\text{CaF}} \) and of the associated gIPSC after the various conditioning depolarizations to determine the extent to which the recovery of \( I_{\text{CaF}} \) from inactivation and the readily releasable pool from depletion could be dissociated. If \( I_{\text{CaF}} \) recovers more rapidly than the gIPSC, it would indicate that a common readily releasable pool was depleted by the conditioning and the test depolarization. We held the presynaptic cell at \(-70\) mV and applied conditioning and test depolarizing pulses to \(-40\) mV. The postsynaptic cell was held at \(-40\) mV.

In the first experiments, depolarizing test pulses, applied 200 ms after conditioning depolarization of either 4-s duration (Fig. 4A1) or 200-ms duration (Fig. 4A2), evoked strongly reduced \( I_{\text{CaF}} \) and corresponding gIPSC. In both cases, increase in time interval between conditioning depolarization and the test depolarization led to progressive recovery of \( I_{\text{CaF}} \) and corresponding gIPSC. \( I_{\text{CaF}}(P) \) and gIPSC (P) were back to their initial amplitudes in 8–12 s [Fig. 4, B1 and B2; \( I_{\text{CaF}}(P) \) and gIPSC (P) were normalized by values obtained during conditioning and the test depolarization]. We held the presynaptic cell at \(-70\) mV and applied conditioning and test depolarizing test pulse depolarizations to \(-40\) mV. The postsynaptic cell was held at \(-40\) mV. The amplitude of \( I_{\text{CaF}}(P) \) and corresponding gIPSC (P) were back to their initial amplitudes in 8–12 s [Fig. 4, B1 and B2; \( I_{\text{CaF}}(P) \) and gIPSC (P) were normalized by values obtained during conditioning and the test depolarization]. We then increased in time interval between conditioning depolarization and the test depolarization. Interval between conditioning depolarization and the test depolarization (200 ms after conditioning depolarization), was significantly different from the amplitude of \( I_{\text{CaF}}(P) \), evoked by the conditioning depolarization and the two other test depolarizations (\( P = 0.00096, P = 0.00883, P = 0.002218 \), respectively; Bonferroni test). A similar result was obtained for the corresponding gIPSC (P) (\( P = 0.001715, P = 0.01535, P = 0.005043 \), respectively; Bonferroni test). In the experiments with a conditioning depolarization of 200-ms duration, the decrease in normalized \( I_{\text{CaF}}(P) \) in response to the first test depolarization was smaller than the decrease in the corresponding normalized gIPSC (P). The amplitudes of both \( I_{\text{CaF}}(P) \) and gIPSC (P) in response to the test depolarizations were strongly dependent on the time interval between conditioning depolarization and the test depolarization (\( P = 0.000001 \), repeated-measures ANOVA), but the time courses of changes in \( I_{\text{CaF}}(P) \) and gIPSC (P) amplitudes were significantly different (\( P = 0.015628 \), repeated-measures ANOVA). The amplitude of \( I_{\text{CaF}}(P) \), evoked by the first test depolarization...
tion (200 ms after conditioning depolarization), was significa-
nantly smaller than the amplitudes of $I_{CaF}$ (P), evoked by conditioning depolarization and the last test depolarizations ($P = 0.0085$, and $P = 0.010393$, respectively; Bonferroni test). The corresponding gIPSC$_F$ (P) was significantly smaller than the gIPSC$_F$ (P) evoked by conditioning depolarization and from the three other test depolarizations ($P = 0.000007$, $P = 0.000694$, $P = 0.000048$, and $P = 0.000019$, respectively; Bonferroni test). The amplitude of this normalized gIPSC$_F$ (P) was significantly smaller than the corresponding normalized $I_{CaF}$ (P) ($P = 0.009843$; Bonferroni test).

The results show that recovery in the strength of graded synaptic transmission between interneurons after a prolonged conditioning depolarization (seconds) followed the recovery of $I_{CaF}$ (with its complete deactivation time not <8–12 s). After a brief conditioning depolarization (hundreds of milliseconds), however, because the recovery of the gIPSC$_F$ lagged the recovery of $I_{CaF}$, the replenishment of a readily releasable vesicle pool appears to limit the recovery of synaptic strength. This finding indicates that $I_{CaF}$ can severely deplete the pool of readily releasable synaptic vesicles available for release by subsequent $I_{CaF}$ (Fig. 1).

We next sought to determine whether $I_{CaS}$ could deplete the readily releasable pool of vesicles available to $I_{CaF}$. We thus modified the voltage-clamp protocols of Fig. 4 so that the depleting effects of these two components of LVA current could be compared. First we performed the experiments ($n = 5$) illustrated in Fig. 5A to more carefully determine the time course of $I_{CaF}$ and gIPSC$_F$ recovery after a brief conditioning depolarization that activated only $I_{CaF}$. In independent traces, we held the presynaptic cell at −70 mV and applied a conditioning depolarizing step of 100 ms to −40 mV. Test depolarizing pulses of 100 ms to −40 mV were applied after a progressively increasing (500-ms increments) time interval of 200 to 3,200 ms. Simultaneous recordings of $I_{CaF}$ and gIPSC$_F$, normalized by $I_{CaF}$ (P) and gIPSC$_F$ (P) recorded during the conditioning step and averaged over the five experiments, are presented in Fig. 5A1. Figure 5A2 presents plots of the test $I_{CaF}$ (P) and gIPSC$_F$ (P), extracted from the individual traces constituting the recordings presented in Fig. 5A1 versus time. The time courses of changes in amplitudes of both the test $I_{CaF}$ (P) and the corresponding gIPSC$_F$ (P) [Fig. 5A1 (middle column, Pre($I_{Ca}$), right column, Post(gIPSC)), and Fig. 5A2] were not significantly different ($P = 0.054333$, repeated-measures ANOVA) but there was a significant difference in the normalized amplitudes of $I_{CaF}$ (P) and gIPSC$_F$ (P) seen in response to the first test depolarization ($P = 0.014218$, Bonferroni test). The gIPSC$_F$ (P) evoked by the sixth test depolarization returns to the level of the gIPSC$_F$ (P) evoked by conditioning depolarization ($P = 1.0$, Bonferroni test). Until this time, the amplitudes of “test” gIPSC$_F$ (P) were significantly smaller than amplitudes of the “conditioning” gIPSC$_F$ (P) ($P$ varied from 0.0000001 to 0.00016, Bonferroni test). At the same time, only $I_{CaF}$ (P) evoked by the first test depolarization was significantly decreased ($P = 0.0000001$, Bonferroni test) compared with the “conditioning” $I_{CaF}$ (P), and after that there were no significant differences ($P$ varied from 0.100913 to 1.0, Bonferroni test). These results corroborate the findings of Fig. 4, indicating that there is significant depletion of the readily releasable pool available for released by $I_{CaF}$ shortly after strong release evoked by $I_{CaF}$.

Next we performed the experiments ($n = 7$) illustrated in Fig. 5B in which we held the presynaptic cell at −70 mV and applied a conditioning depolarizing step to −40 mV of progressively increasing duration, from 100 to 3,100 ms (500-ms increments) and then 200 ms later applied a 100-ms test depolarizing pulse to −40 mV. In this protocol the conditioning step first evokes only $I_{CaF}$ but progressively evokes $I_{CaS}$ for longer intervals, whereas the test pulse evokes only $I_{CaF}$. Simultaneous recordings of LVA $I_{Ca}$ and gIPSC$_F$, normalized in independent traces by $I_{CaF}$ (P) and gIPSC$_F$ (P) recorded during conditioning depolarizing step, and averaged over seven experiments, are presented in Fig. 5B1. Figure 5B2 presents plots of the test $I_{CaF}$ (P) and gIPSC$_F$ (P), extracted from the individual traces constituting the recordings presented in Fig. 5B1, versus time. Although the test $I_{CaF}$ (P) decreases steadily with the increase in duration of conditioning depolarizing step [Fig. 5B1, middle column, Pre($I_{Ca}$), and Fig. 5B2], the gIPSC$_F$ (P) after an initial decrease that bottoms out with the 1,100-ms conditioning pulse [Fig. 5B1, right column, Post(gIPSC), and Fig. 5B2]. The normalized amplitudes of $I_{CaF}$ (P), evoked by test depolarizations, and the corresponding gIPSC$_F$ (P) were significantly different ($P = 0.0000001$; repeated-measures ANOVA) with different time courses ($P = 0.0000001$; repeated-measures ANOVA). Most important, the decrease in the normalized amplitudes of each (except the very last one) $I_{CaF}$ (P) elicited by test depolarizations were significantly smaller than the decrease in the normalized amplitudes of the corresponding gIPSC$_F$ (P) values ($P$ ranged from 0.0000001 to 0.0025671; Bonferroni test). We interpret these differences in normalized amplitude and time course between the test $I_{CaF}$ and gIPSC$_F$ as indicating that release evoked by $I_{CaS}$ depletes the readily releasable pool of vesicles available to be released by $I_{CaF}$, especially for conditioning steps ≤1,100. Nevertheless, as the conditioning pulse exceeds beyond 1,000 ms and both $I_{CaS}$ (inactivation) and gIPSC$_S$ (inactivation of $I_{CaS}$ and pool vesicle depletion) wane, $I_{CaF}$-evoked release begins to recover as a result of recovery of the readily releasable vesicle pool, presumably through increased vesicle recycling or mobilization (Schneggenburger et al. 2002; Thomson 2000, 2003).

This interpretation is supported by a comparison of the time course of normalized amplitudes of the test gIPSC$_F$ (P) for the two voltage-clamp protocols in Fig. 5, A and B. As illustrated in Fig. 5C, the time course of recovery of normalized amplitudes of the gIPSC$_F$ (P) from Fig. 5, A1 and B1 are significantly different ($P = 0.0000001$, repeated-measures ANOVA), with significant differences in amplitudes of gIPSC$_F$ (P) evoked by second and all subsequent test depolarizations ($P$ varied from 0.0000001 to 0.001104, Bonferroni test) (Fig. 5C). Sustained $I_{CaS}$-related synaptic transmission (Fig. 5B) led to much deeper depletion of the readily releasable pool of synaptic vesicles available for release by $I_{CaF}$ than intensive but brief $I_{CaF}$-related synaptic transmission (Fig. 5A), thus indicating that $I_{CaF}$ and $I_{CaS}$ do release vesicles from common release sites.

**Do multichannel Ca domains cooperate in fast graded transmission between heart interneurons?**

In the experiments illustrated in Fig. 6 ($n = 3$), we tried to determine the type of Ca domain (Augustine 2001) that controls the synaptic transmission evoked by $I_{CaF}$ and to evaluate potential cooperativity between $I_{CaF}$ channels. Brief depolar-
FIG. 5. Influence of the duration of the presynaptic conditioning depolarization and of interval between conditioning and test depolarization on time course of recovery of \( I_{\text{CaF}} \) and glIPSC\(_F\) evoked by a brief test pulse depolarization. A: in independent traces, brief test depolarizing steps were applied at progressively increasing time intervals after 100-ms conditioning depolarizing step to \(-40\) mV (1st interval duration 200 ms, increment 500 ms). 1: simultaneous recordings of \( I_{\text{CaF}} \) and glIPSC\(_F\). In each trace, the presynaptic \( I_{\text{CaF}} \) and the corresponding glIPSC\(_F\) were normalized by \( I_{\text{CaF}} \) (\( P \)) and glIPSC\(_F\) (\( P \)), recorded during conditioning depolarization. Recordings were averaged over 5 experiments (different preparations). 2: plots of normalized \( I_{\text{CaF}} \) (\( P \)) and glIPSC\(_F\) (\( P \)) vs. time, extracted from recordings presented in 1. Filled black circles: normalized \( I_{\text{CaF}} \) (\( P \)); empty gray diamonds: normalized glIPSC\(_F\) (\( P \)). Asterisks denote normalized glIPSC\(_F\) (\( P \)) significantly different from the corresponding normalized \( I_{\text{CaF}} \) (\( P \)). B: in independent traces, conditioning depolarizing steps to \(-40\) mV of progressively increasing duration (1st step duration 100 ms, increment 500 ms) were applied. By 200 ms after conditioning depolarization, 100-ms test depolarizing steps were applied. 1: simultaneous recordings of \( I_{\text{CaF}} \) and glIPSC\(_F\). In each trace, the presynaptic \( I_{\text{CaF}} \) and the corresponding glIPSC\(_F\) were normalized by \( I_{\text{CaF}} \) (\( P \)) and glIPSC\(_F\) (\( P \)), recorded during conditioning depolarization. Recordings were averaged over 7 experiments (different preparations). 2: plots of normalized \( I_{\text{CaF}} \) (\( P \)) and glIPSC\(_F\) (\( P \)) vs. time, extracted from recordings presented in 1. Filled black circles: normalized \( I_{\text{CaF}} \) (\( P \)); empty gray diamonds: normalized glIPSC\(_F\) (\( P \)). Asterisks denote normalized glIPSC\(_F\) (\( P \)) significantly different from the corresponding normalized \( I_{\text{CaF}} \) (\( P \)). C: plots of glIPSC\(_F\) (\( P \)) from A2 and B2 superimposed. Filled gray diamonds: glIPSC\(_F\) (\( P \)) from A2; filled black diamonds: glIPSC\(_F\) (\( P \)) from A2. Asterisks denote glIPSC\(_F\) (\( P \)) from A2 significantly different from corresponding glIPSC\(_F\) (\( P \)) from B2.
ized steps from a holding potential of −70 to −40 mV (5 to 100 ms) applied presynaptically evoked a progressively increasing \(I_{\text{CaF}}\) and a corresponding gIPSC\(_F\) (Fig. 6, A and B). Both amplitudes of the gIPSC\(_F\) (P) and \(I_{\text{CaF}}\) (P) (Fig. 6A) significantly increased with increasing pulse duration (\(P = 0.002881\), factorial ANOVA; averaged data not shown) as did both integrated gIPSC\(_F\) and \(I_{\text{CaF}}\) (Fig. 6B) (\(P = 0.0021618\); factorial ANOVA). The \(\log_{10}\) nonaveraged integrated gIPSC\(_F\) plotted versus the \(\log_{10}\) nonaveraged integrated \(I_{\text{CaF}}\) (Hill coordinates) (Fig. 6C), yields an exponent of dependency equal to 1.7 (\(R^2 = 0.76\), \(P = 0.0001\)). The \(\log_{10}\) averaged integrated gIPSC\(_F\) plotted versus the \(\log_{10}\) averaged integrated \(I_{\text{CaF}}\) (Hill coordinates) yields an exponent of dependency equal to 1.95 (\(R^2 = 0.99\), \(P = 0.0048\)). Similar results were obtained for \(\log/\log\) plots of gIPSC\(_F\) (P) versus \(I_{\text{CaF}}\) (P) (nonaveraged data: exponent \(= 1.81\), \(R^2 = 0.54\), \(P = 0.00099\); averaged data: exponent \(= 2.58\), \(R^2 = 0.796\), \(P = 0.0267\); data not shown). These data suggest the involvement of more than one (presumably, at least two) \(I_{\text{CaF}}\) channels in gating transmitter release at the early stages of graded synaptic transmission (Augustine 2001; Augustine et al. 1991; Bertram et al. 1999; Borst and Sakmann 1999; Fedchyshin and Wang 2005; Gentile and Stanley 2005). Thus multichannel calcium domains appear to mediate \(I_{\text{CaF}}\)-related graded synaptic transmission between heart interneurons.

**How do the rate and amount of Ca\(^{2+}\) influx by LVA Ca channels affect graded synaptic transmission between heart interneurons?**

To evaluate how the rate and amount of Ca entering by LVA Ca channels affects graded transmission, we compared graded release at 2 and 5 mM external Ca\(^{2+}\) (Angstadt and Calabrese 1991) with simultaneous recordings of presynaptic intracellular Ca signal, presynaptic LVA \(I_{\text{CaF}}\), and gIPSCs. The presynaptic cell in these experiments was filled with Calcium Orange and held at −70 mV and the postsynaptic cell was held at −40 mV.

As illustrated in Fig. 7A (\(n = 5\)), superfusion (3 min) with 2 mM Ca\(^{2+}\) saline had a significant effect on LVA \(I_{\text{CaF}}\) and gIPSCs, evoked by presynaptic depolarization to −40 mV (\(P = 0.002345\), factorial ANOVA), and their corresponding gIPSCs (\(P = 0.00512\), factorial ANOVA). \(I_{\text{CaF}}\) (P) was reduced in 2 mM Ca\(^{2+}\) saline by roughly 50% (\(P = 0.010533\); Bonferroni test (one-way ANOVA)), but \(I_{\text{CaS}}\) (1 s and 2 s) were both virtually unchanged (\(P = 1.000\) and \(P = 1.000\), respectively; Bonferroni test (factorial ANOVA and one-way ANOVA)) (Fig. 7B, 2a–2c). The corresponding gIPSC\(_F\) (P) was reduced by nearly 50% (\(P = 0.04976\); Bonferroni test (one-way ANOVA)) and gIPSC\(_S\) (1 s and 2 s) were not significantly affected (\(P = 1.000\) and \(P = 1.000\), respectively)

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**FIG. 6.** Time dissection of the development of presynaptic \(I_{\text{CaF}}\) and corresponding gIPSC\(_F\). A: presynaptic \(I_{\text{CaF}}\) and corresponding gIPSC\(_F\), evoked by 5-, 15-, 25-, 50-, and 100-ms presynaptic depolarizations from holding potential of −70 to −40 mV. B1: dependency of \(I_{\text{CaF}}\) (P) and gIPSC\(_F\) (P) on duration of presynaptic depolarization. Data were averaged over 3 similar experiments (individual preparations). B2: dependency of integrated \(I_{\text{CaF}}\) and gIPSC\(_F\) on duration of presynaptic depolarization. Data were averaged over 3 similar experiments (individual preparations). Filled black circles: \(I_{\text{CaF}}\) (P); empty gray diamonds: gIPSC\(_F\) (P). C: dependency of the nonaveraged integrated gIPSC\(_F\) (P) on the integrated \(I_{\text{CaF}}\) (P) (\(\log_{10}/\log_{10}\) plot). Empty black circles: gIPSC\(_F\) (P); Black line: linear fit. Gray dashed lines: upper and lower 95% confidence limits.
the increase in mediating release, corroborating data presented above in Figs. 2 and 3. In the present experiments, the delay of the intracellular Ca fluorescent signal time-to-peak was associated with a prominent decrease in the postsynaptic responses despite only a moderate reduction of ΔF/F. ANCOVA (P = 0.039602) confirms a strong effect of changes in ΔF/F (P) time-to-peak on amplitude of gIPSCF (P).

**Ni**²⁺ (1 mM) is an effective blocker of LVA Ca currents and associated graded synaptic transmission in leech heart interneurons

Whereas HVA Ca channels in heart interneurons can be selectively blocked by 100 μM Cd²⁺ (Lu et al. 1997), no such tool was available for LVA Ca channels. On the other hand, Ni²⁺ has been widely used as a relatively selective blocker of LVA Ca channels (Huguenard 1996; Lee et al. 1999a; Perez-Reyes 2003; Yunker 2003b). To determine whether it is possible to use Ni²⁺ to manipulate LVA Ca currents in leech heart interneurons, we performed the experiments illustrated in Fig. 8. Individual heart interneurons were voltage clamped at −70 mV and 2-s depolarizing steps to −30 mV were applied to evoke LVA Ca currents. Recordings were performed during superfusion with control saline and after 5-min superfusion with saline containing Ni²⁺ at different concentrations.

Preliminary experiments showed that Ni²⁺ at concentrations of ≥2 mM quickly and effectively blocked both HVA and LVA Ca currents, and in concentrations of ≥0.5 mM had little

Bonferroni test (factorial ANOVA and one-way ANOVA). The presynaptic fluorescent Ca signal (ΔF/F) was not significantly affected (P, 1 s and 2s) in 2 versus 5 mM Ca²⁺ saline (Fig. 7B, 1a–1c) [P = 0.110322; Bonferroni test (factorial ANOVA and one-way ANOVA)]. The time-to-peak, however, of I_{CaF}, gIPSC, and ΔF/F were all significantly increased [P = 0.04905, P = 0.01561, and P = 0.006769, respectively; Bonferroni test (one-way ANOVA)] (Fig. 7B, 1d–3d). These observations suggest that the rate of increase in internal Ca²⁺ concentration may be as important as the absolute amount of

**FIG. 7.** Effect of extracellular Ca²⁺ concentration on presynaptic LVA Ca currents, graded postsynaptic currents, and fluorescent Ca signal (ΔF/F). A: simultaneous recordings of presynaptic I_{Ca}, fluorescent Ca signal, and gIPSC, evoked by presynaptic depolarization (2 s) to −40 mV from a holding potential of −70 mV. A1: recordings obtained in 5 mM Ca²⁺/0 mM Na⁺ saline. A2: recordings obtained in 2 mM Ca²⁺/0 mM Na⁺ saline. B1–B3: ΔF/F, I_{Ca}, and gIPSC, respectively, averaged over 5 experiments similar to those presented in A (different preparations). Data recorded in 5 mM Ca²⁺/0 mM Na⁺ are presented in black; data recorded in 2 mM Ca²⁺/0 mM Na⁺ in white. In B1–B3: a, ΔF/F (P), I_{CaF} (P), gIPSC (P); b, ΔF/F (1 s), I_{Ca} (1 s), I_{gCaS} (1 s); c, ΔF/F (2 s), I_{CaS} (2 s), gIPSC (2 s); d, time-to-peak of ΔF/F, I_{Ca}, (P), gIPSC (P). Asterisks denote significant difference between corresponding measurements recorded in 2 mM Ca²⁺/0 mM Na⁺ and in 5 mM Ca²⁺/0 mM Na⁺.

**FIG. 8.** Block of LVA Ca currents by 1 mM Ni²⁺. LVA Ca currents were evoked by depolarization to −30 mV from holding potential of −70 mV. A: I_{Ca}, evoked by a 2-s depolarizing step recorded in 5 mM Ca²⁺/0 mM Na⁺ saline (Control (A)) and 5-min (A2) superfusion with the saline containing 1 mM Ni²⁺. B1–B3: respectively, I_{CaF} (P), I_{gCaS} (277 ms), and I_{CaS} (1 s) as presented in A averaged over 5 similar experiments (different preparations). Black: Control; white: 1 mM Ni²⁺. Asterisks denote I_{CaF} (P) and I_{gCaS} (277 ms) recorded in 1 mM Ni²⁺ significantly different from that recorded in Control.
or no effect ($n > 15$; data not shown). At the same time, 1 mM Ni$^{2+}$ had a very reproducible effect on LVA Ca current, and at least in 75% of experiments strongly blocked $I_{CaF}$ with a somewhat lesser effect on $I_{CaS}$ ($n > 15$). Figure 8A presents typical recordings from experiments demonstrating the effect of 1 mM Ni$^{2+}$ on LVA $I_{CaS}$ ($n = 5$). Depolarization to $-30$ mV under control conditions evoked typical $I_{CaF}$ and $I_{CaS}$ (Angstadt and Calabrese 1991; Lu et al. 1997; Olsen and Calabrese 1996). The LVA $I_{CaS}$ recorded were very stable and reproducible: responses to two depolarizing steps applied with a 3-min interval were virtually indistinguishable. Ni$^{2+}$ (1 mM) progressively blocked all LVA Ca current. Superfusion with 1 mM Ni$^{2+}$ progressively blocked $I_{CaF}$ (Fig. 8A2), by almost 50% at 5 min [P = 0.000054; Bonferroni test (one-way ANOVA)] (Fig. 8B1). $I_{CaS}$ was more resistant to the effect of 1 mM Ni$^{2+}$, but nevertheless 1 mM Ni$^{2+}$ had a significant blocking effect ($P = 0.008534$; factorial ANOVA). $I_{CaS}$, recorded at 277 ms from the beginning of depolarization [$I_{CaS}$(277 ms), estimated maximum of $I_{CaS}$; see above], was reduced by 40% ($P = 0.048294$; Bonferroni test) and $I_{CaS}$ (1 s) was reduced insignificantly by about 20% ($P = 1.00000$; Bonferroni test) (Fig. 8, B2 and B3). A differential effect of 1 mM Ni$^{2+}$ on $I_{CaF}$ versus $I_{CaS}$ was confirmed by factorial ANOVA ($P = 0.0000638$). These observations show that 1 mM Ni$^{2+}$ is an effective blocker of LVA Ca currents with a predominant effect on $I_{CaF}$.

To evaluate the effect of block of LVA Ca channels by 1 mM Ni$^{2+}$ on graded synaptic transmission, we held the presynaptic cell at $-70$ mV (the postsynaptic cell was held at $-40$ mV) and applied presynaptic depolarizing steps (2 s) to $-55$ mV and up to $-30$ mV in 5-mV increments (Fig. 9). Similar to our previous results (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000), under control conditions (superfusion with 5 mM Ca$^{2+}$/0 mM Na$^{+}$ saline), incremental depolarization evoked a progressive concomitant increase in presynaptic $I_{CaF}$ and $I_{CaS}$ [for $I_{CaF}$ (P), $I_{CaS}$ (277 ms), and $I_{CaS}$ (1 s) $P = 0.0000001$ for all three; repeated-measures ANOVA], and in the gIPSC [for gIPSC$_F$ (P) and gIPSC$_S$ (1 s) $P = 0.0000001$, and for gIPSC$_S$ (405 ms) $P = 0.000001$; repeated-measures ANOVA] (see Fig. 9A for examples of recordings and Fig. 9B for a summary).
for averages over seven experiments). Some decrease in LVA Ca current at depolarizing potentials > -40 mV is probably a result of contamination with outward currents (Angstadt and Calabrese 1991; Lu et al. 1997). Superfusion (3 min) with 1 mM Ni\textsuperscript{2+} (Fig. 9, A and B, 1 mM Ni\textsuperscript{2+}) strongly blocked both $I_{\text{Ca,HT}}$ and $I_{\text{Ca,AS}}$ (277 ms) [for both $I_{\text{Ca,HT}}$ (P) and $I_{\text{Ca,AS}}$ (277 ms) $P = 0.000001$; repeated-measures ANOVA], with a smaller effect on $I_{\text{Ca,AS}}$ (1 s) ($P = 0.051321$; repeated-measures ANOVA), significantly reduced synaptic transmission [for gIPSC\textsubscript{F} (P), gIPSC\textsubscript{S} (405 ms), and gIPSC\textsubscript{C} (1 s), $P = 0.000001$, and $P = 0.008419$, respectively; repeated-measures ANOVA], and up-shifted the dependency of the postsynaptic response on presynaptic depolarization: a much more depolarizing potential had to be applied presynaptically to evoke release than in the absence of Ni\textsuperscript{2+}. Whereas in control presynaptic depolarization to -55 mV evoked a detectable gIPSC\textsubscript{F}, and depolarization to -50 mV evoked detectable gIPSCs of both kinds, in 1 mM Ni\textsuperscript{2+}-containing solutions, the first gIPSCs were detectable only at depolarization to -45 mV (Fig. 9B); at any given presynaptic depolarizing potential the evoked gIPSC\textsubscript{F} (P) values were reduced, compared with control ($P = 0.035947$; repeated-measures ANOVA). This effect on gIPSC\textsubscript{S} (405 ms) and gIPSC\textsubscript{C} (1 s) was not significant ($P = 0.099433$ and $P = 0.204156$; repeated-measures ANOVA). Moreover, the time-to-peak for $I_{\text{Ca,HT}}$ and the corresponding gIPSC were greatly increased ($P = 0.009406$ and $P = 0.008015$, respectively; repeated-measures ANOVA) (Fig. 9B).

Thus the effect of 1 mM Ni\textsuperscript{2+} on graded synaptic transmission follows from its ability to block presynaptic LVA Ca currents.

**Can 1 mM Ni\textsuperscript{2+} be used to separate synaptic transmission mediated by LVA and HVA Ca currents in leech heart interneurons?**

LVA Ca currents are completely inactivated at holding potentials above -50 mV (Angstadt and Calabrese 1991), but additional depolarization to much higher potentials evokes a postsynaptic response that has been attributed to presynaptic HVA Ca current ($I_{\text{Ca,HT}}$) (Simon et al. 1994) that is selectively blocked by 150 μM Cd\textsuperscript{2+} (Ivanov and Calabrese 2000; Lu et al. 1997). We took advantage of these findings to confirm the selective block of LVA Ca currents by 1 mM Ni\textsuperscript{2+} and to corroborate that the postsynaptic response to HVA depolarization is mediated by activation of an $I_{\text{Ca,HT}}$. In these experiments ($n = 4$), we also monitored the intracellular Ca fluorescence signal by filling the presynaptic cell with Calcium Orange. The presynaptic LVA Ca currents were inactivated by clamping the presynaptic cell at a potential of -40 mV for 20 s, after which 2-s depolarizations to -10 mV were applied. The postsynaptic cell was held at -35 mV. Superfusion with 1 mM Ni\textsuperscript{2+} did not noticeably affect IPSCs evoked by high-threshold presynaptic depolarization (Fig. 10, A and B). Averaged across these experiments ($n = 4$), the hIPSC integrated over time in 1 mM Ni\textsuperscript{2+}-containing saline [0.49 nA·s (SD 0.33)] versus in Control saline [0.44 nA·s (SD 0.31)] was not significantly different ($P = 0.821372$; Bonferroni test (one-way ANOVA)]. Subsequent superfusion with 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}, however, nearly completely blocked the postsynaptic response to high-threshold depolarization (Fig. 10, B and C). Averaged across these experiments ($n = 4$), the hIPSC integrated over time in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+} [0.01 nA·s (SD 0.01)] versus in 1 mM Ni\textsuperscript{2+}-containing saline [0.49 nA·s (SD 0.33)] was significantly smaller; $P = 0.027204$; Bonferroni test (one-way ANOVA)]. The block of IPSCs evoked by high-threshold depolarization by Cd\textsuperscript{2+} confirms that this synaptic response is $I_{\text{Ca,HT}}$ dependent (Fig. 10, B and C). The absence of any effect of 1 mM Ni\textsuperscript{2+} on this hIPSC compared with Control (Fig. 10, A and B) indicates that Ni\textsuperscript{2+} at least at 1 mM is a selective blocker of LVA Ca currents and does not have any sizable effect on synaptic transmission evoked by HVA Ca current, and thus on HVA $I_{\text{Ca,HT}}$.

Cd\textsuperscript{2+} increases background fluorescence in heart interneurons and this effect has been attributed to Cd\textsuperscript{2+} influx into cells (Ivanov and Calabrese 2000). Our current data confirm these findings (Fig. 10, D and E). Ni\textsuperscript{2+} had no significant effect on background fluorescence [the changes in background fluorescence in 1 mM Ni\textsuperscript{2+}-containing saline compared with Control vs. changes in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline compared with Control were significantly different: $P = 0.016158$; Bonferroni test (one-way ANOVA)]. But did significantly increase the amplitude of fluorescent signal in response to depolarization [the changes in fluorescence evoked by high-
threshold depolarization in 1 mM Ni\textsuperscript{2+}-containing saline compared with Control vs. changes in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline compared with Control were significantly different: \( P = 0.015574 \); Bonferroni test (one-way ANOVA) (Fig. 10, B, D, E1, and E2), indicating that Ni\textsuperscript{2+} can enter the cell during depolarization and after cessation of depolarization can somehow be bound up or eliminated/extruded from the cytoplasm.Like almost all other Ca indicators, Calcium Orange can bind a range of di- and trivalent cations with corresponding changes in the fluorescent signal; the ratio of the change in fluorescence of Ca Orange to 5 μM Ni\textsuperscript{2+} versus 100 μM Ca\textsuperscript{2+} is 71/96 (Haugland 1996).

**Partial blockade of LVA Ca current with 1 mM Ni\textsuperscript{2+} affects modulation of spike-mediated synaptic transmission between heart interneurons**

In leech heart interneurons, spike-mediated synaptic transmission (smIPSC) is independent of previous spike activity (Nicholls and Wallace 1978a,b) and is modulated by changes in presynaptic background Ca\textsuperscript{2+} concentration, which depends in turn on LVA Ca currents (Ivanov and Calabrese 2003). Here we wanted to determine the effect of blockade of LVA Ca currents by 1 mM Ni\textsuperscript{2+} on modulation of smIPSCs. Isolated third or fourth segmental ganglia were bathed in 5 mM Ca\textsuperscript{2+}/20 mM Mg\textsuperscript{2+} saline to prevent spontaneous spiking activity (Nicholls and Wallace 1978a). The presynaptic cell was current clamped at \(-55\) to \(-50\) mV, and the postsynaptic cell was voltage clamped at \(-40\) mV. During a 2-s subthreshold depolarizing current step, a train of brief (6-ms) suprathreshold current pulses was superimposed to evoke presynaptic spikes; suprathreshold current pulses were also applied before and after the step. Postsynaptic responses were recorded as graded and spike-mediated IPSCs. After control recordings of cells activity were made, ganglia were superfused for 3–6 min with 1 mM Ni\textsuperscript{2+} added to the saline, tested, and then superfused for another 3–6 min with both 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+} added to the saline and tested again (Fig. 11A). In some experiments, immediately after control recordings, ganglia were superfused for 3–6 min with only 150 μM Cd\textsuperscript{2+} added to the saline (Fig. 11B). Under control condition, typical postsynaptic responses with clearly distinguishable graded and spike-mediated components were recorded (Fig. 11, A1 and B1); the time course of the modulation in spike-mediated synaptic transmission reproduced our previous findings (Fig. 11A4a, Control) where changes in smIPSC amplitude were attributed to changes in intracellular Ca\textsuperscript{2+} concentration (Ivanov and Calabrese 2003). Addition of 1 mM Ni\textsuperscript{2+} blocked gIPSCs and slightly delayed the buildup of synaptic modulation (\( P = 0.000108 \); repeated-measures ANOVA) (Fig. 11, A2 and A4, 1 mM Ni\textsuperscript{2+}). The time constant for a single exponential fit to the rise of the amplitude of smIPSCs equaled 324.8 ms in control saline and 499.5 ms in 1 mM Ni\textsuperscript{2+}-containing saline. In addition, the time from the beginning of the first high-threshold depolarization to the maximal smIPSC achieved was significantly shorter in control versus 1 mM Ni\textsuperscript{2+}-containing saline [\( P = 0.00103 \); Bonferroni test (one-way ANOVA)] (Fig. 11A, 4a and 4b). Addition of 150 μM Cd\textsuperscript{2+} (both in the absence and in the presence of 1 mM Ni\textsuperscript{2+}) completely blocked smIPSCs (Fig. 11, A3 and B2). gIPSCs were only moderately affected by addition of 150 μM Cd\textsuperscript{2+} in the absence of 1 mM Ni\textsuperscript{2+} (Fig. 11A4a). Addition of 150 μM Cd\textsuperscript{2+} in the absence of 1 mM Ni\textsuperscript{2+} significantly different from recorded in control. B: postsynaptic responses (IPSCs) in control saline (B1) and saline containing 150 μM Cd\textsuperscript{2+} (B2). CM, current monitor trace for Pre cell.
11B2), but were completely blocked when both 1 mM Ni\textsuperscript{2+} and 150 \textmu M Cd\textsuperscript{2+} were added to the saline (Fig. 11A).

These results further corroborate our previous work (Ivanov and Calabrese 2000; Lu et al. 1997), where we showed that Cd\textsuperscript{2+} is a highly selective blocker of HVA Ca currents in leech heart interneurons, and confirms the critical role of I_{Ca\text{F}} in spike-mediated transmission (Lu et al. 1997; Simon et al. 1994). Changes in the time course of synaptic plasticity produced by 1 mM Ni\textsuperscript{2+} likely result from a reduction in intracellular background Ca\textsuperscript{2+} caused by partial block of LVA Ca current by 1 mM Ni\textsuperscript{2+}, with a resultant delay in binding of Ca\textsuperscript{2+} to the modulatory Ca binding site (Ivanov and Calabrese 2003). This effect of 1 mM Ni\textsuperscript{2+} on the time course of synaptic plasticity resembles the effect of the slow Ca\textsuperscript{2+} chelator EGTA (Ivanov and Calabrese 2003).

DISCUSSION

There is now strong evidence that LVA (low-threshold) Ca channels are involved in synaptic transmission in a variety of different systems (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000; Lu et al. 1997; Olsen and Calabrese 1996; Pan 2000; Pan et al. 2001; Uhrenbuhl and Nedergaard 2005), expanding the view that LVA Ca channels are localized mainly in dendrites and serve mainly to drive spiking activity or generate the pacemaker activity supporting bursting activity (Erickson et al. 1993; Fisher and Bourque 2001; Huguenard 1996; McCormick and Bal 1997; Yunker 2003a). In some cases the involvement of LVA Ca channels in synaptic transmission may be masked by much stronger HVA Ca channel-dependent synaptic transmission and thus underestimated. Therefore the characterization of the involvement of LVA Ca channels in synaptic transmission in leech heart interneurons is important not only for understanding heartbeat central pattern generator but also for defining a role of LVA Ca channels in synaptic transmission that is potentially applicable to other systems.

In the current study, we were able to partially separate the effects of I_{Ca\text{F}} and I_{Ca\text{S}} on inhibitory synaptic transmission between heart interneurons, describe the power relation between LVA currents and the strength of synaptic transmission, and more precisely evaluate the effects of HVA and LVA Ca currents on inhibitory synaptic transmission between heart interneurons.

Comparison of LVA Ca channels in leech heart interneurons and known cloned T-type Ca channels

Similar to its effect on T-type Ca channels in other systems (Huguenard 1996; Lee et al. 1999a; Perez-Reyes 2003; Yunker 2003b; but see Zamponi et al. 1996), we found that Ni\textsuperscript{2+} in relatively high concentration (1 mM) is an effective blocker of I_{Ca\text{F}} and I_{Ca\text{S}} LVA Ca currents in heart interneurons; Cloning and expression studies (Perez-Reyes 2003) have identified three cloned T-type Ca channels (pore-forming subunits): \alpha_{L,3.1}, \alpha_{H,3.2}, and \alpha_{H,3.3}. All these channels are Ni\textsuperscript{2+} sensitive, but Ca\textsubscript{3.1} and Ca\textsubscript{3.3} are 20 times less sensitive than Ca\textsubscript{3.2} to Ni\textsuperscript{2+} (Lacínová 2000; Lee et al. 1999a; Perez-Reyes 2003). Ni\textsuperscript{2+} shifts I–V curves to more depolarized potentials and Ni\textsuperscript{2+} block is reduced at more depolarized test potentials (Lee et al. 1999a,b; Perez-Reyes 2003). The low sensitivity to Ni\textsuperscript{2+} (effective concentration 1 mM) of both I_{Ca\text{F}} and I_{Ca\text{S}} (Fig. 8) and the observation that Ni\textsuperscript{2+} block was less effective at higher test potentials (Fig. 9) make these channels similar to Ca\textsubscript{3.1} and Ca\textsubscript{3.3}.

Kinetics also indicate similarity of I_{Ca\text{F}} and I_{Ca\text{S}} to the cloned T-type Ca channels; the time constants of activation and inactivation of Ca\textsubscript{3.1} and Ca\textsubscript{3.3} (6 and 30 ms, and 30 and 137 ms, respectively) (Klöckner et al. 1999) are in the same range as I_{Ca\text{F}} and I_{Ca\text{S}} respectively (Angstadt and Calabrese 1991). The time course of recovery of I_{Ca\text{F}} current from inactivation described here is also on the same scale as that in T-type currents (Klöckner et al. 1999). All these comparisons suggest that the I_{Ca\text{F}} channel of heart interneurons is similar to Ca\textsubscript{3.1}, and the I_{Ca\text{S}} channel to Ca\textsubscript{3.3}.

Comparison of the properties and functional role of low-threshold Ca channels of heart interneurons and T-type Ca channels of retinal bipolar cells

The properties of LVA Ca currents in heart interneurons (Angstadt and Calabrese 1991) and in vertebrate retinal bipolar cells, especially in rod bipolar cells (Pan 2000), are very similar. In both heart interneurons and bipolar cells, LVA Ca currents consist of two components (fast and slow), have similar kinetics, and are blocked by Ni\textsuperscript{2+} in the millimolar range. In bipolar cells, LVA channels are localized in synaptic terminals and are responsible for tonic (asynchronous, low-threshold) synaptic transmission (Pan 2001). The similarity between these two very different cell types extends further; the HVA Ca channels that are responsible for ultrafast synaptic transmission in retinal bipolar cells are of the L-type, and the HVA Ca channels in heart interneurons appear to be similar, being highly sensitive to Cd\textsuperscript{2+} (Lu et al. 1997). Moreover, Ma and Pan (2003) found that T-type channels appear to be essential for the initiation of the spontaneous pacemaker-like activity recorded in the majority of bipolar cells. Similarly in heart interneurons, LVA Ca channels generate the plateau potentials that drive bursts of action potentials (Arbas and Calabrese 1987; Olsen and Calabrese 1996). Thus the main types of Ca channels in vertebrate retinal bipolar cells and in heart interneurons are similar and share corresponding physiological/functional properties.

Localization of LVA Ca channels at release sites and their involvement in graded synaptic transmission between heart interneurons

Graded synaptic transmission between heart interneurons mediated by LVA currents (I_{Ca\text{S}} and I_{Ca\text{F}}) is delayed and partially suppressed by intracellular EGTA (Ivanov and Calabrese 2003), suggesting that these channels are located relatively far from the release trigger compared with HVA channels, because spike-mediated transmission is unaffected by EGTA. The difference between I_{Ca\text{F}} and I_{Ca\text{S}} in mediating transmitter release (Fig. 1), where the rapid initial transfer of a smaller amount of charge by I_{Ca\text{F}} (i.e., smaller Ca\textsuperscript{2+} entry) evokes a larger postsynaptic response than the delayed transfer of a larger amount of charge by I_{Ca\text{S}} (i.e., larger Ca\textsuperscript{2+} entry) is explained most easily, if I_{Ca\text{F}} and I_{Ca\text{S}} channels share the same release sites. The massive transmitter release mediated by Ca\textsuperscript{2+} entering by I_{Ca\text{F}} channels appears to result from the
involvement of multichannel Ca\(^{2+}\) domains of (probably) clustered \(I_{\text{CaF}}\) channels in this fast graded inhibitory synaptic transmission (Fig. 6). The initial \(I_{\text{CaF}}\)-evoked transmitter release partially depletes the readily releasable pool of synaptic vesicles that is common for Ca\(^{2+}\) entering by both LVA Ca currents, without sufficient replenishment by vesicles from reserve pools for depolarizations of the order of 2–5 s. The very long time lag between the peaking of Ca\(^{2+}\) and the gIPSCs (Fig. 1) argues against cooperativity among \(I_{\text{CaS}}\) channels in mediating transmitter release and suggests that single (individual) \(I_{\text{CaS}}\) channels are distributed uniformly and not specifically in the vicinity of release sites, possibly exerting their effect through radial diffusion of Ca\(^{2+}\) (Augustine 2001; Neher 1998). The possibility that \(I_{\text{CaS}}\) might evoke transmitter release at sites different from the \(I_{\text{CaF}}\)-controlled release sites seems unlikely because the release sites from which \(I_{\text{CaF}}\) releases transmitter are depleted by Ca\(^{2+}\) entering as \(I_{\text{CaS}}\) (Fig. 5). Apparently, only a fraction of Ca\(^{2+}\) delivered by \(I_{\text{CaS}}\) during prolonged depolarization is involved in transmitter release, possibly attributable to depletion of the pool of readily releasable vesicles by \(I_{\text{CaF}}\), and possibly arising from strong Ca\(^{2+}\) buffering in proximity of the release trigger. 

After a short (1- to 200-ms) low-threshold presynaptic depolarization, the replenishment of the readily releasable vesicle pool is rate limiting for recovery of graded synaptic transmission, not recovery of \(I_{\text{CaF}}\) from inactivation that proceeds more rapidly (Figs. 4 and 5). The recovery of graded synaptic transmission after prolonged (3- to 5-s) low-threshold presynaptic depolarization occurs simultaneously with the recovery of \(I_{\text{CaF}}\) (with its complete deinactivation time of 8–12 s) (Fig. 4). For intermediate durations (0.5- to 3-s depolarizations) recovery of \(I_{\text{CaF}}\) outstrips the recovery of graded transmission, indicating that \(I_{\text{CaS}}\) depletes a pool of readily releasable vesicles normally available for release by \(I_{\text{CaF}}\). The factors that contribute to the replenishment of the readily releasable pool by mobilization of vesicles from a reserve pool, vesicle recycling, or priming reluctant vesicles (Congar and Trudeau 2002; Harata et al. 2001; Kelly 1993; Neher 1998; Neher and Zucker 1993; Schneggenburger et al. 2002; Voets 2000; Wang and Kaczmarek 1998; Wang and Zucker 1998; Wu and Borst 1999) remain to be determined in heart interneurons.

Overall our results indicate that \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\) channels evoke transmitter release from the same release sites. \(I_{\text{CaF}}\) channels are probably clustered near release sites but not intimately associated with the release trigger. Multichannel Ca\(^{2+}\) domains and a rapid increase in intracellular Ca\(^{2+}\) concentration (Figs. 1, 2, 6, and 7) produce fast and massive synaptic release by \(I_{\text{CaF}}\). In contrast, \(I_{\text{CaS}}\) channels appear to be distributed more uniformly and not specifically in the vicinity of release sites, and they probably interact with the release trigger by radial diffusion of Ca\(^{2+}\).

The versatile intimate interrelations between fast and slow LVA Ca channels in regulating graded transmitter release together with the continuously changing dependency of graded synaptic transmission on the availability of synaptic vesicles and intracellular Ca\(^{2+}\) (Figs. 2 and 3)—demonstrated here as well as the previously described modulation (homosynaptic enhancement) of spike-mediated transmission mediated by Ca\(^{2+}\) entering by LVA Ca channels (see Fig. 12 in Ivanov and Calabrese 2003)—form the basis for a unique dynamics of reciprocally inhibitory synaptic transmission between heart interneurons that are further explored in a companion paper.

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-24072.

REFERENCES


