Spike-Mediated and Graded Inhibitory Synaptic Transmission Between Leech Interneurons: Evidence for Shared Release Sites

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Ivanov, Andrei I. and Ronald L. Calabrese. Spike-mediated and graded inhibitory synaptic transmission between leech interneurons: evidence for shared release sites. J Neurophysiol 96: 235–251, 2006. First published April 26, 2006; doi:10.1152/jn.01094.2005. Inhibitory synaptic transmission between leech heart interneurons consist of two components: graded, gated by Ca$^{2+}$ entering by low-threshold [low-voltage–activated (LVA)] Ca channels and spike-mediated, gated by Ca$^{2+}$ entering by high-threshold [high-voltage–activated (HVA)] Ca channels. Changes in presynaptic background Ca$^{2+}$ produced by Ca$^{2+}$ influx through LVA channels modulate spike-mediated transmission, suggesting LVA channels have access to release sites controlled by HVA channels. Here we explore whether spike-mediated and graded transmission can use the same release sites and thus how Ca$^{2+}$ influx by HVA and LVA Ca channels might interact to evoke neurotransmitter release. We recorded pre- and postsynaptic currents from voltage-clamped heart interneurons bathed in 0 mM Na$^{+}$/5 mM Ca$^{2+}$ saline. Using different stimulating paradigms and inorganic Ca channel blockers, we show that strong graded synaptic transmission can occlude high-threshold/spike-mediated synaptic transmission when evoked simultaneously. Suppression of LVA Ca currents diminishes graded release and concomitantly increases the ability of Ca$^{2+}$ entering by HVA channels to release transmitter. Uncaging of Ca chelator corroborates that graded release occludes spike-mediated transmission. Our results indicate that both graded and spike-mediated synaptic transmission depend on the same readily releasable pool of synaptic vesicles. Thus Ca$^{2+}$, entering cells through different Ca channels (LVA and HVA), acts to gate release of the same synaptic vesicles. The data argue for a closer location of HVA Ca channels to release sites than LVA Ca channels. The results are summarized in a conceptual model of a heart interneuron release site.

INTRODUCTION

At many synapses more than one kind of Ca channel is involved in neurotransmitter release and clusters of different Ca channel types may overlap and cooperate to trigger fusion (Meinrenken et al. 2003; Mintz et al. 1995; Reid et al. 1998; Wu and Shaggau 1994, 1997). On other hand, Ca$^{2+}$ entering the cell through different types of Ca channels may have different effects on synaptic transmission. For example, in rat calyx-type synapses, P/Q-type Ca channels trigger release more effectively than N- and R-type Ca channels, which appear to be located more distantly from release sites (Wu et al. 1998, 1999). Mouse cortical CA3–CA1 synapses appear to be similarly organized (Qian and Noebels 2001).

Ca channel cooperativity in neurotransmitter release depends on the kind of Ca channels involved and the location of these channels with respect to active zones (Meinrenken et al. 2002, 2003; but see Gentile and Stanley 2005). Ca channels at calyx of Held synapses are clustered and located at different distances from the synaptic vesicle release trigger in different active zones, leading to variability in the release efficacy from one active zone to another (Meinrenken et al. 2002). Involvement of different Ca channels in neurotransmitter release may change during development (Fedchyshin and Wang 2005). Moreover, distant Ca channels that are not involved in Ca domain(s) that trigger release can nevertheless provide bulk (background) intracellular Ca$^{2+}$ that may modulate neurotransmitter release (Bertram et al. 1999).

Synapses may be either phasic (spike-mediated and fast) or tonic (non-spike-mediated and slow, or graded) and involve synchronous and/or asynchronous release. At the same synapse, transmission may be both phasic (spike-mediated, synchronous) and tonic (graded, asynchronous) (Angstadt and Calabrese 1991; Ayali et al. 1998; Ivanov and Calabrese 2000, 2006; Pan et al. 2001; Warzecha et al. 2003), and in some cases the involvement of low-voltage–activated (LVA) Ca channels in transmission is well documented (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000, 2006; Pan et al. 2001). Transmitter release in these neurons resembles catecholamine secretion in chromaffin cells (Artalejo et al. 1994), where activation of different Ca channels leads to a different mode of neurosecretion, and different Ca channels control catecholamine secretion with different efficacies as a result of their different proximity to release sites. At other neuronal synapses asynchronous (tonic, bulk Ca-dependent) release can often be recorded after spike-evoked (phasic, synchronous) release (Atluri and Regehr 1998) and be in a competitive relation with synchronous release (Otsu et al. 2004).

In leeches, reciprocally inhibitory synaptic transmission between heart interneurons is both spike-mediated [depends on high-voltage–activated (HVA) Ca channels] and graded (depends on two types of LVA Ca channels termed $I_{CaS}$ and $I_{CaF}$) (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000; Lu et al. 1997; Olsen and Calabrese 1996). Previously we showed that LVA Ca channels, which are widely distributed throughout heart interneurons, not only generate the plateau potential that drives the burst of action potential and mediate graded transmission, but also provide intracellular background Ca for modulation of spike-mediated transmission (Angstadt and Calabrese 1991; Arbas and Calabrese 1987; Ivanov and Calabrese 2000, 2003; Lu et al. 1997; Olsen and Calabrese 1996). Moreover, we demonstrated in the companion paper that both $I_{CaS}$ and $I_{CaF}$ mediate release from the same release sites (Ivanov and Calabrese 2006). We also showed that manipulat-
ing intracellular Ca\(^{2+}\) concentration with Ca chelators had a stronger effect on graded synaptic transmission than on spike-mediated transmission and its plasticity, indicating either different active zones for spike-mediated and graded synaptic transmission or a different location of HVA and LVA Ca channels in the same active zones (Ivanov and Calabrese 2003). These findings led us to propose two questions that we address here. Are the vesicle pools and release sites for high-mediated transmission and its plasticity, indicating either different Ca channels (low- and high-threshold) to act to gate release the same synaptic vesicles. Our results further argue for a closer association of HVA Ca channels to cells through different Ca channels (low- and high-threshold) acting on the same releasable pool of synaptic vesicles. Ca\(^{2+}\) ions entering cells through different Ca channels (low- and high-threshold) act to gate release the same synaptic vesicles. Our results further argue for a closer association of HVA Ca channels to release sites than LVA Ca channels and are summarized in a conceptual model of a heart interneuron release site.

**Methods**

**Animals**

Adult leeches (H. medicinalis) were obtained from Leeches USA and Biopharm and maintained in artificial pond water (Leeches USA) at approximately 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 microscapelers. 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\(^{-2}\)-hydroxyethylpiperazine-N\(^{-2}\)-ethanesulfonic (HEPES) acid buffer, adjusted to pH 7.4 with NaOH 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 pre- and postsynaptic cell was recorded in voltage-clamp mode. Voltage-clamp recordings were made with an Axoclamp-2A amplifier (Axon Instruments) in single-electrode voltage-clamp mode (SEVC) 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 a 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 a different holding potential to a fixed depolarizing potential. Various approaches were used, from single voltage pulses/steps to combined pulses/steps, and to trains of “artificial spikes,” which were copied from recordings of spontaneously active heart interneurons, recorded in separate experiments. Software-controlled leak subtraction was implemented as previously described (Ivanov and Calabrese 2003). More details on all stimulus protocols used are provided in RESULTS.

**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 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 postero-lateral 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). 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, 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, and 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.

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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 camera’s DC mode.

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 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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**UV photolysis of caged Ca$^{2+}$ chelator**

In some experiments Ca imaging/electrophysiology was combined with UV photolysis. For experiments of this kind, the optical system was modified and used as described by Ivanov and Calabrese (2003). Briefly, for UV photolysis we used a 100-W mercury lamp, equipped with a UV transmitting fused-silica condenser, an electronic shutter (Oriel Instruments), and a glass UV filter (U-360, Edmunds Industrial Optics). The lamp was connected by a UV transmitting fused-silica fiber (core diameter 1.000 μm, numerical aperture 0.22; Oriel Instruments) to an “ablation laser unit” (Photonic Instruments), attached to the microscope. The location and focusing of the spot of “uncaging” light were adjusted with controls on the ablation laser unit so the spot was centered in the image plane and as close to the estimated center of synaptic contact region as possible.

Diazo-2 (derived from BAPTA, “caged BAPTA”; Molecular Probes, diazo-2, tetrapotassium salt, “cell impermeant,” MW 710.86, cat no. D-3034) is a photoactivatable Ca$^{2+}$ scavenger; the nominal $K_d$ of diazo-2 for Ca$^{2+}$ changes on UV illumination from 2.2 μM to about 80 nM (Adams and Tsien 1993; Delaney 2000); diazo-2 photo-release Ca$^{2+}$ chelator on UV illumination was <360 nm.

To fill cells with diazo-2, the same techniques as for filling with Calcium Orange but different microelectrode solutions were used. Filling electrodes for diazo-2, contained (in mM): 5 Calcium Orange, 40 diazo-2, and 40 KOH/HEPES, pH 7.2.

All protocols used for photo-release of caged Ca$^{2+}$ chelator were generated using the pCLAMP program CLAMPFIT, which controlled the shutter of the release lamp through the Digidata 1200/1320A connected to the shutter control unit. Typically, Ca$^{2+}$/Ca$^{2+}$ chelator were photo-released for 800–1,600 ms during electrophysiological and Ca fluorescence data acquisition.

**Data analysis**

All stored data were analyzed on the same computer using the 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, and repeated-measures ANOVA 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**

LVA channels are widely distributed throughout heart interneurons and serve to generate plateau potentials, support the formation of bursts of action potential during normal rhythmic activity, mediate graded synaptic transmission, and provide intracellular background Ca$^{2+}$ for modulation of spike-mediated transmission (Angstadt and Calabrese 1991; Arbas and Calabrese 1987; Ivanov and Calabrese 2000, 2003; Lu et al. 1997; Olsen and Calabrese 1996). Spike-mediated transmission, on the other hand, is produced by HVA Ca channels of unknown distribution (Ivanov and Calabrese 2000, 2003; Simon et al. 1994). These findings left unanswered two questions addressed in the present study. Are the vesicle pools and release sites for high-threshold (thus spike-mediated) and low-threshold (graded) transmission shared? To the extent that these vesicle pools and release sites are shared, does Ca$^{2+}$ entering by HVA and LVA Ca channels have equivalent access to the release trigger?

Do spike-mediated and graded synaptic transmission deplete the same pool of readily releasable vesicles in heart interneurons?

To determine whether vesicle pools and thus release sites are shared, we performed experiments designed to test whether depletion of releasable transmitter by low-threshold stimulation occluded release by high-threshold stimulation. In all experiments presented in this paper, ganglia were bathed in 5 mM Ca$^{2+}$/0 mM Na$^{+}$ saline, and both pre- and postsynaptic heart interneurons were voltage clamped with sharp microelectrodes. To test for occlusion (Fig. 1, n = 7), we elicited low-threshold (graded) transmission using depolarizing steps from a holding potential of −70 to −40 mV that evoked the two kinetically distinct LVA Ca currents presynaptically ($I_{C_AF}$ and $I_{C_S}$) (Angstadt and Calabrese 1991) and the corresponding postsynaptic responses (gIPSC$_F$ and gIPSCs) (Fig. 1C). We also elicited high-threshold transmission, using a train of “artificial spikes” at 2.5 Hz from a holding potential of −40 mV that evoked HVA Ca current presynaptically and the corresponding postsynaptic (spike-mediated) responses (sIPSCs). The train of artificial spikes evoked robust sIPSCs with typical (compare with Ivanov and Calabrese 2003) stochastic variations in their peak amplitude (Fig. 1A). Artificial spikes elicited during a 2-s depolarizing step to −40 mV from a holding potential of −70 mV, however, failed to elicit sIPSCs during the intense graded transmission early in the step, but as the gIPSC waned (concomitantly with the inactivation of LVA Ca current), they began to elicit sIPSCs comparable in amplitude to those elicited from −40 mV (Fig. 1B). The “absolute” peak amplitude of these IPSCs (i.e., amplitude relative to holding current; see Fig. 1D) was almost constant (with some weak tendency to increase during the depolarizing step) and did not exceed the peak value of gIPSC [gIPSC (P)] (Fig. 1, A2 and B2). The peak amplitude of sIPSCs (i.e., amplitude relative to the baseline postsynaptic current recorded just before the onset of any given sIPSC; see Fig. 1D), however, increased concomitantly with the decrease in the gIPSC (P). Although changes in peak amplitudes of sIPSCs elicited from holding potential of −40 mV and “absolute” peak amplitude of IPSCs elicited during the depolarizing step to −40 mV did not depend on the time from the beginning of train/depolarization ($P = 1.0$ and 0.99989, respectively, one-way ANOVA), changes in peak amplitude of sIPSCs elicited during the depolarization to −40 mV and in gIPSC were strongly time dependent ($P$ values of 0.000013 and 0.001, respectively, one-way ANOVA).

Thus it appears that strong graded synaptic transmission can occlude sIPSCs when they are simultaneously evoked. The simplest explanation for this occlusion is that HVA and LVA Ca currents evoke neurotransmitter release from common vesicle pools and release sites. Depletion of the readily releasable vesicle pool by intense low-threshold Ca currents occludes release by Ca$^{2+}$ entering by high-threshold channels; inactivation of the low-threshold currents diminished graded release and when accompanied by replenishment of the readily releasable pool restores the ability of Ca$^{2+}$ entering by HVA Ca channels to release transmitter. As shown in the companion
paper, the pool of vesicles available for release by LVA currents is indeed being replenished at this time (see Fig. 5 of Ivanov and Calabrese 2006). Similar results were obtained in experiments in which we used brief (10- to 15-ms pulses to 0 and 10 mV) pulses instead of artificial spikes (see, e.g., Fig. 5A). In most subsequent experiments, we preferred to use brief pulses or even 1-s-long steps instead of artificial spikes because more stable results were obtained using this approach. Moreover, we now simply refer to the postsynaptic responses to low- and high-threshold depolarization as gIPSCs and, hIPSCs respectively, assuming their correspondence to graded and spike-mediated inhibitory postsynaptic currents.

**Does block of low-threshold synaptic transmission with Ni2+ relieve occlusion of high-threshold synaptic transmission between heart interneurons?**

As a next step (Fig. 2, n = 3 different preparations), we used Ni2+ (1 mM), a blocker of LVA Ca currents in heart interneurons (Ivanov and Calabrese 2006), to selectively block low-threshold transmission and determine whether this relieved the above-observed occlusion of high-threshold transmission. We kept both the presynaptic and the postsynaptic heart interneurons voltage clamped. The presynaptic cell was held at −70 mV and a depolarizing step protocol, first to −40 mV for 5 s (to activate LVA Ca channels) and then to +10 mV for 1 s (to activate HVA Ca channels), was applied. Additionally, two brief pulses of 10 ms to +10 mV were applied during depolarizing step to −40 mV. The postsynaptic cell was held at −35 mV.

In control saline (without 1 mM Ni2+), low-threshold depolarization evoked typical LVA Ca current (Fig. 2A) and corresponding gIPSCs. The hIPSCs evoked by the 1-s high-threshold depolarization delivered at the end of the low-threshold depolarization step were small but clearly noticeable and persistent (see Ivanov and Calabrese 2006), although no hIPSCs evoked by the brief high-threshold depolarizing pulses superimposed on the low-threshold step were detected. [HVA Ca

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**FIG. 1.** Spike-mediated IPSCs (smlIPSCs) are occluded by strong graded IPSCs (gIPSCs). A1: smlIPSCs, evoked by a train of artificial spikes, applied from presynaptic holding potential of −40 mV; A2: data, as presented in A1, averaged over 7 similar experiments (different preparations) are plotted vs. the time. B1: smlIPSCs and gIPSC, evoked by a train of artificial spikes generated during a depolarizing step to −40 mV; B2: data as presented in B1, averaged over 7 similar experiments (different preparations) are plotted vs. the time. In A2 and B2: black filled circles: peak amplitude of smlIPSCs [smlIPSCs (P)]; gray filled diamonds: absolute peak amplitude of IPSCs [absolute IPSC (P)]; empty boxes: amplitude of gIPSC evoked by depolarization to −40 mV measured immediately before presynaptic application of each artificial spike. C: typical example of a gIPSC evoked by step depolarization to −40 mV, for comparison. A1, B1, C1: recordings from different preparations. D: method used to calculate amplitudes of IPSCs for A2 and B2. Absolute peak amplitude of IPSC (P) = current at the peak of a given smlIPSC (holding current); amplitude of smlIPSC (P) = current at the peak of a given smlIPSC (postsynaptic current just before the onset of the smlIPSC); amplitude of gIPSC = current just before the onset of a given smlIPSC (holding current). Here and in subsequent figures the following abbreviations are used: Pre: presynaptic cell, the cell that was stimulated and was thus functionally presynaptic. Post: postsynaptic cell, the opposite heart interneuron, where postsynaptic responses to the presynaptic cell stimulation were recorded. HVA Ca current: high-voltage–activated Ca current (high-threshold Ca current). LVA Ca current: low-voltage–activated Ca current (low-threshold Ca current). I_{CaF}: presynaptic Ca current; I_{CaS, and I_{CaHT}: fast and slow LVA calcium currents, and HVA calcium current, respectively. IPSC: inhibitory postsynaptic current; gIPSC and smlIPSC: graded and spike-mediated inhibitory postsynaptic current; gIPSC and hIPSC: inhibitory postsynaptic current, evoked by I_{CaF} + I_{CaS, and I_{CaHT}, respectively. (P): peak signal; thus smlIPSC (P) means peak spike-mediated postsynaptic current.
current could not be recorded because of contamination with K current (Angstadt and Calabrese 1991) and here and in subsequent figures, current artifacts arising from high-threshold (to \( \geq -10 \) mV) depolarizations were not used for analysis and are presented only to indicate when these depolarizations occurred during LVA currents recorded. Subsequent superfusion with saline containing 1 mM Ni\(^{2+}\) led to noticeable changes (Fig. 2B). The LVA Ca currents were significantly blocked \([I_{\text{CaF}} (P)]\) in control: \(-1.265 (SD 0.115)\) nA, in 1 mM Ni\(^{2+}\)-containing saline: \(-0.385 (SD 0.011)\) nA, \(P = 0.000197\), one-way ANOVA, Bonferroni test] as were the corresponding gIPSCs \([gIPSC (P), as above: 0.704 (SD 0.073)\) nA and 0.071 (SD 0.070) nA, \(P = 0.000413\), one-way ANOVA, Bonferroni test], but the htIPSCs to 1-s high-threshold depolarizing step were significantly increased \([htIPSC (P), as above: 0.360 (SD 0.120)\) nA and 0.752 (SD 0.048) nA, \(P = 0.006383\), one-way ANOVA, Bonferroni test] (Fig. 2C) with a corresponding decrease in time-to-peak \([htIPSC time-to-peak, as above: 329.6 (SD 63.0)\) ms and 157.2 (SD 43.4) ms, \(P = 0.017509\), one-way ANOVA, Bonferroni test]. In addition htIPSCs to the brief high-threshold depolarizing pulses appeared. These results stated above indicate again that intense graded release mediated by LVA Ca currents can occlude high-threshold release.

For the next experiments (Fig. 3, \(n = 5\), different preparations), we slightly modified the protocol to demonstrate that release by HVA and LVA Ca currents can occur independently, if they are separated temporally. Both the presynaptic and postsynaptic heart interneurons were voltage clamped. The presynaptic cell was held at \(-70\) mV and the postsynaptic cell was held at \(-35\) mV. The time between the beginning of the low-threshold depolarization and the high-threshold depolarization was increased to 12 s from 5 s in the previous protocol. The duration of high-threshold depolarization was 2 s, and the brief high-threshold depolarizations were excluded. Under these conditions \((n = 5)\), whereas LVA Ca currents \([I_{\text{CaF}} (P)]\) in control: \(-1.109 (SD 0.434)\) nA, in 1 mM Ni\(^{2+}\)-containing saline: \(-0.512 (SD 0.231)\) nA, \(P = 0.000332\), one-way ANOVA, Bonferroni test] and corresponding gIPSCs \([gIPSC (P), as above: 0.874 (SD 0.310)\) nA and 0.364 (SD 0.365) nA, \(P = 0.0014\), one-way ANOVA, Bonferroni test] were significantly blocked by 1 mM Ni\(^{2+}\) as in previous experiments, the response to high-threshold depolarization remained unchanged (Fig. 3, A and B, compare with Fig. 2). Ni\(^{2+}\) (1 mM) blocked LVA Ca currents and the corresponding gIPSCs, but htIPSCs were not affected \([htIPSC (P), as above: 0.582 (SD 0.250)\) nA and 0.572 (SD 0.379) nA, \(P = 1.0\), one-way ANOVA, Bonferroni test] (Fig. 3C).

Under control conditions, the relatively shorter low-threshold depolarization, used in experiments illustrated in Fig. 2, depletes the pool of readily releasable synaptic vesicles and occludes neurotransmitter release by subsequent high-threshold depolarization. Inhibition of LVA Ca channels by 1 mM Ni\(^{2+}\) by decreasing low-threshold release reduced this depletion, increasing the availability of these synaptic vesicles for high-threshold depolarization-evoked release. Under control conditions, the longer low-threshold depolarization used in the experiments illustrated in Fig. 3 sufficiently inactivates LVA Ca, and thus low-threshold transmission with 1 mM Ni\(^{2+}\), relieves this occlusion. High-threshold depolarizations (to 10 mV) were applied during a 5-s depolarizing step to \(-40\) mV from holding potential of \(-70\) mV. A: presynaptic \(I_{\text{Ca}}\) and IPSCs recorded in Control saline (5 mM Ca\(^{2+}\)/70 mM Na\(^{+}\)). B: presynaptic \(I_{\text{Ca}}\) and IPSCs recorded 4 min after beginning superfusion with 1 mM Ni\(^{2+}\)-containing saline. C: \(I_{\text{CaF}} (P)\), gIPSC (P), and htpSC (P), as illustrated in A and B, averaged over three similar experiments (different preparations). C: data recorded in Control saline are presented in black and data recorded in the 1 mM Ni\(^{2+}\)-containing saline in white. Asterisks indicate that \(I_{\text{CaF}} (P)\), gIPSC (P), and htpSC (P) recorded in 1 mM Ni\(^{2+}\)-containing saline are significantly different from those recorded in Control saline. Here and in subsequent figures, see the text for the probability value \(P\).
Ca currents so that low-threshold transmitter release decays completely by the fourth second of depolarization, similar to the experiments illustrated in Fig. 2, but there is enough time where release is halted before the high-threshold depolarization to restore the readily available vesicles for release. The htIPSCs in control and in the presence of 1 mM Ni$^{2+}$ are of the same amplitude because there was sufficient time for vesicle pool replacement to overcome the effect of vesicle depletion by unblocked LVA Ca current.

The experiments illustrated in Fig. 4 (n = 5 different preparations) show that the changes in postsynaptic responses to high-threshold depolarization during low-threshold depolarization caused by 1 mM Ni$^{2+}$ were not the result of some additional activation of LVA Ca channels but solely the result of Ca$^{2+}$ entering the cell by HVA Ca channels. We used 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$ to separate LVA and HVA Ca currents (see Figs. 10 and 11 in Ivanov and Calabrese 2006). Both presynaptic and postsynaptic heart interneurons were voltage clamped. The presynaptic cell was held at −70 mV and complex depolarizing step to −40 mV for 8 s and to +10 mV for 2 s was applied. The postsynaptic cell was held at −35 mV. In the presence of 1 mM Ni$^{2+}$, the postsynaptic response to the high-threshold depolarization (which followed 8-s low-threshold depolarization) was significantly increased (Fig. 4B; compare with Fig. 4A), but subsequent addition of 150 µM Cd$^{2+}$ that selectively blocks HVA Ca current (Ivanov and Calabrese 2000; Lu et al. 1997) significantly blocked this response [Fig. 4D; htIPSC (P) in control: 0.737 (SD 0.083) nA, in 1 mM Ni$^{2+}$-containing saline: 1.322 (SD 0.304) nA (P = 0.016746, one-way ANOVA, Bonferroni test), in 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$-containing saline: 0.386 (SD 0.356) nA (P = 0.000491, compared with 1 mM Ni$^{2+}$-containing saline, one-way ANOVA, Bonferroni test). The peak amplitude of htIPSCs was significantly influenced by the experimental conditions used (control, 1 mM Ni$^{2+}$-containing saline, 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$-containing saline) (P = 0.0000001, one-way ANOVA). htIPSC time-to-peak in 1 mM Ni$^{2+}$ compared with 150 µM Cd$^{2+}$-containing saline was significantly increased [htIPSC (P) time-to-peak in control: 103.80 (SD 36.03) ms, in the saline containing 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$: 698.40 (SD 435.73) ms, P = 0.030711, one-way ANOVA, Bonferroni test].

We next performed a similar analysis using 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$ to separate LVA and HVA Ca currents but with a simulated burst protocol in the presynaptic heart interneuron (n = 3 different preparations) (Fig. 5). The postsynaptic cell was held at −40 mV, and the presynaptic cell was held at −70 mV, while a train of brief 15-ms depolarizing pulses to 10 mV superimposed on a 2-s depolarizing step to −40 mV was applied. In control saline (Fig. 5A), strong low-threshold (graded) transmission (gIPSC) occluded high-threshold transmission (htIPSCs) as in Fig. 1. In 1 mM Ni$^{2+}$ saline, LVA Ca currents were strongly reduced as was the corresponding gIPSP, but the htIPSCs were considerably increased, indicating an absence of occlusion (Fig. 5B). In saline containing both 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$, htIPSCs were completely eliminated (Fig. 5C). As illustrated in Fig. 5D, gIPSC (P), in control saline, the amplitude of gIPSC (P) was equal to 1.117 (SD 0.213); in 1 mM Ni$^{2+}$-containing saline, 0.531 (SD 0.235); in both 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$-containing saline, 0.619 (SD 0.229) nA. The amplitude of gIPSC (P) was significantly different from the control (P = 0.000238, one-way ANOVA, Bonferroni test) and from the saline containing 1 mM Ni$^{2+}$ alone (P = 0.016746). In the presence of 1 mM Ni$^{2+}$ and 150 µM Cd$^{2+}$, the amplitude of gIPSC was decreased compared with 1 mM Ni$^{2+}$-containing saline, whereas htIPSP was not changed. Asterisks indicate that htIPSC (P) and gIPSC (P) recorded in 1 mM Ni$^{2+}$-containing saline were significantly different from those recorded in Control saline.

**FIG. 3.** Occlusion of high-threshold transmission (htIPSC) by strong low-threshold transmission (gIPSC) is relieved if low-threshold depolarization, which inactivates LVA $I_{Ca}$, is prolonged, indicating that with low-threshold release terminated for a sufficient period a shared vesicle pool can be restored. Under these conditions, 1 mM Ni$^{2+}$ does not further relieve occlusion of htIPSCs by strong low-threshold transmission (gIPSC). A: in Control saline, a prolonged depolarizing step to −40 mV from holding potential of −70 mV for 12 s and subsequently to +10 mV for 2 s evokes a typical biphasic postsynaptic response with the low-threshold depolarization-evoked gIPSC decaying completely by the 4th second after the beginning of depolarizing step. B: in 1 mM Ni$^{2+}$ strongly reduced LVA $I_{Ca}$, and the corresponding gIPSC, but the response to high-threshold depolarization (htIPSC) remained unchanged. C: $I_{Ca}$ (P), gIPSC (P), and htIPSC (P), as illustrated in A and B, averaged over 5 different experiments (different preparations). In C data recorded in Control saline are presented in black; data recorded in 1 mM Ni$^{2+}$-containing saline, in white. LVA $I_{Ca}$ current and the corresponding gIPSP in response to low- and high-threshold depolarization in 1 mM Ni$^{2+}$-containing saline were decreased compared with Control saline, whereas htIPSP was not changed. Asterisks indicate that $I_{Ca}$ (P) and gIPSC (P) recorded in 1 mM Ni$^{2+}$-containing saline were significantly different from those recorded in Control saline.
influenced by the experimental conditions used (control, 1 mM Ni\(^{2+}\)-containing saline, both 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline) \((P = 0.020252,\) one-way ANOVA) with significant differences between gIPSCs (P), recorded in control and both experimental salines \((P = 0.029903 for 1\) mM Ni\(^{2+}\)-containing saline, and \(P = 0.039093 for 1\) mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline, one-way ANOVA, Bonferroni test). Amplitudes of htIPSCs (Fig. 5D, htIPSC) were also significantly influenced by experimental conditions used (control, 1 mM Ni\(^{2+}\)-containing saline, 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline) \((P = 0.0000001,\) repeated-measures ANOVA). Taken collectively, these data strongly support the hypothesis that low- and high-threshold Ca currents evoke release of shared pool of transmitter vesicles at common release sites.

**Effects of uncaging of caged Ca\(^{2+}\) chelator on the interaction between spike-mediated and graded synaptic transmission between heart interneurons**

To understand further the interaction between LVA and HVA Ca currents in mediating synaptic transmission between heart interneurons, we used photo-uncaging of caged Ca\(^{2+}\) chelator (diazo-2). We reasoned that by reducing/blocking graded transmission by uncaging Ca\(^{2+}\) chelator, we should augment the response to high-threshold depolarization during concurrent low threshold and high-threshold depolarization.

In these experiments \((n = 5\) different preparations) (Fig. 6), the presynaptic cell was filled with diazo-2 (caged Ca\(^{2+}\) chelator) and Calcium Orange (to monitor changes in intracellular Ca\(^{2+}\) concentration) and held at \(-70\) mV, whereas a train of artificial spikes was superimposed on a 5-s depolarizing step to \(-40\) mV. The stimulus protocol evoked a robust gIPSC and greatly diminished htIPSCs that recovered somewhat at the end of the step (Fig. 6A). Diazo-2 itself is a weak Ca chelator (nominal \(K_d = 2.2\) \(\mu\)M (Adams and Tsien 1993; Delaney 2000), and thus intracellular injection of diazo-2 changed LVA Ca currents, apparently by slowing the inactivation of \(I_{\text{CaL}}\), similarly to intracellularly injected BAPTA (see Ivanov and Calabrese 2003; Lu et al. 1997). Each of two subsequent releases of Ca chelator (Fig. 6, B and C) led to suppression of the gIPSC with a simultaneous increase in htIPSCs amplitudes. Two minutes after the last light flash, the gIPSC was strongly suppressed, whereas htIPSCs amplitudes were strongly increased (Fig. 6D). To quantify the effects of Ca\(^{2+}\) chelator uncaging on gIPSCs and htIPSCs, we averaged the amplitudes of gIPSCs (P) and htIPSCs (P) evoked before the first and after the last Ca\(^{2+}\) chelator uncaging \((n = 1–3\) uncagings in each preparation) over \(n = 5\) different preparations (Fig. 6E). After Ca\(^{2+}\) chelator uncaging, the gIPSC (P) amplitudes were significantly smaller and htIPSC (P) amplitudes were significantly larger \((P = 0.040792 and P = 0.0000001,\) respectively; repeated-measures ANOVA). The simultaneous increase in strength of spike-mediated (high-threshold) transmission and suppression of graded transmission by uncaged Ca\(^{2+}\) chelator further supports the existence of a common vesicle pool and release sites for both spike-mediated and graded synaptic transmission between heart interneurons.

**FIG. 4.** Using the stimulus paradigm of Fig. 2, 150 \(\mu\)M Cd\(^{2+}\) blocks the Ni\(^{2+}\)-augmented htIPSCs (relief of occlusion by low-threshold transmission), evoked by 2-s depolarization to 0 mV at the end of 8-s depolarization to \(-40\) mV from holding potential of \(-70\) mV. Compared with Control saline (A), 1 mM Ni\(^{2+}\)-containing saline (B) blocked both \(I_{\text{CaL}}\) and the corresponding gIPSC evoked by low-threshold depolarization and increased the htIPSC after high-threshold depolarization. C, in 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline, the htIPSP was substantially blocked. D, \(I_{\text{CaL}}\), gIPSC (P), and htIPSC (P), as illustrated in A, B, and C, respectively, averaged over 5 similar experiments (different preparations). In D data recorded in Control saline are presented in black, data recorded in 1 mM Ni\(^{2+}\) containing saline in gray, and data recorded in 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline in white, and data recorded in 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline in gray. Black asterisk indicates htIPSC (P) recorded in 1 mM Ni\(^{2+}\)-containing saline significantly different from that recorded in Control saline, and gray filled circle indicates htIPSC (P) recorded in 1 mM Ni\(^{2+}\) and 150 \(\mu\)M Cd\(^{2+}\)-containing saline was significantly different from that recorded in 1 mM Ni\(^{2+}\)-containing saline.
transmission. The inability of diazo-2 (both caged and uncaged) to block hIPSCs argues that HVA Ca channels are closer to release sites than LVA Ca channels.

Effects of inactivation of LVA Ca currents by incremental depolarization of the presynaptic holding potential on high-threshold postsynaptic responses

Incremental depolarization of presynaptic holding potential leads to inactivation of both LVA Ca currents evoked by low-threshold depolarization, and the corresponding gIPSCs are attributed to a progressive decrease in LVA Ca channel-mediated Ca\(^{2+}\) influx (Ivanov and Calabrese 2005). Because of an increase in presynaptic intracellular background Ca\(^{2+}\) associated with a more depolarized holding potential, however, the mean amplitude of hIPSCs, evoked from progressively depolarizing presynaptic holding potential increases (Ivanov and Calabrese 2003). In these experiments, we used presynaptic holding potential to affect the balance between high- and low threshold transmission (n = 6) (Fig. 7) with the aim of gaining further evidence in support of our hypothesis that LVA and HVA currents evoke release at shared release sites. We used the protocol used in our companion paper (Ivanov and Calabrese 2006) with some modifications. We held postsynaptic cell at −35 mV and applied 2-s depolarizing steps to −40 mV presynaptically from incrementally depolarized holding potentials (from −70 to −45 mV in 5-mV increments). Additionally, 15-ms depolarizing pulses to 10 mV were applied before, during, and after the 2-s depolarizing step.

With an increase of presynaptic holding potential to more depolarized values, LVA Ca currents and corresponding gIPSCs progressively decreased, as described in the companion paper (Ivanov and Calabrese 2006), and the amplitudes of the hIPSCs progressively increased [Fig. 7A, bottom (6) to top (1)]. The amplitude of the hIPSC, evoked by the pulse applied before the depolarizing step (hIPSC\(_1\)) from a presynaptic holding potential of −45 mV was no more than twofold the amplitude of the hIPSC\(_1\) evoked from −70 mV. This modest increase in amplitude of hIPSC\(_1\) with increasing holding potential (Fig. 7B) can be attributed to an increase in intracellular background Ca\(^{2+}\) (cf. Ivanov and Calabrese 2003.) On the other hand, the amplitude of hIPSC evoked by pulse applied after depolarizing step (hIPSC\(_3\)) from a presynaptic holding potential of −45 mV was sixfold greater than that from −70 mV, and the hIPSC evoked by pulse applied during depolarizing step (hIPSC\(_2\)) from −45 mV was more than tenfold greater than that from −70 mV. Similar results were obtained in n = 6 preparations and averaged data are presented in Fig. 7B. Amplitudes of hIPSCs were significantly affected by presynaptic holding potential (repeated-measures ANOVA; P = 0.000003) and by when the hIPSCs were evoked (before, during, or after) with respect to the low-threshold depolarization (−40 mV) (repeated-measures ANOVA; P = 0.0000001). Moreover, the interaction between these factors had a significant effect on hIPSCs amplitude (repeated-measures ANOVA; P = 0.0000001). A strong negative correlation was observed between the amplitudes of both binned (according to holding potential) nonnormalized hIPSC\(_{CS}\) (Fig. 7C, top) and hIPSC\(_{CS}\) with the peak amplitude of the binned (according to holding potential) nonnormalized gIPSC\(_{FS}\) [gIPSC\(_{FS}\) (P)]; correlation coefficients (R) were equal to −0.997 and −0.971, respectively (P = 0.0001 and P = 0.00123, respectively). A linear fit of nonbinned nonnormalized hIPSC\(_{CS}\) versus nonbinned nonnormalized gIPSC\(_{CS}\) (P) also showed a significant negative correlation (R = −0.7571, P = 0.0001) (Fig. 7C, bottom). The prominent increase in the amplitude of both hIPSC\(_2\) and hIPSC\(_3\) is consistent with decreased depletion of readily releasable synaptic vesicles for high-threshold transmission arising from the progressive decrease in low-threshold (graded) transmitter release. The modest increase in amplitude of hIPSC\(_1\) with increasing holding potential (Fig. 7B) was most

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**FIG. 5.** Effects of 1 mM Ni\(^{2+}\) and 150 μM Cd\(^{2+}\) on postsynaptic responses to a train of brief depolarizing pulses to 10 mV superimposed on 2-s depolarization to −40 mV from presynaptic holding potential of −70 mV. Compared with Control saline (A), in 1 mM Ni\(^{2+}\)-containing saline (B), LVA \(I_{Ca}\) and the corresponding gIPSC evoked by depolarization to −40 mV were substantially blocked, and the amplitudes of hIPSCs, elicited by a superimposed train of brief depolarizing pulses to 10 mV, were increased (relief of occlusion, see text). 150 μM Cd\(^{2+}\) and 1 mM Ni\(^{2+}\)-containing saline blocked hIPSCs with a progressive decrease in \(I_{Ca}\) and gIPSC resulting from a lengthened exposure to 1 mM Ni\(^{2+}\) (C). D: gIPSC (P) and hIPSC (P), as illustrated in A, B, and C, averaged over 3 similar experiments (different preparations). In D data recorded in Control saline are presented in black, data recorded in 1 mM Ni\(^{2+}\)-containing saline in white, and data recorded in 1 mM Ni\(^{2+}\) and 150 μM Cd\(^{2+}\)-containing saline in gray. Black asterisk indicates gIPSC (P) recorded in 1 mM Ni\(^{2+}\)-containing saline significantly different from that recorded in Control saline, and filled gray circle indicates gIPSC (P) recorded in 1 mM Ni\(^{2+}\) and 150 μM Cd\(^{2+}\)-containing saline significantly different from that recorded in Control saline.
likely the result of an increase in intracellular background Ca\(^{2+}\) (cf. Ivanov and Calabrese 2003).

These conclusions were further supported in experiments, similar to those illustrated in Fig. 7, in which after control recordings, we applied 1 mM Ni\(^{2+}\)-containing saline to selectively block LVA Ca currents (Fig. 9) \(n = 7\). Control recordings were similar to Fig. 7 (Fig. 8A). As expected, 1 mM Ni\(^{2+}\) blocked (significantly reduced) both LVA Ca currents and the corresponding gIPSCs at all presynaptic holding potentials (Fig. 8B, 1a and 1b). [For the effect of presynaptic holding potential on \(I_{\text{CaF}}\) (P) and gIPSC\(_{\text{s}}\) (P), respectively, \(P = 0.005734\) and \(P = 0.0000001\), factorial ANOVA; for the effect of 1 mM Ni\(^{2+}\) on \(I_{\text{CaF}}\) (P) and gIPSC\(_{\text{s}}\) (P), \(P = 0.0000001\) and \(P = 0.048534\), respectively, factorial ANOVA.] Amplitudes of the htIPSC\(_{\text{s}}\) (Fig. 8B2) and the htIPSC\(_{\text{s}}\) were greater in 1 mM Ni\(^{2+}\)-containing saline than in Control saline at all presynaptic holding potentials tested [For the effect of presynaptic holding potential on htIPSC\(_{\text{s}}\) (P) and htIPSC\(_{\text{s}}\) (P), \(P = 0.0000005\) and \(P = 0.005734\), respectively, factorial ANOVA; for the effect of 1 mM Ni\(^{2+}\)-containing saline were as strong as those in Control saline. In Control saline, \(R = -0.997\) \(P = 0.00282\) for htIPSC\(_{\text{s}}\), and in 1 mM Ni\(^{2+}\)-containing saline, \(R = -0.999\) \(P = 0.0007636\). The increase of htIPSCs measured during and after low-threshold depolarization is consistent with a decreased depletion of readily releasable synaptic vesicles as a result of the decrease in low-threshold synaptic transmission brought on by Ni\(^{2+}\) blockade of LVA Ca channels.

![FIG. 6. Effects of uncaging (photo-release) of caged Ca\(^{2+}\) chelator (DM-Nitrophen) on spike-mediated (high-threshold) and graded synaptic transmission between heart interneurons. htIPSCs were elicited by a train of artificial spikes superimposed on a 5-s step depolarization to \(-40\) mV from a holding potential of \(-70\) mV that elicited a gIPSP (simulated burst), and the effect of uncaging of caged Ca\(^{2+}\) chelator on synaptic transmission was assessed. All recordings (A–D) are from the same preparation. A1: with no uncaging of caged Ca\(^{2+}\) chelator, evoked htIPSCs were occluded by strong graded transmission (gIPSP) but gradually began to recover toward the end of the simulated burst. B1: first uncaging of Ca\(^{2+}\) chelator prematurely terminated the gIPSP and promoted recovery of the htIPSPs. C1: in this run, the previous uncaging of Ca\(^{2+}\) chelator continued to suppress the gIPSP and relieved some occlusion of the htIPSPs. Second uncaging of caged Ca\(^{2+}\) chelator further promoted recovery of the htIPSPs. D1: in this run, no further uncaging of caged Ca\(^{2+}\) chelator was performed, but the 2 previous uncagings of Ca\(^{2+}\) chelator continued to suppress the gIPSP and substantially relieved occlusion of the htIPSPs. A2–D2: plots of htIPSCs (P) (empty circles) and gIPSCs (black line), extracted from A1–D1. Gray bar indicates the time and duration of the uncaging light flash. E: amplitudes of htIPSCs (P) before and after Ca\(^{2+}\) chelator uncaging (as illustrated in A1 and D1), averaged over 5 similar experiments (different preparations). htIPSCs recorded before Ca\(^{2+}\) chelator uncaging are shown in black; htIPSCs recorded after Ca\(^{2+}\) chelator uncaging are shown in white.]}
Ca$^{2+}$ saline ($n = 6$ different preparations). The presynaptic cell was filled with Ca Orange to monitor changes in intracellular Ca$^{2+}$. Recordings were performed first in 2 mM Ca$^{2+}$ saline and then in 5 mM Ca$^{2+}$ saline or vice versa. The presynaptic cells were held at $-70$ mV and the postsynaptic cells were held at $-40$ mV. Trains of 15-ms pulses to 10 mV were applied either from the holding potential or during a 5-s depolarization to $-40$ mV. In 2 mM Ca$^{2+}$ saline, amplitudes of htpIPSCs evoked from $-70$ mV were smaller and the time course to attainment of maximal htpIPSC amplitude delayed compared with those in 5 mM Ca$^{2+}$ saline. Amplitudes of htpIPSCs in 5 mM Ca$^{2+}$ were significantly larger than those in 2 mM Ca$^{2+}$ saline ($P = 0.000001$, repeated-measures ANOVA) (Fig. 9, A and C). The time from the beginning of the first depolarizing pulse to maximal htpIPSC in 5 mM Ca$^{2+}$ was significantly shorter than that in 2 mM Ca$^{2+}$ saline: 1,450.00 (SD 219.09) ms and 2,170.00 (SD 219.09) ms, $P = 0.000826$ (one-way ANOVA, Bonferroni test). The simultaneously recorded presynaptic Ca signal was smaller and its buildup delayed. [The time from the beginning of the first depolarizing pulse to the maximal $\Delta F/F$ in 5 mM Ca$^{2+}$ was significantly shorter than that in 2 mM Ca$^{2+}$ saline: 2,450.80 (SD 1,334.62) ms and 3,966.80 (SD 596.16) ms, respectively, $P = 0.048987$ (one-way ANOVA, Bonferroni test).] In 2 mM Ca$^{2+}$ saline, the LVA Ca currents evoked by the 5-s depolarization to $-40$ mV and the corresponding gIPSCs were decreased and delayed (Fig. 9B), whereas the presynaptic Ca signals were slightly diminished and strongly delayed (see Fig. 8 in Ivanov and Calabrese 2006). The htpIPSCs evoked during the depolarization to $-40$ mV, however, were increased. Amplitudes of htpIPSCs in 5 mM Ca$^{2+}$ saline were significantly smaller than those in 2 mM Ca$^{2+}$ saline ($P = 0.000008$, repeated-measures ANOVA) (Fig. 9, B and D). Factorial ANOVA indicates a significant cooperative effect ($P = 0.000001$) of Ca$^{2+}$ driving force and presynaptic depolarization on htpIPSCs amplitudes. Thus although a decrease in Ca driving force reduced Ca$^{2+}$ influx by LVA and presumably also HVA Ca channels, leading to decreased gIPSCs and htpIPSCs evoked from $-70$ mV, htpIPSCs evoked during a depolarizing step to $-40$ mV (i.e., during low-threshold stimulation) increased. This increase in htpIPSCs is consistent with the hypothesis that reduced low-threshold release results in greater availability (decreased depletion) of readily releasable synaptic vesicles for high-threshold release at shared release sites. The decreased depletion of readily releasable synaptic vesicles in 2 mM Ca$^{2+}$ saline uncovers the enhancing effect of Ca$^{2+}$ entering by LVA Ca channels on htpIPSCs. In 5 mM Ca$^{2+}$ saline, this effect was masked by severe depletion of the readily releasable pool.

Different latencies of brief high- and low-threshold depolarization-evoked release may reflect a differential localization of HVA and LVA Ca channels at shared release sites

The experiments described above support our main hypothesis that LVA and HVA Ca channels mediate transmitter release at common sites. To probe how these common sites might be organized, we compared the effectiveness of HVA and LVA Ca currents in mediating release. High-threshold depolarizations of different shapes and durations from hyperpolarized presynaptic holding potentials are widely used to

**FIG. 7.** Effect of progressive incremental depolarization of the presynaptic holding potential on postsynaptic responses, elicited by brief high-threshold depolarizing pulses. Inactivation of LVA $I_{Ca}$ prevents occlusion of high-threshold transmission by graded transmission. A1–A6: $I_{Ca}$ and IPSCs evoked by 2-s step depolarization to $-35$ mV from different holding potentials ($-45$ to $-70$ mV, respectively) with brief depolarizing pulses to 10 mV applied before, during, and after the step depolarization. B: htpIPSC (P) evoked before (htIPSC$_1$, filled black circles) during (htIPSC$_2$, empty circles), and after (htIPSC$_3$, filled gray circles) low-threshold depolarization to $-35$ mV plotted vs. presynaptic holding potential. C: top: nonbinned nonnormalized htpIPSC$_2$ (P) plotted vs. nonbinned nonnormalized gIPSC$_2$ (P); black line: linear fit. C: bottom: binned averaged nonnormalized htpIPSC$_2$ (P) plotted vs. binned averaged nonnormalized gIPSC$_2$ (P). In B and C data were averaged over 6 similar experiments (different preparations).

**Reducing Ca$^{2+}$ driving force affects the balance between low- and high-threshold transmission**

To further elucidate the interaction between high- and low threshold transmission between heart interneurons, we altered the driving force on Ca$^{2+}$ entry into the neurons and compared both high-threshold transmission and a concomitant high- and low-threshold transmission in 5 (control) and 2 (reduced) mM Ca$^{2+}$ saline.

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mimic action-potential-mediated synaptic transmission (Borst and Sakmann 1999; Fedchyshin and Wang 2005; Gentile and Stanley 2005). We used a similar approach here: the presynaptic cell was depolarized from a holding potential of $-70$ to $-30$ mV (low-threshold depolarization) and to 10 mV (high-threshold depolarization) with pulses of increasing duration (5 to 50 ms in 5-ms increments), whereas the postsynaptic cell was held at $-35$ mV ($n = 8$ different preparations). Across the different pulse durations, htlIPSCs were larger in amplitude ($P = 0.0000001$, repeated-measures ANOVA) and had on average an 11.87-ms shorter synaptic delay (measured from the beginning of presynaptic depolarization to the beginning of the postsynaptic response) [gIPSC latency was 22.23 (SD 4.29) ms and htlIPSC latency was 10.36 (SD 3.53 ms); $P = 0.0000001$, repeated-measures ANOVA] than the corresponding gIPSCs (Fig. 11A). The latency between the pulse and the first recorded

FIG. 8. Effect of progressive incremental depolarization of the presynaptic holding potential and addition of 1 mM Ni$^{2+}$ on postsynaptic responses elicited by brief high-threshold depolarizing pulses. By blocking LVA $I_{\text{Ca}}$, 1 mM Ni$^{2+}$ amplifies the effect of inactivation of LVA $I_{\text{Ca}}$ in preventing occlusion of high-threshold transmission by graded transmission. $A1$–$A3$: $I_{\text{Ca}}$ and IPSCs evoked by a 2-s step depolarization to $-35$ mV from different holding potentials ($-45$, $-50$, and $-70$ mV, respectively) with brief depolarizing pulses to 10 mV applied before, during, and after the step depolarization. $Left$: recordings in Control saline; $right$: recordings in 1 mM Ni$^{2+}$-containing saline. $B$: data, as illustrated in $A$, averaged over 7 similar experiments (different preparations) plotted vs. presynaptic holding potential. $B1a$: $I_{\text{Ca}}F$ ($P$). $B1b$: $g_{\text{IPSC}}F$ ($P$). $B2$: htlIPSC$_2$ ($P$). Data recorded in Control saline are shown in black and data recorded in 1 mM Ni$^{2+}$-containing saline in white.
latency of 2.0 (SD 0.2) ms, significantly different from the latency of $I_{\text{Ca,F}}$ to low-threshold depolarization (one-way ANOVA, $P = 0.010686$, Bonferroni test). htIPSCs were observed in some preparations in response to the 5-ms pulse and in all preparations in response to the 10-ms pulse, whereas gIPSCs were first recorded only in response to 20-ms pulses, at which point the simultaneously monitored LVA Ca current was nearly at its maximal peak value (90% of peak value for the 50-ms depolarization) (Fig. 10B). Subsequent, 3-min superfusion with 1 mM Ni2+ -containing saline suppressed LVA Ca current and corresponding IPSCs, but had no effect on the amplitude of high-threshold depolarization-evoked IPSCs (data not shown). These data indicate that very brief high-threshold depolarizations ($\leq 15$ ms) from a hyperpolarized presynaptic holding potential evoke postsynaptic responses that depend mainly or exclusively on Ca2+ entry by $I_{\text{CaHT}}$, whereas depolarizations of longer duration lead to more complex mixed responses with low-threshold Ca currents contributing to release. The consistently shorter synaptic delay of htIPSCs suggests a closer localization of high-threshold Ca channels to the release trigger.

**DISCUSSION**

Widely distributed throughout neuritic tree of heart interneurons, LVA Ca channels are responsible for graded transmission and providing background Ca2+ for enhancement of spike-mediated transmission (Ivanov and Calabrese 2003), whereas HVA Ca channels are responsible for spike-mediated (high-threshold) synaptic transmission. Ultrastructural morphology suggests that there are 10 to 50 synaptic connections between heart interneurons in ganglia 3 and 4 (Calabrese et al. 1989). Taking into account different possible characteristics of synchronous and asynchronous synapses (Millar et al. 2005; Msghina et al. 1999; Quigley et al. 1999), these multiple synaptic contacts between heart interneurons allow for the possibility that some of them are specialized for graded and some for spike-mediated synaptic transmission. Thus there are two compatible alternatives: 1) HVA and LVA Ca channels share the same release sites or 2) their release sites are different, but some LVA Ca channels are present near (more distantly localized from release trigger) HVA Ca channel-operated release sites. The different degrees of effectiveness of fast and slow LVA Ca channels in mediating graded transmission that indicates different access of these channels to the same release trigger (Ivanov and Calabrese 2006) make the situation potentially more complex. We addressed two primary questions in the present paper: are the vesicle pools and release sites for high-threshold (spike-mediated) and low-threshold (graded) transmission shared and to the extent that these vesicle pools and release sites are shared, do LVA and HVA channels have equivalent access to the release trigger?

Through the use of different stimulating paradigms, inorganic Ca channels blockers, and photo-release Ca2+ chelator, we were able to support the hypothesis that both graded synaptic transmission and spike-mediated synaptic transmission use the same release sites by using the same readily releasable pool of synaptic vesicles. Ca2+ ions entering cells through different Ca channels (LVA and HVA) act to gate release the same synaptic vesicles. Moreover, our observations on the relative latency of htIPSCs versus gIPSCs further
supports the hypothesis that HVA Ca channels are more closely associated than LVA Ca channels with the release trigger. In addition, as argued in Ivanov and Calabrese (2006), the location and distribution of fast and slow LVA Ca channels near release sites are also different.

**Mitigating factors**

Here we consider some potential pitfalls in our analysis that may mitigate the interpretations of our data. Imperfect space clamp (both pre- and postsynaptic) cannot be completely ruled out, although these voltage-clamp methods have been tested and used extensively in our laboratory (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000, 2003). Imperfect space clamp could lead to different voltage control of different synaptic terminals with subsequent effects on transmitter release and postsynaptic responses. Nevertheless, the data presented (e.g., in Fig. 5) suggest that such potential problems cannot account for the results of our experiments. The relief from occlusion of high-threshold transmission by low-threshold transmission in the presence of 1 mm Ni²⁺, which blocks LVA Ca channels, with an almost uniform increase in amplitude of htIPSCs and a simultaneous decrease in the gIPSC is difficult to explain based on imperfect space clamp. The same may be said of the experiments illustrated in Fig. 6; block of gIPSCs by uncaged Ca²⁺ chelator immediately leads to an increase in amplitudes of htIPSCs.

Another potential concern is an uneven intracellular Ca²⁺ distribution throughout the neuritic tree, particularly release sites. Ivanov and Calabrese (2000) showed that the time course of changes in intracellular Ca fluorescence arising from activation of LVA Ca channels is uniform throughout the entire neuritic tree and synaptic contact region. This finding suggests a wide and more-or-less uniform distribution of LVA Ca channels in the neuritic tree and synaptic contact region, and thus it is likely that LVA Ca channels have access to most or all of the release sites. A Ca signal related to activation of HVA Ca channels could not be recorded with our current methods (Ivanov and Calabrese 2000), suggesting a more strict local-
ization of a relatively small number of HVA channels to release sites and very spatially restricted Ca domains near the mouths of these channels (see also Ivanov and Calabrese 2003).

Postsynaptic effects, such as changes in number and sensitivity of postsynaptic receptors and diffusion and removal of transmitter from the cleft, might influence our recorded responses. Nevertheless, the results presented in the paper, especially the results of experiments that involved the block of HVA and LVA channels (e.g., Figs. 5 and 8), uncaging of caged Ca2+ chelator (Fig. 6), and changes in Ca2+ driving force (Fig. 9) (as well results of Ivanov and Calabrese 2000, 2003) indicate that the time course of postsynaptic responses is governed by transmitter release (i.e., by presynaptic Ca currents and vesicle availability) and that any potential influence of postsynaptic changes on synaptic transmission on the timescale used here is small.

Association of Ca channels and the release trigger

The structural organization of active zones and thus their functional properties vary from one synaptic connection to another (Mshina et al. 1999; Poage and Meriney 2002; Sätzler et al. 2002; Stanley 1997; Wachman et al. 2004). The spatial relations between Ca channels and the readily releasable synaptic vesicles and their associated release trigger within an active zone determine the release mode and reliability of a given synaptic connection. There are thought to be three main modes of relation between Ca channels and the release trigger (Augustine 2001; see also Neher 1998).

1) Ca nanodomains arise from local diffusion from single open Ca channel located in intimate proximity to a release trigger (few nanometers). Activation of just a single Ca channel can release acetylcholine at calyx-type presynaptic nerve terminal of the chick ciliary ganglion with the distance from Ca channel to release trigger being about 20 nm and the Ca2+ concentration near release trigger being about 10 μM (Stanley 1993). Secretion at such a release site is triggered by Ca2+ that enters through a discrete, specifically associated cluster of Ca channels. In such a cluster the members nearest the release trigger are the most effective. Such an organization has the advantage of allowing a molecular interaction between the release mechanism and its triggering Ca channels (Gentile and Stanley 2005; Stanley 1993, 1997).

2) Ca microdomains arise from multiple open Ca channels, clustered together but relatively distant from the release trigger. The involvement of multiple Ca channels, in some cases of different types, creating overlapping Ca2+ influx to produce microdomains gating transmitter release has been widely described (Fossier et al. 1993; Mintz et al. 1995; Qian and Noebels 2001; Reid et al. 1998; Wu and Shaggau 1994, 1997; Wu et al. 1998, 1999) and summarized and modeled by Meinrenken et al. (2002, 2003) for the rat calyx of Held synapse. The model assumes that the distance from a vesicle to Ca channel clusters varies across multiple release sites of a single calyx synapse from 30 to 300 nm, with Ca2+ peak concentrations from 0.5 to 40 μM. Such a topography leads to release probability ranging from <0.01 to 1. One to a few clusters can be present at an active zone, and one to a few vesicles (release triggers) may be under control of one cluster.

3) Radial gradients of Ca2+ are the result of Ca influx through Ca channels, randomly/uniformly distributed at ≥1 μm from a release trigger. In chromaffin cells, where transmitter release is much slower than at synapses, Ca channels and vesicles are not closely localized and radial diffusion of Ca2+ from distant Ca channels activates release triggers (Chow et al. 1994; Marengo and Monck 2000; Neher 1998; Neher and Augustine 1992). The inhibitory synapses between heart interneurons appear to incorporate all three of these types of organizations.

**Proposed organization of Ca channels at release sites for the synaptic connections between heart interneurons**

A proposed organization of Ca channels at release sites within an active zone of synaptic connections between heart interneurons is presented in Fig. 11. We hypothesized earlier (Ivanov and Calabrese 2003) that release sites for spike-mediated (high-threshold) transmission in heart interneurons are conventional with high-threshold Ca channels closely as-
associated with a low-affinity secretory trigger (see Augustine et al. 1992; Linhas et al. 1995; Neher 1998; Stanley 1993, 1997) (possibly synaptotagmin 1) closely associated with synaptic vesicle membranes (Sudhoff 2002, 2004; Sugita et al. 2002). This hypothesis is supported by our current findings. High-threshold synaptic transmission (spike-mediated transmission and transmission evoked by brief or prolonged depolarizations to \(-10\) mV and higher) is insensitive to intracellularly injected EGTA (Ivanov and Calabrese 2003) and is not very sensitive to caged and uncaged diazo-2 (which is built on the fast Ca\(^{2+}\) chelator BAPTA) (Fig. 6; see also Ivanov and Calabrese 2003). These responses to fast and slow Ca\(^{2+}\) chelators suggest a very close association (<40 nm) of the release trigger and high-threshold Ca channels (see Adler et al. 1991; Augustine et al. 1992; Fedchynshin and Wang 2005; Meinrenken et al. 2002, 2003; Neher 1998).

Such a close association of high-threshold Ca channels and the release trigger may argue for direct binding interactions between synaptic proteins and Ca channels that may be an essential determinant of synaptic transmission, although the necessity of such coupling for synaptic transmission in invertebrates is still unclear (Arien et al. 2003; Atlas 2001; Catterall 1999; Jarvis and Zamponi 2001, 2005; Spafford and Zamponi 2003; Spafford et al. 2003). A narrowly restricted localization of a small number of HVA Ca channels to release sites is consistent with our almost complete inability to record changes in intracellular Ca fluorescence in response to brief high-threshold depolarizations (see Ivanov and Calabrese 2000). The same conclusion follows from the much shorter synaptic delay for htIPSCs compared with the synaptic delay for gIPSCs with substantially smaller differences between latencies for \(I_{\text{CaHT}}\) and \(I_{\text{CaF}}\) (Fig. 10).

Because synaptic modulation of spike-mediated transmission depends on background Ca\(^{2+}\) arising from LVA Ca channels but is independent of spiking activity (thus from Ca\(^{2+}\) entering by high-threshold Ca channels), the high-affinity facilitation binding site (enhancer) appears to be localized more distant from HVA Ca channels than the release trigger (Ivanov and Calabrese 2003), perhaps to prevent its saturation by Ca\(^{2+}\) entering the cell by HVA Ca channels. The separation of HVA Ca channels from the enhancer by a docked vesicle, suggested for activity-dependent facilitation by Zucker and colleagues (Tang et al. 2000; Zucker 1999; Zucker and Regehr 2002) and Shahrezaei and Delaney (2004), is an attractive possibility. We have been able yet to determine the cooperativity of HVA channels in mediating release; i.e., how many HVA Ca channels contribute to transmitter release. Nevertheless, HVA Ca channel–dependent release appears to be based on typical nanodomains (Augustine 2001) with Ca channel(s) localized in very close proximity to docked/fusion ready vesicle.

The ability of \(I_{\text{CaF}}\) to effectively occlude high-threshold transmission argues for release sites shared by LVA and HVA channels. The sensitivity of \(I_{\text{CaF}}\)-dependent release to both fast and slow Ca\(^{2+}\) chelators (Ivanov and Calabrese 2003; present findings), the cooperativity of \(I_{\text{CaF}}\) channels in triggering release (Ivanov and Calabrese 2006), the longer synaptic delay (compared with HVA Ca channel–dependent synaptic transmission) suggests a more distant localization of \(I_{\text{CaF}}\) channel clusters from release trigger than for the HVA Ca channels. This hypothesized localization—clustered \(I_{\text{CaF}}\) channels within active zone at a distance from the release trigger—resembles the clustering of Ca channels proposed for rat calyx of Held synapse where Ca\(^{2+}\) microdomains are thought to trigger release (Meinrenken et al. 2002, 2003). Because of the massive influx of Ca\(^{2+}\) by \(I_{\text{CaF}}\) channels, Ca\(^{2+}\) concentration at the release enhancer is sufficient to evoke enhancement of high-threshold synaptic transmission. The release cooperativity of about 2 (see Fig. 6 in Ivanov and Calabrese 2006) for \(I_{\text{CaF}}\) channels does not necessarily mean that the clusters consists of just two Ca channels, but rather that not less than two \(I_{\text{CaF}}\) channels in any given cluster have to be open to evoke transmitter release. Wachman et al. (2004) found that in frog motor nerve terminal there is a remarkably low probability of a given Ca channel opening within an active zone after an action potential. Thus Ca\(^{2+}\) microdomains arising from clustered Ca channels appear to be responsible for \(I_{\text{CaF}}\) channel–dependent transmitter release.

The lower effectiveness of slow low-threshold Ca channels in transmitter release suggests that they are widely distributed throughout active zone, nonclustered, and evoke release and enhancement of high-threshold synaptic transmission by radial diffusion of Ca\(^{2+}\) from relatively distant Ca channels to the release trigger and the release enhancer, respectively (Ivanov and Calabrese 2006).

Such an organization of release sites appears optimal for economical reciprocal inhibitory synaptic transmission between heart interneurons that increases the robustness of rhythmic bursting. A burst is initiated when LVA Ca currents activate supporting depolarization and spiking and produce a gIPSC, which serves to terminate the burst of the opposite heart interneuron. As the burst progresses, LVA Ca currents inactivate, leading to a progressively decreasing gIPSC, and spike-mediated (-HVA) synaptic transmission, which serves to keep the opposite heart interneuron silent, increases; increased background Ca\(^{2+}\) resulting from LVA Ca currents enhances this transmission.

These considerations do not exclude the possibility that there are release sites that are purely under control of LVA Ca channels or that there are release sites with different secretory triggers, such as synaptotagmin 7, which has relatively high Ca\(^{2+}\) affinity and slow dynamics and seemingly subserves asynchronous release (Sudhoff 2002, 2004; Sugita et al. 2002). Such release sites must be in the minority, however, or the occlusion between high- and low-threshold synaptic transmission would not be so complete.

Shared release sites for spike-mediated and graded transmission in leech heart interneurons is inconsistent with the hypothesis of Matthews (2000) that low Ca\(^{2+}\) affinity is associated with sustained neurotransmitter release, whereas the brevity of Ca\(^{2+}\) signals driven by action potentials allows for higher Ca\(^{2+}\) affinity and greater integration of local Ca\(^{2+}\) signals. In heart interneurons, coexisting Ca channels of different types appear to be localized differently relative to the low-affinity release trigger and to the high-affinity enhancer at release sites, and thus there is the potential for both kinetic competition between the release trigger and Cr\(^{2+}\) buffers for Ca\(^{2+}\) (Augustine et al. 1991) and the interfering effect of Ca\(^{2+}\) buffers on Ca\(^{2+}\) that diffuses from distant Ca channels to the release trigger (Meinrenken et al. 2003). Ultimately, our data support the hypothesis of Jones (2003) that the role of a Ca channel in neurotransmitter release is determined less by gating kinetics than by the channel location. The occurrence of multiple
calcium channel types may indicate that channels are specialized for different functions (Jones 2003), but we have yet to determine the specific functions of the different Ca channels types at release sites.

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