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Spike-mediated and graded inhibitory synaptic transmission between leech interneurons: Evidence for shared release sites.

Andrei I. Ivanov and Ronald L. Calabrese
Department of Biology, Emory University, Atlanta GA 30322 USA

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Shared sites for spike-mediated and graded transmission

Correspondence to:
Andrei I. Ivanov
Department of Biology
Emory University
1510 Clifton Road N.E.
Atlanta, GA 30322 USA
Andrei.Ivanov@emory.edu
Phone: (404)727-4202
Fax: (404)727-2880
Inhibitory synaptic transmission between leech heart interneurons consist of two components; graded, gated by Ca\(^{2+}\) entering via low-threshold (LVA) Ca channels, and spike-mediated, gated by Ca\(^{2+}\) entering via high-threshold (HVA) Ca channels. Changes in presynaptic background Ca\(^{2+}\) produced by Ca\(^{2+}\) influx through LVA channels does modulates 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 employ the same release sites and, thus, how Ca\(^{2+}\) influx via 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. Employing different stimulating paradigms and inorganic Ca channels 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 via HVA channels to release transmitter. Uncaging of Ca chelator corroborate that graded release occludes spike-mediated.

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 (Wu and Shaggau 1994, 1997; Mintz et al. 1995; Reid et al. 1998; Meinrenken et al. 2003). On other hand, Ca$^{2+}$ entering the cell through Ca channels of different type 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 organized similarly (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, 2005; Pan et al. 2001; Warzecha et al. 2003), and in some cases the involvement of LVA Ca channels in transmission is well documented (Angstadt and Calabrese 1991; Ivanov and Calabrese 2000, 2005; Pan et al. 2001).

Transmitter release in these neurons resembles catecholamine secretion in chromaffine cells (Artalejo et al. 1994), where activation of different Ca channels leads to different mode of neurosecretion, and different Ca channels control catecholamine secretion with different efficacies due to 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 HVA Ca channels) and graded (depends on two types of LVA Ca channels termed $I_{CaS}$ and $I_{CaF}$) (Angstadt and Calabrese 1991; Olsen and Calabrese 1996; Lu et al. 1997; Ivanov, Calabrese 2000). 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 (Arbas and Calabrese 1987; Angstadt and Calabrese 1991; Olsen and Calabrese 1996; Lu et al. 1997; Ivanov and Calabrese 2000, 2003). Moreover, we demonstrated in the preceding paper that both $I_{CaS}$ and $I_{CaF}$ mediate release from the same release sites (Ivanov and Calabrese 2005). We also showed that manipulating intracellular $Ca^{2+}$ concentration with Ca chelators had a stronger
effect on graded synaptic transmission than on spike-mediated transmission and its plasticity, indicating that 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-threshold (so, spike-mediated) and low-threshold (graded) transmission shared? To the extent that these vesicle pools and release sites are shared, does Ca\(^{2+}\) entering via HVA and LVA Ca channels have equivalent access to the release trigger?

In this study, we recorded pre- and postsynaptic currents from voltage-clamped heart interneurons bathed in 0 mM Na\(^{+}\)/5 mM Ca\(^{2+}\) saline. Employing different stimulating paradigms, inorganic Ca channels blockers, and photo-release of Ca\(^{2+}\) and Ca chelator, we were able to show that both graded and spike-mediated synaptic transmission depend on transmitter release from the same release sites using the same releasable pool of synaptic vesicles. Ca\(^{2+}\) ions entering cells through different Ca channels (low- and high-threshold) act to gate release of 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.
MATERIALS AND METHODS

Animals. Adult leeches (H. medicinalis) were obtained from Leeches USA and Biopharm and maintained in artificial pond water (Leeches USA) at ~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 Corp.) 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 CaCl₂, 10 glucose, and 10 N’-2-hydroxyethylpiperazine - N’-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 Olympus BX50WI fluorescent microscope with an Olympus 40x/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 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/5mM Ca²⁺ saline (in mM: 110.0 N-methyl-D-glucamine (NMDG), 4.0 KCl, 5.0 CaCl₂, 10.0 Glucose, 10.0 HEPES acid buffer, adjusted to pH 7.4 with KOH or HCl. In a few of these experiments, Ca²⁺ in the saline was reduced to 2 mM with suitable osmotic adjustment of NMDG to 115.0 mM. In some cases, 150 μM Cd²⁺, 1 mM Ni²⁺, 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 (SEVC) 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 chlorided 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, 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* section.

**Ca Imaging.** In some experiments, we monitor 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 minutes with a recording microelectrode. Changes of Calcium Orange fluorescence were continuously monitored and recorded with ICCD-350f CCD camera (Video Scope International, Ltd.), connected to the fluorescent microscope mentioned above, equipped with 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 40x/0.80W water immersion objective and Axon Imaging Workbench 4.0 (AIW 4.0) software with a Digidata 2000 interface (Axon Instruments, Inc.) on a Pentium III (Intel) computer. Intensifier gain and black (baseline) levels were adjusted to achieve minimal background fluorescence, convenient visualization of the filled neuron, and sufficient dynamic range for monitoring fluorescence changes.

Our setup permits the acquisition of full frame images of 640 x 480 pixels size at a resolution of 0.379 \(\mu\text{m}^2\) for 1 pixel (395 \(\mu\text{m}\) x 295 \(\mu\text{m}\) for full frame) with the Olympus 40x/0.80W water immersion objective. Changes of fluorescence were recorded from the approximate synaptic contact region of a heart interneuron (600-1200 pixels, 235-
470 $\mu$m$^2$), as described by Ivanov, Calabrese (2003). In all experiments, the maximal available acquisition rate (video rate, 30 Hz) was used, yielding a time resolution of 33 msec. Video signals were accumulated for 33 msec per image, without any kind of gating, using 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, Inc.) 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.

**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, 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 1000 $\mu$m, numerical aperture 0.22, Oriel Instruments) to an “ablation laser unit” (Photonic Instruments, Inc.), 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 # D-3034) is a photoactivatable
Ca$^{2+}$ scavenger; the nominal $K_d$ of Diazo-2 for Ca$^{2+}$ changes upon UV illumination from 2.2 $\mu$M to approximately 80 nM (Adams and Tsien 1993; Delaney 2000). Diazo-2 photo-release Ca$^{2+}$ chelator upon UV illumination at $<$360nm.

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, 40 KOH/HEPES, pH 7.2.

All protocols used for photo-release of caged Ca$^{2+}$ chelator were generated using the pCLAMP program CLAMPEX, 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 800-1600 msec during electrophysiological and Ca fluorescence data acquisition.

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 ($\Delta F/F$), 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 employed One-way ANOVA, Factorial ANOVA, and Repeated Measures ANOVA with post hoc comparisons made by Student T-test with Bonferonni correction for multiple comparisons (Bonferonni 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 Mean ± 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 (Arbas and Calabrese 1987; Angstadt and Calabrese 1991; Olsen and Calabrese 1996; Lu et al. 1997; Ivanov and Calabrese 2000, 2003). Spike-mediated transmission, on the other hand, is produced by HVA Ca channels of unknown distribution (Simon et al. 1994; Ivanov and Calabrese 2000, 2003). These findings left unanswered two questions addressed in the present study. Are the vesicle pools and release sites for high-threshold (so, spike-mediated) and low-threshold (graded) transmission shared? To the extent that these vesicle pools and release sites are shared, does Ca\(^{2+}\) entering via 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 mV to -40 mV that
evoked the two kinetically distinct LVA Ca currents presynaptically (I_{CaF} and I_{CaS}) (Angstadt and Calabrese 1991) and the corresponding postsynaptic responses (gIPSC_F and gIPSC_S) (Fig. 1C). We also elicited high-threshold transmission, using a train of “artificial spikes” at 2.5 Hz from holding potential of -40 mV that evoked HVA Ca current presynaptically and the corresponding postsynaptic (spike-mediated) responses (smIPSC’s). The train of artificial spikes evoked robust smIPSC’s with typical (compare to 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 holding potential of -70 mV, however, failed to elicit smIPSC’s 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 smIPSC’s comparable in amplitude to those elicited from -40 mV (Fig. 1B). The “absolute” peak amplitude of these IPSC’s (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. 1A2 and B2). The peak amplitude of smIPSC’s (i.e., amplitude relative to the baseline postsynaptic current recorded just before the onset of any given smIPSC; see Fig. 1D), however, increased concomitantly with the decrease in the gIPSC (P). While changes in peak amplitudes of smIPSC’s elicited from holding potential of -40 mV and “absolute” peak amplitude of IPSC’s 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 smIPSC’s elicited during the depolarization to -40 mV and in gIPSC
were strongly time-dependent ($p = 0.000013$ and 0.001, respectively, One-way ANOVA).

Thus it appears that strong graded synaptic transmission can occlude smIPSC’s 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 via 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 via HVA Ca channels to release transmitter. As shown in the preceding paper, the pool of vesicles available for release by LVA currents is indeed being replenished at this time (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 for example 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 gIPSC’s and, htlIPSC’s respectively, assuming their correspondence to graded and high-threshold transmission respectively.
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 2005), 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 gIPSC’s. The htIPSC’s evoked by the 1-s high-threshold depolarization delivered at the end of the low-threshold depolarizing step were small but clearly noticeable and persistent (see Ivanov and Calabrese 2006), but no htIPSC’s evoked by the brief high-threshold depolarizing pulses superimposed on the low-threshold step were detected. (HVA Ca current could not be recorded due to contamination with K current (Angstadt and Calabrese 1991) and here and in subsequent figures, current artifacts due to high-threshold (to ≥ -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 Ni²⁺, led to noticeable changes (Fig. 2B). The LVA Ca currents were significantly blocked (I_{	ext{CaF}} (P) in control: -1.265 (SD 0.115) nA, in 1 mM Ni²⁺-
containing saline: -0.385 (SD 0.011) nA, p = 0.000197, One-way ANOVA, Bonferroni test), as were the corresponding glIPSC’s (glIPSC (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 htIPSC’s 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 htIPSC’s 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), while LVA Ca currents (ICaF (P) in control: -1.109 (SD 0.434) nA, in 1 mM Ni²⁺-containing saline: -0.512 (SD 0.231) nA, p = 0.000332, One-way ANOVA, Bonferroni test), and corresponding glIPSC’s (glIPSC (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\textsuperscript{2+} as in previous experiments, the response to high-threshold depolarization remained unchanged (Fig. 3A and B, compare to Fig. 2). 1 mM Ni\textsuperscript{2+} blocked LVA Ca currents and the corresponding gIPSC’s, but htIPSC’s 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\textsuperscript{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 currents so that low-threshold transmitter release decays completely by 4\textsuperscript{th} s 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 htIPSC’s in control and in the presence of 1 mM Ni\textsuperscript{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\textsuperscript{2+} were not the result of some additional activation of LVA Ca channels but solely the result of Ca\textsuperscript{2+} entering the cell by HVA Ca
channels. We used 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+} to separate LVA and HVA Ca currents (see Fig. 10 & 11 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\textsuperscript{2+}, the postsynaptic response to the high-threshold depolarization (which followed 8-s low-threshold depolarization) was significantly increased (Fig. 4B, compare to Fig. 4A), but subsequent addition of 150 μM Cd\textsuperscript{2+} that selectively blocks HVA Ca current (Lu et al. 1997; Ivanov and Calabrese 2000) significantly blocked this response (Fig. 4D: \(ht\text{IPSC (P)}\) in control: 0.737 (SD 0.083) nA, in 1 mM Ni\textsuperscript{2+}-containing saline: 1.322 (SD 0.304) nA (p = 0.016746, One-way ANOVA, Bonferroni test), in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline: 0.386 (SD 0.356) nA (p = 0.000491, compared to 1 mM Ni\textsuperscript{2+}-containing saline, One-way ANOVA, Bonferroni test). The peak amplitude of \(ht\text{IPSC}'s\) was significantly influenced by the experimental conditions used (control, 1 mM Ni\textsuperscript{2+}-containing saline, 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline) (p = 0.0000001, One-way ANOVA). \(ht\text{IPSC time-to-peak in 1 mM Ni\textsuperscript{2+} compared to 150 μM Cd\textsuperscript{2+}-containing saline was significantly increased ((ht\text{IPSC (P) time-to-peak in control: 103.80 (SD 36.03) ms, in the saline containing 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{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\textsuperscript{2+} and 150 μM Cd\textsuperscript{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-msec
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 (htIPSC’s) as in Fig. 1. In 1 mM Ni^{2+} saline, LVA Ca currents were strongly reduced as was the corresponding gIPSC, but the htIPSC’s were greatly increased indicating an absence of occlusion (Fig. 5B). In saline containing both 1 mM Ni^{2+} and 150 μM Cd^{2+}, htIPSC’s 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 influenced by the experimental conditions used (control, 1 mM Ni^{2+}-containing saline, both 1 mM Ni^{2+} and 150 μM Cd^{2+}-containing saline) (p = 0.020252, One-way ANOVA) with significant differences between gIPSC’s (P), recorded in control and both experimental salines (p = 0.029803 for 1 mM Ni^{2+}-containing saline, and p = 0.039093 for 1 mM Ni^{2+} and 150 μM Cd^{2+}-containing saline, One-way ANOVA, Bonferroni test). The amplitude of htIPSC’s (Fig. 5D, htIPSC) were also significantly influenced by experimental conditions used (control, 1 mM Ni^{2+}-containing saline, 1 mM Ni^{2+} and 150 μM 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 while a train of artificial spikes was superimposed on a 5-s depolarizing step to -40 mV. The stimulus protocol evoked a robust glIPSC and greatly diminished htIPSC’s that recovered somewhat at the end of the step (Fig. 6A). Diazo-2 itself is a weak Ca chelator (nominal $K_d$ = 2.2 μM) (Adams and Tsien 1993; Delaney 2000), thus intracellular injection of Diazo-2 changed LVA Ca currents, apparently by slowing the inactivation of $I_{Ca_F}$ similarly to intracellularly injected BAPTA (see Lu et al. 1997; Ivanov and Calabrese 2003). Each of two subsequent releases of Ca chelator (Fig. 6B and C) led to suppression of the glIPSC with a simultaneous increase in htIPSC’s amplitudes. Two minutes after the last light flash, the glIPSC was strongly suppressed, while htIPSC’s amplitudes were strongly increased (Fig. 6D). To quantify the effects of Ca$^{2+}$ chelator uncaging on glIPSC’s and htIPSC’s, we averaged the amplitudes of glIPSC’s (P) and htIPSC’s (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 glIPSC (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. The inability of Diazo-2 (both caged and uncaged) to block htIPSC’s, argues that HVA Ca channels are closer to release sites than LVA Ca channels.

The 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 gIPSC’s due to a progressive decrease in LVA Ca channel mediated Ca$^{2+}$ influx (Ivanov and Calabrese 2005). Due to an increase in presynaptic intracellular background Ca$^{2+}$ associated with a more depolarized holding potential, however, the mean amplitude of htIPSC’s, evoked from progressively depolarizing presynaptic holding potential, increases (Ivanov and Calabrese 2003). In these experiments, we used presynaptic holding potential 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 employed the protocol used in our preceding 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 mV to -45
mV with increments of 5mV). 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 gIPSC’s progressively decreased, as described in the
preceding paper (Ivanov and Calabrese 2006), and the amplitudes of the htIPSC’s
progressively increased (Fig 7A, bottom (6) to top (1)). The amplitude of the htIPSC,
evoked by the pulse applied before depolarizing step (htIPSC₁) from a presynaptic
holding potential of -45 mV was no more than 2 times the amplitude of the htIPSC₁
evoked from -70 mV. (This modest increase in amplitude of htIPSC₁ with increasing
holding potential (Fig. 7B) can be attributed to an increase in intracellular background
Ca²⁺ (c.f. Ivanov and Calabrese 2003).) On the other hand, the amplitude of htIPSC
evoked by pulse applied after depolarizing step (htIPSC₃) from a presynaptic holding
potential of -45 mV was 6 times greater than that from -70 mV, and the htIPSC evoked
by pulse applied during depolarizing step (htIPSC₂) from -45 mV was >10 times greater
than that from -70 mV. Similar results were obtained in n = 6 preparations and
averaged data is presented in Fig. 7B. The amplitude of htIPSC’s were significantly
affected by presynaptic holding potential (Repeated Measures ANOVA; p = 0.000003)
and by when the htIPSC’s were evoked (before, during, or after) with respect to the low
threshold depolarization (-40 mV) (Repeated Measures ANOVA; p = 0.000001).
Moreover, the interaction between these factors had a significant effect on htIPSC’s
amplitude (Repeated Measures ANOVA; p = 0.000001). A strong negative correlation
was observed between the amplitudes of both binned (according to holding potential)
non-normalized htIPSC₂’s (Fig. 7C, top panel) and htIPSC₃’s with the peak amplitude of
the binned (according to holding potential) non-normalized $g_{IPSC_F}$'s ($g_{IPSC_F}$ (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 non-binned non-normalized $ht_{IPSC_2}$ vs. non-binned non-normalized $g_{IPSC_F}$ (P) also showed a significant negative correlation (R = -0.7571, $p = 0.0001$) (Fig. 7C, bottom panel). The prominent increase in the amplitude of $ht_{IPSC_2}$ and of $ht_{IPSC_3}$ is consistent with decreased depletion of readily releasable synaptic vesicles for high-threshold transmission due to the progressive decrease in low-threshold (graded) transmitter release. The modest increase in amplitude of $ht_{IPSC_1}$ with increasing holding potential (Fig. 7B) was most likely the result of an increase in intracellular background Ca$^{2+}$ (c.f. 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 block selectively 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 $g_{IPSC}$'s at all presynaptic holding potentials (Fig. 8B1a, 8B1b). (For the effect of presynaptic holding potential on $I_{CaF}$ (P) and $g_{IPSC_F}$'s (P), respectively, $p = 0.005734$ and $p = 0.0000001$, Factorial ANOVA; for effect of 1 mM Ni$^{2+}$ on $I_{CaF}$ (P) and $g_{IPSC_F}$'s (P), $p = 0.0000001$ and $p = 0.048534$, respectively, Factorial ANOVA.) The amplitude of the $ht_{IPSC_2}$'s (Fig. 8B2) and the $ht_{IPSC_3}$'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 $ht_{IPSC_2}$'s (P) and $ht_{IPSC_3}$'s (P), $p = 0.000005$ and $p = 0.005734$, respectively, Factorial ANOVA; for effect of 1 mM Ni$^{2+}$ on $ht_{IPSC_2}$'s (P) and $ht_{IPSC_3}$'s (P), respectively, $p = 0.011216$ and $p = 0.000676,$
Factorial ANOVA), but at all holding potentials htlPSC₁’s were not significantly different than in Control saline (p = 0.505282, Factorial ANOVA) (Fig. 8A). The negative correlations between the binned (according to holding potential) non-normalized amplitude of both htlPSC₂’s and htlPSC₃’s with the binned (according to holding potential) non-normalized gIPSCᵣ’s (P) in the presence of 1 mM Ni²⁺-containing saline were as strong as in Control saline. In Control saline, R = -0.997 (p = 0.00282) for htlPSC₂’s, and in 1 mM Ni²⁺-containing saline, R = -0.999 (p = 0.0007636). The increase of htlPSC’s measured during and after low-threshold depolarization is consistent with a decreased depletion of readily releasable synaptic vesicles due to decrease in low-threshold synaptic transmission brought on by Ni²⁺ blockade of LVA Ca channels.

*Reducing Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ saline (n = 6 different preparations). The presynaptic cell was filled with Ca Orange to monitor changes in intracellular Ca²⁺. Recordings were performed first in 2 mM Ca²⁺ saline and then in 5 mM Ca²⁺ 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 Ca²⁺
saline, the amplitude of htIPSC’s evoked from -70 mV were smaller and the time-course to attainment of maximal htIPSC amplitude delayed compared to those in 5 mM Ca\textsuperscript{2+} saline. The amplitude of htIPSC’s in 5 mM Ca\textsuperscript{2+} was significantly larger than in 2 mM Ca\textsuperscript{2+} saline (p = 0.0000001, Repeated Measures ANOVA) (Fig. 9A and C). The time from the beginning of first depolarizing pulse to maximal htIPSC in 5 mM Ca\textsuperscript{2+} was significantly shorter than in 2 mM Ca\textsuperscript{2+} saline: 1450.00 (SD 219.09) ms and 2170.00 (SD 219.09) ms, p = 0.000826 (One-way ANOVA, Bonferroni test). The simultaneously recorded presynaptic Ca signal was smaller and its build up delayed (The time from the beginning of the first depolarizing pulse to the maximal ΔF/F in 5 mM Ca\textsuperscript{2+} was significantly shorter than in 2 mM Ca\textsuperscript{2+} saline: 2450.80 (SD 1334.62) ms and 3966.80 (SD 596.16) ms, respectively, p = 0.048987 (One-way ANOVA, Bonferroni test). In 2 mM Ca\textsuperscript{2+} saline, the LVA Ca currents evoked by the 5-s depolarization to -40 mV and the corresponding gIPSC’s were decreased and delayed (Fig. 9B), while the presynaptic Ca signals were slightly diminished and strongly delayed (See Fig. 8 in Ivanov and Calabrese 2006). The htIPSC’s evoked during the depolarization to -40 mV, however, were increased. The amplitudes of htIPSC’s in 5 mM Ca\textsuperscript{2+} saline was significantly smaller than in 2 mM Ca\textsuperscript{2+} saline (p = 0.000008, Repeated Measures ANOVA) (Fig. 9B and D). Factorial ANOVA indicates a significant cooperative effect (p = 0.0000001) of Ca\textsuperscript{2+} driving force and presynaptic depolarization on htIPSC’s amplitudes. Thus, although a decrease in Ca driving force reduced Ca\textsuperscript{2+} influx via LVA and presumably also HVA Ca channels leading to decreased in gIPSC’s and htIPSC’s evoked from -70 mV, htIPSC’s evoked during a depolarizing step to -40 mV (i.e. during low-threshold stimulation) increased. This increase in htIPSC’s 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 via LVA Ca channels on htIPSC’s. In 5 mM Ca\(^{2+}\) saline, this effect was masked by severe depletion of the readily releasable pool.

The different latency 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 mimic action-potential-mediated synaptic transmission (Fedchyshin and Wang 2005; Gentile and Stanley 2005; Borst and Sakmann 1999). We used a similar approach here: the presynaptic cell was depolarized from a holding potential of -70 mV to -30 mV (low-threshold depolarization) and to 10 mV (high-threshold depolarization) with pulses of increasing duration (5 ms to 50 ms in increments of 5 ms), while the postsynaptic cell was held at -35 mV (n = 8 different preparations). Across the different pulse durations, htIPSC’s 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 htlPSC latency was 10.36 (SD 3.53 ms); p = 0.0000001, Repeated Measures ANOVA) than the corresponding glPSC’s (Fig. 11A). The latency between the pulse and the first recorded I$_{CaF}$ was 4.1 (SD 1.8) ms (n = 8 different preparations). Although we had difficulty recording I$_{CaHT}$ to determine its latency, it is unlikely that the large difference in synaptic delay between glPSC’s and htlPSC’s can be accounted for solely by a difference in latency of I$_{CaF}$ and I$_{CaHT}$. A small brief inward current was detected in 7 of the 8 preparations during high-threshold depolarization (data not shown) at a latency of 2.0 (SD 0.2) ms, significantly different from the latency of I$_{CaF}$ to low-threshold depolarization (One-way ANOVA, p = 0.010686, Bonferroni test). htlPSC’s were observed in some preparations in response to the 5-ms pulse and in all preparations in response to the 10-ms pulse, while glPSC’s 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 Ni$^{2+}$-containing saline suppressed LVA Ca current and corresponding IPSC’s, but had no effect on amplitude of high-threshold depolarization-evoked IPSC’s (data not shown). These data indicate that very brief high-threshold depolarizations (up to 15 ms) from a hyperpolarized presynaptic holding potential evoke postsynaptic responses that depend mainly or exclusively on Ca$^{2+}$ entry via I$_{CaHT}$, while depolarizations of longer duration lead to more complex mixed responses with low-threshold Ca currents contributing to release. The consistently shorter synaptic delay of htlPSC’s 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 Ca\(^{2+}\) for enhancement of spike-mediated transmission (Ivanov and Calabrese 2003), while 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 (Msghina et al. 1999; Quigley et al. 1999; Millar et al. 2005), 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 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) makes 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?

Employing different stimulating paradigms, inorganic Ca channels blockers, and photo-release Ca\(^{2+}\) chelator, we were able to support the hypothesis that both graded
synaptic transmission and spike-mediated synaptic transmission employ the same release sites using the same readily releasable pool of synaptic vesicles. Ca\(^{2+}\) ions entering cells through different Ca channels (LVA and HVA) act to gate release of the same synaptic vesicles. Moreover, our observations on the relative latency of htIPSC’s vs. gIPSC’s further supports the hypothesis that HVA Ca channels are more closely associated with the release trigger than LVA Ca channels. 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) can not be completely ruled out, though 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 Ni\(^{2+}\), which blocks LVA Ca channels, with an almost uniform increase in amplitude of htIPSC’s 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 glPSC’s by uncaged Ca\(^{2+}\) chelator immediately leads to an increase in amplitudes of htlPSC’s.

Another potential concern is an uneven intracellular Ca\(^{2+}\) distribution throughout neuritic tree and, especially, release sites. Ivanov and Calabrese (2000) showed that the time course of changes in intracellular Ca fluorescence due to activation of LVA Ca channels is uniform throughout 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 the access to the most or all 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 localization 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, diffusion and removal of transmitter from the cleft, etc. 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. Fig. 5 and 8), uncaging of caged Ca\(^{2+}\) chelator (Fig. 6), changes in Ca\(^{2+}\) 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 time scale used here is small.
Association of Ca channels and the release trigger

The structural organization of active zones and thus their functional properties varies from one synaptic connection to another (Stanley 1997; Msghina et al. 1999; Poage and Meriney 2002; Sätzler et al. 2002; 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 approximately 20 nm and the Ca\textsuperscript{2+} concentration near release trigger being about 10 μM (Stanley 1993). Secretion at such a release site is triggered by Ca\textsuperscript{2+} 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 (Stanley 1993, 1997; Gentile and Stanley 2005).

(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 Ca\textsuperscript{2+} influx to produce microdomains gating transmitter release has been widely described (Fossier et al. 1993; Wu and Shaggau 1994, 1997; Mintz et al. 1995; Reid et al. 1998; Wu et al. 1998, 1999;
Qian and Noebels 2001), 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 Ca\(^{2+}\) peak concentrations from 0.5 to 40 \(\mu\text{M}\). 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 Ca\(^{2+}\) are the result of Ca influx through Ca channels, randomly/uniformly distributed at 1 \(\mu\text{m}\) or further from a release trigger. In chromaffine cells, where transmitter release is much slower than at synapses, Ca channels and vesicles are not closely localized and radial diffusion of Ca\(^{2+}\) from distant Ca channels activates release triggers (Neher and Augustine 1992; Chow et al. 1994; Neher 1998; Marengo and Monck 2000). 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 associated with a low-affinity secretory trigger (see: Augustine et al. 1992; Stanley 1993, 1997; Llinas et al. 1995; Neher 1998) (possibly synaptotagmin 1) closely associated with synaptic vesicle membranes (Sugita et al. 2002; Sudhoff
2002, 2004). 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 suggests a very close association (less than 40 nm) of the release trigger and high-threshold Ca channels (see: Adler et al. 1991; Augustine et al. 1992; Neher 1998; Meinrenken et al. 2002, 2003; Fedchyshin and Wang 2005).

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, though the necessity of such coupling for synaptic transmission in invertebrates is still unclear (Catterall 1999; Atlas 2001; Jarvis and Zamponi 2001; Arien et al. 2003; Spafford and Zamponi 2003; Spafford et al. 2003; Jarvis and Zamponi 2005). A narrowly restricted localization of 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 htIPSC’s compared to the synaptic delay for gIPSC’s with substantially smaller differences between latencies for $I_{CaHT}$ and $I_{CaF}$ (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 via 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 via 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 (Zucker 1999; Tang et al. 2000; Zucker RS, Regehr WG 2002), and Shahrezaei and Delaney (2004), is an attractive possibility. We have been unable 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\(_{CaF}\) to effectively occlude high-threshold transmission argues for release sites shared by LVA and HVA channels. The sensitivity of I\(_{CaF}\)-dependent release to both fast and slow Ca\(^{2+}\) chelators (Ivanov and Calabrese 2003; current findings), the cooperativity of I\(_{CaF}\) channels in triggering release (Ivanov and Calabrese 2006), the longer synaptic delay (compared to HVA Ca channel dependent synaptic transmission) suggests a more distant localization of I\(_{CaF}\) channel clusters from release trigger than for the HVA Ca channels. This hypothesized localization - clustered I\(_{CaF}\) 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). Due to the massive influx of Ca\(^{2+}\) via I\(_{CaF}\) channels, Ca\(^{2+}\) concentration at the release enhancer is sufficient to evoke enhancement of high-threshold synaptic transmission. The release
cooperativity of ~2 (see Fig. 6 in Ivanov and Calabrese 2006) for I_{CaF} channels does not necessarily mean that the clusters consists of just two Ca channels, but rather that not less than two I_{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_{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, non-clustered, 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, which are purely under control of LVA Ca channels or that there are release sites with
different secretory triggers, e.g., synaptotagmin 7, which has relatively high Ca\textsuperscript{2+} affinity and slow dynamics and seemingly subserves asynchronous release (Sugita et al. 2002; Sudhoff 2002, 2004). 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\textsuperscript{2+} affinity is associated with sustained neurotransmitter release whereas the brevity of Ca\textsuperscript{2+} signals driven by action potentials allows for higher Ca\textsuperscript{2+} affinity and greater integration of local Ca\textsuperscript{2+} signals. In heart interneurons, coexisting Ca channels of different type appear to be localized differently relative to the low-affinity release trigger and to the high-affinity enhancer at release sites, thus there is the potential for both kinetic competition between the release trigger and Ca\textsuperscript{2+} buffers for Ca\textsuperscript{2+} (Augustine et al. 1991) and the intercepting effect of Ca\textsuperscript{2+} buffers on Ca\textsuperscript{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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FIG. 1. Spike-mediated IPSC’s (smIPSC’s) are occluded by strong graded IPSC’s (gIPSC). A1 - smIPSC’s, evoked by a train of artificial spikes, applied from presynaptic holding potential of -40 mV; A2 - data, as presented in A1, averaged over seven similar experiments (different preparations) are plotted vs. the time. B1 - smIPSC’s 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 seven similar experiments (different preparations) are plotted vs. the time. In A2 & B2: black filled circles – peak amplitude of smIPSC’s (smIPSC’s (P)); grey filled diamonds – absolute peak amplitude of IPSC’s (absolute IPSC’s (P)); empty boxes – amplitude of gIPSC’s 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 are recordings from different preparations. D: Method used to calculate amplitudes of IPSC’s for A2 and B2. Absolute peak amplitude of IPSC (P) = current at the peak of a given smIPSC – holding current; amplitude of smIPSC (P) = current at the peak of a given smIPSC – postsynaptic current just before the onset of the smIPSC; amplitude of gIPSC = current just before the onset of a given smIPSC – 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). $I_{Ca}$ – presynaptic Ca current; $I_{CaF}$, $I_{CaS}$. 
and $I_{CaHT}$ – fast and slow LVA Calcium currents, and HVA Calcium current, respectively. IPSC – inhibitory postsynaptic current; $gIPSC$ and $smIPSC$ – graded and spike-mediated inhibitory postsynaptic current; $gIPSC$ and $htIPSC$ - inhibitory postsynaptic current, evoked by $I_{CaF} + I_{CaS}$, and $I_{CaHT}$, respectively. $(P)$ – peak signal, thus $smIPSC (P)$ means peak spike-mediated postsynaptic current.

FIG. 2. High-threshold (depolarization-evoked) transmission (htIPSC) is occluded by strong low-threshold transmission (gIPSC) and blockade of LVA $I_{Ca}$, and thus low-threshold transmission with 1 mM Ni$^{2+}$, relives 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 $I_{Ca}$ and IPSC’s recorded in Control saline (5 mM Ca$^{2+}$/0 mM Na$^{+}$). B: presynaptic $I_{Ca}$ and IPSC’s recorded 4 min after beginning superfusion with 1 mM Ni$^{2+}$-containing saline. C: $I_{CaF} (P)$, $gIPSC (P)$, and $htIPSC (P)$, as illustrated in A & B, averaged over three similar experiments (different preparations). In 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 $I_{CaF} (P)$, $gIPSC (P)$, and $htIPSC (P)$ recorded in 1 mM Ni$^{2+}$-containing saline are significantly different than those recorded in Control saline. Here and in subsequent figures, for p value, see the text.

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 htIPSC’s 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 bi-phasic postsynaptic response with the low-threshold depolarization-evoked gIPSC decaying completely by the 4th s after the beginning of depolarizing step. **B**: 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_{CaF} (P), gIPSC (P), and htIPSC (P), as illustrated in **A & B**, averaged over five similar experiments (different preparations). In **C** data recorded in Control saline are presented in black and 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 to Control saline, while htIPSP was not changed. Asterisks indicate that I_{CaF} (P) and gIPSC (P) recorded in 1 mM Ni^{2+} -containing saline were significantly different than those recorded in Control saline.

**FIG. 4.** Using the stimulus paradigm of Fig. 2, 150 μM Cd^{2+} blocks the Ni^{2+}-augmented htIPSC’s (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 to Control saline (A), 1 mM Ni^{2+}-containing saline (B) blocked both I_{Ca} and the corresponding gIPSC evoked by low-threshold depolarization and increased the htIPSC following high-threshold depolarization. **C**: In 1 mM Ni^{2+} and 150 μM Cd^{2+}-containing saline, the htIPSP was substantially blocked. **D**: I_{CaF} (P), gIPSC (P), and htIPSC (P), as illustrated in **A, B & C**, averaged over five 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 grey. The black asterisk indicates htIPSC (P)
recorded in 1 mM Ni\textsuperscript{2+}-containing saline significantly different than that recorded in Control saline, and grey filled circle indicates htIPSC (P) recorded in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline was significantly different than that recorded in 1 mM Ni\textsuperscript{2+}-containing saline.

FIG. 5. Effects of 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{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 to Control saline (A), in 1 mM Ni\textsuperscript{2+}-containing saline (B), LVA I\textsubscript{Ca} and the corresponding gIPSC evoked by depolarization to -40 mV were substantially blocked, and the amplitudes of htIPSC’s, elicited by a superimposed train of brief depolarizing pulses to 10 mV were increased – relief of occlusion, see text. 150 μM Cd\textsuperscript{2+} and 1 mM Ni\textsuperscript{2+}-containing saline blocked htIPSC’s with a progressive decrease in I\textsubscript{Ca} and gIPSC due to lengthened exposure to 1 mM Ni\textsuperscript{2+} (C). D: gIPSC (P) and htIPSC (P), as illustrated in A, B & C, averaged over three similar experiments (different preparations). In D data recorded in Control saline are presented in black, data recorded in 1 mM Ni\textsuperscript{2+}-containing saline in white, and data recorded in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline in grey. Black asterisk indicates gIPSC (P) recorded in 1 mM Ni\textsuperscript{2+}-containing saline significantly different than that recorded in Control saline, and filled grey circle indicates gIPSC (P) recorded in 1 mM Ni\textsuperscript{2+} and 150 μM Cd\textsuperscript{2+}-containing saline significantly different than that recorded in Control saline.

FIG. 6. Effects of uncaging (photo-release) of caged Ca\textsuperscript{2+} chelator (DM-Nitrophen) on spike-mediated (high-threshold) and graded synaptic transmission between heart
interneurons. htIPSC’s were elicited by a train of artificial spikes superimposed upon a step depolarization (5 s) 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 htIPSC’s were occluded by strong graded transmission (gIPSP) but gradually begin to recover toward the end of the simulated burst. B1: The first uncaging of Ca\(^{2+}\) chelator prematurely terminated the gIPSP and promoted recovery of the htIPSP’s. C1: In this run, the previous uncaging of Ca\(^{2+}\) chelator continued to suppress the gIPSP and relieved some occlusion of the htIPSP’s. The second uncaging of caged Ca\(^{2+}\) chelator further promoted recovery of the htIPSP’s. D1: In this run, no further uncaging of caged Ca\(^{2+}\) chelator was performed, but the two previous uncagings of Ca\(^{2+}\) chelator continued to suppress the gIPSP and substantially relieved occlusion of the htIPSP’s. A2 – D2: Plots of htIPSC’s (P) (empty circles) and gIPSC’s (black line), extracted from A1 – D1. The grey bar indicates the time and duration of the uncaging light flash. E: Amplitudes of htIPSC’s (P) before and after Ca\(^{2+}\) chelator uncaging (as illustrated in A1, and D1), averaged over five similar experiments (different preparations). htIPSC’s recorded before Ca\(^{2+}\) chelator uncaging are shown in black, htIPSC’s recorded after Ca\(^{2+}\) chelator uncaging are shown in white.

FIG. 7. The effect of progressive incremental depolarization of the presynaptic holding potential on postsynaptic responses, elicited by brief high-threshold depolarizing pulses. Inactivation of LVA \(I_{\text{Ca}}\) prevents occlusion of high-threshold transmission by graded transmission. A1-6: \(I_{\text{Ca}}\) and IPSC’s evoked by 2-s step depolarization to -35 mV
from different holding potentials (-45 mV to -70 mV, respectively) with brief depolarizing pulses to 10 mV applied before, during and after the step depolarization. B: htlIPSC (P) evoked before (htlIPSC\(_1\), filled black circles) during (htlIPSC\(_2\), empty circles) and after (htlIPSC\(_3\), filled grey circles) low-threshold depolarization to -35 mV plotted vs. presynaptic holding potential. C: top panel: non-binned non-normalized htlIPSC\(_2\) (P) plotted vs. non-binned non-normalized peak gIPSC\(_F\) (gIPSC\(_F\) (P); black line – linear fit; bottom panel: binned averaged non-normalized htlIPSC\(_2\) (P) plotted vs. binned averaged non-normalized peak gIPSC\(_F\) (gIPSC\(_F\) (P). In B and C data were averaged over six similar experiments (different preparations).

FIG. 8. The 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\(_{Ca}\), 1 mM Ni\(^{2+}\) amplifies the effect of inactivation of LVA I\(_{Ca}\) in preventing occlusion of high-threshold transmission by graded transmission. A1-3: I\(_{Ca}\) and IPSC’s evoked by 2-s a 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 seven similar experiments (different preparations) plotted vs. presynaptic holding potential. B1a: I\(_{CaF}\) (P). B1b: gIPSC\(_F\) (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.

FIG. 9. The effect of extracellular Ca\(^{2+}\) concentration on graded and high-threshold synaptic transmission between heart interneurons. A: IPSC’s and fluorescent Ca signal
evoked by a train of 15-ms depolarizing pulses to 10 mV from presynaptic holding potential of -70 mV. B: IPSC’s and fluorescent Ca signal evoked by a train of 15-ms depolarizing pulses to 10 mV superimposed on 5-s depolarizing step to -40 mV from a presynaptic holding potential of -70 mV (simulated burst); In A and B, on the left recordings were made in 5 mM Ca\(^{2+}\)/0 mM Na\(^+\) saline (control saline) and on the right in 2 mM Ca\(^{2+}\)/0 mM Na\(^+\) saline. C: Amplitudes of htIPSC’s, evoked by a train of 15-ms depolarizing pulses to 10 mV from presynaptic holding potential of -70 mV. D: Amplitudes of htIPSC’s evoked by a train of 15-ms depolarizing pulses to 10 mV, superimposed on 5-s depolarizing step to -40 mV from presynaptic holding potential of -70 mV. Data were averaged over six similar experiments (different preparations). In C and D, data recorded in 5 mM Ca\(^{2+}\)/0 mM Na\(^+\) saline are shown in black and data recorded in 2 mM Ca\(^{2+}\)/0 mM Na\(^+\) saline in white.

FIG. 10. The effect of brief high-threshold and low-threshold presynaptic depolarizations of different duration on I\(_{ca}\) and corresponding IPSC’s. The presynaptic cell was depolarized from holding potential of -70 mV to -30 mV and to 10 mV with brief pulses of 5 to 50 ms duration, with a time increment of 5 ms. A1-4: Recordings of I\(_{ca}\) and IPSC’s evoked by presynaptic depolarizations of 5, 15, 25, and 35 ms, respectively. In blue are recordings in response to depolarization to -30 mV and in red recordings in response to depolarization to 10 mV. B: LVA I\(_{ca}\), htIPSC’s, and glIPSC’s evoked by presynaptic depolarizations of 5 to 50 ms, as partially illustrated in A, averaged over eight similar experiments (different preparations) vs. duration of the applied depolarization. B1: IPSC’s, ht IPSC’s (red) and glIPSC’s (blue); B2: I\(_{caF}\) (P) (black) recorded in response to depolarizations to -30 mV.
FIG. 11. Schematic of proposed organization of a release site at synaptic connections between leech heart interneurons. A HVA Ca channel (or a small cluster of such channels) is localized in very close (nearest) proximity to a docked fusion competent vesicle and release trigger (10-40 nm). Associated with the vesicle-trigger complex is an enhancer site responsible for homosynaptic enhancement of spike-mediated transmission by background Ca$^{2+}$ (see Ivanov and Calabrese 2003). The HVA channel(s) provides enough Ca$^{2+}$ to activate the release trigger but not sufficient to significantly activate the enhancer. Clusters of fast LVA Ca channels (I_{CaF} channels) are localized more distant (up to several hundred nm) from the release trigger and cooperate to produce Ca$^{2+}$ microdomains that provide enough Ca$^{2+}$ to activate the release trigger and to activate strongly the enhancer. Widely distributed around the release site, non-clustered slow LVA Ca channels (I_{CaS} channels) evoke release by radial diffusion of Ca$^{2+}$ from distant Ca channels to release trigger, and thus much less effectively that fast low-threshold Ca channels. They also provide background Ca$^{2+}$ that can activate the enhancer. The circle filled with red-to-blue color gradient is a fusion-competent synaptic vesicle. The yellow hexagon is the release trigger. The empty triangle is the enhancer. Light gray lines are the presynaptic membrane, and bright red, bright blue, and bright-green doubled elongated ovals, respectively are an HVA Ca channel, I_{CaF} channels, and I_{CaS} channels (shown in side view). Solid and dashed lines of the color corresponding to the Ca channel(s) are isolines of Ca$^{2+}$ concentration triggering and enhancing release, respectively.
Figure 1
Figure 2
Figure 3
A  Control

Post (IPSC)
→ -35 mV

Pre (I_{Ca})

Pre (V_m)
→ -70 mV

B  1 mM Ni^{2+}, 3 min

Post (IPSC)
→ -35 mV

Pre (I_{Ca})

Pre (V_m)
→ -70 mV

C  1 mM Ni^{2+} and 150 μM Cd^{2+}, 3 min

Post (IPSC)
→ -35 mV

Pre (I_{Ca})
→ -40 mV 0 mV 100 mV

Pre (V_m)
→ -70 mV 2 s

D  Currents, peaks

Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
**Figure 10**

**A**

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```
-40 mV
```

```
Preme (V_m)
```

```
-70 mV
```

**B**

1. htlPSC (P) & glPSC (P)

```
IPSC (nA)
```

```
0 0.2 0.4 0.6
```

```
5 msec 10 msec 20 msec 30 msec 40 msec 50 msec
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2. $I_{CaF}$ (P)

```
$I_{CaF}$ (nA)
```

```
0 0.4 0.8 1.2 1.6
```

```
0 5 max 10 max 15 max 20 max 25 max 30 max 35 max 40 max 45 max 50 max
```

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Ivanov & Calabrese

Shared sites for spike-mediated and graded transmission
Figure 11