Functional Role of Ca\textsuperscript{2+} Currents in Graded and Spike-Mediated Synaptic Transmission Between Leech Heart Interneurons

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Lu, Jin, John F. Dalton, IV, Darrell R. Stokes, and Ronald L. Calabrese. Functional role of Ca\textsuperscript{2+} currents in graded and spike-mediated synaptic transmission between leech heart interneurons. J. Neurophysiol. 77: 1779–1794, 1997. We used intracellular recording and single electrode voltage-clamp techniques to explore Ca\textsuperscript{2+} currents and their relation to graded and spike-mediated synaptic transmissions in leech heart interneurons. Low-threshold Ca\textsuperscript{2+} currents (activation begins below −50 mV) consist of a rapidly activating component (I_{Ca}\textsuperscript{r}) and a slowly activating component (I_{Ca}\textsuperscript{s}). The apparent inactivation kinetics of I_{Ca}\textsuperscript{r} appear to be influenced by Ca\textsuperscript{2+}: both the substitution of Ca\textsuperscript{2+} (5 mM) with Ba\textsuperscript{2+} (5 mM) in the saline and the intracellular injection of the rapid Ca\textsuperscript{2+} chelator, bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid (BAPTA), from the recording microelectrode, significantly increase its apparent inactivation time constant. The use of saline with a high concentration of Ba\textsuperscript{2+} (37.5 mM) permitted exploration of divalent ion currents over a broader activation range, by acting as an effective charge carrier and significantly blocking outward currents. Ramp and pulse voltage-clamp protocols both reveal a rapidly activating and inactivating Ba\textsuperscript{2+} current (I_{Ba}\textsuperscript{r}) and a less rapidly activating and slowly inactivating Ba\textsuperscript{2+} current with a broad activation range (I_{Ba}\textsuperscript{s}). Low concentrations of Cd\textsuperscript{2+} (100–150 μM) selectively block I_{Ba}\textsuperscript{r}, without significantly diminishing I_{Ca}\textsuperscript{r}. The current that remains in Cd\textsuperscript{2+} lacks the characteristic delayed activation peak of I_{Ba}\textsuperscript{r} and inactivates with two distinct time constants. I_{Ba}\textsuperscript{r} appears to correspond to a combination of I_{Ca}\textsuperscript{r} and I_{Ca}\textsuperscript{s}, i.e., to low-threshold Ca\textsuperscript{2+} currents, that can be described as T-like. I_{Ba}\textsuperscript{s} appears to correspond to a Ca\textsuperscript{2+} current with a broad activation range, which can be described as L-like. Cd\textsuperscript{2+} (100 μM) selectively blocks spike-mediated synaptic transmission between heart interneurons without significantly interfering with low-threshold Ca\textsuperscript{2+} currents and plateau formation in or graded synaptic transmission between heart interneurons. Blockade of spike-mediated synaptic transmission between reciprocally inhibitory heart interneurons with Cd\textsuperscript{2+} (150 μM), in otherwise normal saline, prevents the expression of normal oscillations (during which activity in the two neurons consists of alternating bursts), so that the neurons fire tonically. We conclude that graded and spike-mediated synaptic transmission may be relatively independent processes in heart interneurons that are controlled predominantly by different Ca\textsuperscript{2+} currents. Moreover, spike-mediated synaptic inhibition appears to be required for normal oscillation in these neurons.

INTRODUCTION

Networks of central neurons contribute to the patterning of almost all rhythmic movements in animals (Delcomyn 1980). Oscillation within these motor-pattern-generating networks results from the combination of intrinsic electrical properties of the component neurons and their synaptic interactions (Dean and Cruse 1995; Harris-Warrick 1993; Jacklet 1989; Rossignol and Dubuc 1994). In several pattern-generating networks, especially those found in invertebrate ganglia, graded synaptic transmission (almost invariably inhibitory) —release of transmitter by slow changes in membrane potentials and independent of action potentials per se (Burrows 1985; Siegler 1985)—is thought to play an important, if not dominant role in network function (Büsches 1995; DiCaprio 1989; Heitler and Pearson 1980; Mendelson 1971; Paul and Mulloney 1986a,b; Pearson and Fourtner 1975; Wolf and Büsches 1995) even when the usual spike-mediated transmission is present (e.g., see Granzow et al. 1985; Graubard et al. 1983; Johnson and Harris-Warrick 1990; Johnson et al. 1991; Mangan et al. 1994; Raper 1979). It is not clear whether spike-mediated and graded transmissions represent separate processes or form a continuum (Hartline and Graubard 1992). Because graded and spike-mediated transmissions are active in different ranges of presynaptic membrane potential, one possibility is that they are controlled by Ca\textsuperscript{2+} entry through different populations of Ca\textsuperscript{2+} channels with different activation ranges.

The motor pattern generating network for heartbeat in the leech, Hirudo medicinalis, is paced by the rhythmic alternating activity of two pairs of reciprocally inhibitory heart interneurons, which are located in the third and fourth segmental ganglia (Fig. 1) (for a recent review, see Calabrese et al. 1995). These neurons inhibit one another via both graded and spike-mediated transmissions (Fig. 1C) (Arbas and Calabrese 1987a,b; Nicholls and Wallace 1978a,b). Spike-mediated inhibitory postsynaptic potentials (IPSPs) are large and prominent during normal oscillations, and graded transmission is particularly noticeable when hyperpolarizing perturbations elicit rebound plateaus in one of the neurons (Fig. 1B). Indeed, in high Ca\textsuperscript{2+} (≥5 mM) low Na\textsuperscript{+} (20 mM) salines, spikes are suppressed and oscillations based solely on graded inhibition occur (Arbas and Calabrese 1987b; Nadim et al. 1995).

We have presented evidence from experiments in which two reciprocally inhibitory interneurons were voltage clamped, that graded transmission between heart interneurons is mediated by low-threshold Ca\textsuperscript{2+} currents with two kinetic components, one rapidly inactivating (I_{Ca}\textsuperscript{r}) and one slowly inactivating (I_{Ca}\textsuperscript{s}) (Angstadt and Calabrese 1991). In these experiments, the low-threshold Ca\textsuperscript{2+} currents were well characterized, but contamination with outward currents prevented measurement of Ca\textsuperscript{2+} currents at presynaptic voltages above −30 mV, so that the possibility of Ca\textsuperscript{2+} currents activated at more depolarized potentials, that might underlie spike-mediated transmissions remained unexplored. However, the low-threshold currents we described were inactivated by holding the presynaptic cell at −40 mV or above...
Cells are indexed by ganglion number and body side in this and subsequent figures. HN cells of 3rd and 4th segmental ganglia make reciprocal inhibitory connections across ganglionic midline, and each pair constitutes an independent neural oscillator. These oscillators are coordinated through their connections with HN(1) and HN(2) interneurons, which are here lumped together because they are functionally equivalent. B: typical alternating bursting activity of a pair of oscillator interneurons in an isolated ganglion in normal saline. HN(L,3) cell responds to a 6-s hyperpolarizing current pulse (DCC) with a pronounced sag potential and a robust plateau and intense impulse burst on release from the current pulse. The plateau is so intense that spikes are inactivated for a brief period, and associated with the plateau is strong graded inhibition of opposite HN neuron. C: transition to a burst in HN(L,3) cells and corresponding synaptic inhibition in the opposite HN neurons during normal oscillation (C1) and following a hyperpolarizing pulse (C2). Arrows indicate $-50 \text{ mV}$.

(Angstadt and Calabrese 1991), yet even from a holding potential of $-35 \text{ mV}$, depolarizing voltage pulses to $0 \text{ mV}$ elicited strong synaptic inhibition of the postsynaptic cell (robust IPSCs) (Simon et al. 1994), thus providing indirect evidence for other Ca$^{2+}$ currents that might underlie spike-mediated synaptic transmissions in these neurons.

Here we report on experiments in which we further explore low-threshold Ca$^{2+}$ currents and use saline with a high concentration of Ba$^{2+}$ to explore divalent cation currents over a broad voltage range. We then use selective block of a component of the Ca$^{2+}$ currents with Cd$^{2+}$ (100–150 mM) to provide direct evidence that graded and spike-mediated
synaptic transmissions are separate processes, controlled by different components of the Ca\(^{2+}\) current. We also use selective block of spike-mediated transmission with Cd\(^{2+}\) to demonstrate the necessity of this transmission in sustaining oscillation in heart interneurons in normal saline.

**METHODS**

**Animals**

Adult leeches (*H. medicinalis*) were obtained from Leeches USA and Biopharm and stored at 15°C.

**Preparation**

Leeches were anesthetized in cold saline, after which individual ganglia (midbody ganglion 3 or 4) were dissected and pinned in small, clear, Sylgard-coated Petri dishes. The sheath on the ventral surface of the ganglion was removed with fine scissors or microscalps. 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 acid buffer, adjusted to pH 7.4 with NaOH or HCl. Heart interneurons were identified by the posterolateral position of their somata on the ventral surface of the ganglion and by their characteristic pattern of rhythmical bursting. Once a cell was identified, the superfusate usually was switched to a Na\(^+\)-free (0 Na\(^+\)) saline with altered divalent cation concentrations appropriate to a given experiment. The Na\(^+\) was replaced with the appropriate concentration of N-methyl-D-glucamine (NMDG) necessary to maintain the osmotic strength of normal saline, given the alterations in divalent cation concentrations. Anions were provided by neutralizing the NMDG with HCl. Zero Na\(^+\) salines containing 5 mM Ca\(^{2+}\), or 5 or 37.5 mM Ba\(^{2+}\) were used. Cd\(^{2+}\) (100–150 mM), other divalent ions and organic channel blockers were added to some salines to block Ca\(^{2+}\) currents. Stock solutions of nitrendipine (5 mM) and nifedipine (10 mM) were prepared as ethanol stocks and diluted to final concentrations with 0 Na\(^+\), 37.5 mM Ba\(^{2+}\) saline just before use. The ethanol concentration in saline was <1%, and no noticeable effects of this concentration of ethanol on electrical properties of neurons were detected. The stocks were kept in the dark at 4°C. Calciumuline and \(\omega\)-conotoxin GVIA were dissolved in water and diluted to final concentrations with 0 Na\(^+\), 37.5 mM Ba\(^{2+}\) saline just before use. Stock solutions in water were stored in the dark at <20°C. All physiological salines were adjusted to pH 7.4.

Heart interneurons were penetrated with thin-walled (1 mm OD, 0.75 mm ID) borosilicate microelectrodes (A-M Systems). For the recording of normal activity and in current-clamp experiments, microelectrodes filled with 4 M K-acetate, 20 mM KCl (unbuffered, pH 8.4) were used. In voltage-clamp experiments, microelectrodes were filled with 2 M K-acetate, 2 M tetraethylammonium acetate (TEA-acetate) (unbuffered, pH 7.9), which blocks both \(I_h\)-like current (\(I_h\)) and delayed rectifier-like outward currents (\(I_{Ca}\) and \(I_{Na}\)) ([Simon et al. 1992](http://jn.physiology.org/)) and 20 mM KCl. In some experiments, electrodes containing the Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; potassium salt; Sigma Chemicals) were used. These microelectrodes were filled with 0.2 M BAPTA, 2 M K-acetate, and 20 mM KCl, or with 0.2 M BAPTA, 2 M K-acetate, 2 M TEA-acetate, and 20 mM KCl. These BAPTA-containing solutions were stored at <20°C. Microelectrodes had resistances of 30–45 MΩ and time constants of 0.2–0.5 ms.

**Voltage-clamp recordings** were made using an Axoclamp-2A (Axon Instruments) or a NPI SEC-05L (NPI) used in single-electrode voltage-clamp mode with a sampling rate of 2.5 and 20 kHz, respectively. Current clamp recordings were made with an Axoclamp-2A or a NPI SEC-05L used in discontinuous current-clamp (DCC) mode with a sampling rate of 2.5 and 20 kHz, respectively. In each case, the electrode potential was monitored on an oscilloscope to ensure that the potential settled between current injection cycles. Some current recordings were made with the same amplifiers in bridge mode. All recordings were referenced to a bath microelectrode of the same composition as the recording microelectrode; the bath itself was grounded through a chloridized silver wire. After a voltage-clamp experiment, microelectrodes were withdrawn from the cell, and only in experiments in which the electrode was within ±5 mV of bath potential were the data accepted. Thus all membrane potentials reported for voltage clamp experiments are accurate within a 10-mV range.

Data were digitized, stored, and analyzed on a 486 or Pentium (Intel) computer using Axotape or PCLAMP suite of software (Axon Instruments). All voltage-clamp data were acquired and voltage protocols 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. Each of these positive pulses was preceded by four negative pre-pulses of one-fourth magnitude and equal duration. The interval between the pre-pulses in the sequence, the delay between the pre-pulse sequence and the test pulse and the interval between pre-pulse-test pulse episodes were all adjusted for each different test pulse protocol so that the holding current returned to baseline between all pulses and/or pre-pulses by monitoring the holding current on-line with a chart recorder. The sum of the currents for these four pre-pulses was used as a measure leak current. All currents shown were leak subtracted automatically using this procedure in CLAMPEX unless otherwise noted. Although the raw (unsubtracted) currents were not digitized by CLAMPEX, they were monitored on-line with the chart recorder, so that we could verify that the estimated leak currents were time-invariant and approximately linear. In previous studies of low-threshold Ca\(^{2+}\) currents (Angstadt and Calabrese 1991), we digitized leak currents (using single negative voltage pre-pulses of equal magnitude) directly and subtracted them off-line. The results using the automatic procedure employed are similar to these previous results. Ramp protocols also were used, the height and duration of which were chosen to explore the currents optimally through reasonable voltage and time parameters.

Although the dendritic tree of the HN cell appears to be rather extensive, the cells are thought to be compact electrically, especially under the experimental conditions used here. When bathed in Na\(^+\)-free saline the membrane resistance of the cells is relatively large (50–100 MΩ), and many of the voltage-dependent currents were suppressed in our experiments. Use of Na\(^+\)-free saline eliminates Na\(^+\) currents, and K\(^+\) currents (\(I_{K}\) and \(I_{Na}\)) were suppressed partially with TEA, and/or BAPTA and/or Ba\(^{2+}\). These conditions support acceptable space-clamp. On the other hand, particularly for the fast currents (\(I_{Ca}\) and \(I_{Na}\)), the steepness of the activation curve indicates that there may have been some problems holding the clamp potential in distal areas of the dendritic arbor where these currents reside. Still, these fast currents were graded with pulse potential. Moreover, the focus of these studies was on the function role of Ca\(^{2+}\) currents rather than on a quantitative description of their biophysical properties.

Voltage-clamp data were analyzed and filtered (RC filter with a user defined time constant of >4 ms) using the PClAMP program CLAMPFIT. CLAMPFIT allows least-squares fits of exponential equations to current traces for the analysis of inactivation (or activation) time constants. Two different methods can be applied to make these fits in CLAMPFIT, the Simplex and Chebyshev methods. The former method is slower, requires a seed value, and is regarded as more accurate though subject to selecting local minima with certain seed values, while the later method is faster and does not require a seed value. Some of these data were fit with both methods and no differences were noted.
Inward currents recorded in 0 Na$^+$ saline with 5 mM Ca$^{2+}$ or 5 mM Ba$^{2+}$

Our previous experiments on low-threshold Ca$^{2+}$ currents demonstrated two kinetic components, one rapidly inactivating ($I_{CaF}$) and one slowly inactivating ($I_{CdF}$). Our data suggested that $I_{CaF}$ showed Ca$^{2+}$-mediated inactivation; inactivation was more rapid in elevated Ca$^{2+}$ saline (5 mM) compared with normal Ca$^{2+}$ saline (1.8 mM). To explore this possibility, we compared the inward currents of cells (penetrated with TEA microelectrodes—2 M K-acetate, 20 mM KCl) recorded first in 0 Na$^+$ elevated Ca$^{2+}$ saline (5 mM) and then in 0 Na$^+$ Ba$^{2+}$ saline (5 mM), using a pulse protocol ($V_{hold}$ of −70 mV, successive 1,500-ms pulses incremented by 2.5 mV) (Fig. 2, A and B). Currents were measured at peak amplitude and at 1 s from pulse onset to quantify both the rapidly and slowly inactivating components of the inward current (Fig. 2, C and D). Peak currents were greater with Ca$^{2+}$ as the charge carrier than with Ba$^{2+}$, but Ca$^{2+}$ and Ba$^{2+}$ currents were comparable in amplitude at 1 s. These results indicate that Ba$^{2+}$ is not as effective as a charge carrier for the rapidly inactivating inward current ($I_{CaF}$) as is Ca$^{2+}$.

The apparent inactivation kinetics of the rapidly inactivating component of the inward current differed markedly in Ca$^{2+}$ and Ba$^{2+}$ salines. To quantify this difference, we determined the inactivation time constant of the rapidly inactivating component of the inward current by fitting a single exponential to the rapidly inactivating portion of the current trace (Fig. 3). The inactivation time constants in Ba$^{2+}$ saline were on average about two times slower than those in Ca$^{2+}$ saline at each voltage pulse, and the time constant values in the two salines corresponding to the same voltage pulse showed a statistically significant difference (paired t-test; $P < 0.01$).

These results indicate that Ca$^{2+}$ entry influences apparent inactivation kinetics of $I_{CaF}$. To explore this latter possibility further, we compared the inactivation time constant of the rapidly inactivating component in elevated Ca$^{2+}$ saline (5 mM) of cells penetrated with TEA-filled microelectrodes and of cells penetrated with similar electrodes but with added BAPTA (0.2 M), a fast Ca$^{2+}$ chelator. With BAPTA-containing microelectrodes, the outward currents appeared to be more effectively blocked in 0 Na$^+$ 5 mM Ca$^{2+}$ saline than with the normal TEA-filled electrodes. Current records began to be contaminated with outward current with depolarizing pulses above −35.5 mV with normal TEA-filled electrodes (Fig. 2), but they remained apparently uncontaminated with pulses up to −25 mV when BAPTA was added to the microelectrodes (Fig. 4) ($n = 5$). This effect on outward currents was apparently due to BAPTA itself, because in separate experiments using microelectrodes containing BAPTA but not TEA (2 M K-acetate, 20 mM KCl, and 0.2 M BAPTA) comparable results were obtained ($n = 5$, data not shown). Under these conditions $I_{CaF}$’s activation appeared shifted to the right with the maximum current observed at about −35 mV (Fig. 4), as compared with −42.5 mV, when the current was recorded with normal TEA-filled microelectrodes. These results indicate that $I_{CaF}$ is indeed ‘low-threshold’ and transient. The late current, measured at 1 s after the onset of the pulse ($n = 5$), however, continued to increase (Fig. 4), indicating that $I_{CaF}$’s inactivation was slowed sufficiently so that it contributed to the current at 1 s, or that the more slowly inactivating $I_{CaF}$’s activation range might be broader than previously thought or that another different current was being activated. The inactivation time constant of $I_{CaF}$ was increased dramatically by BAPTA throughout its activation range (Fig. 4). At −37.5 mV, the inactivation time constant of $I_{CaF}$ was significantly greater when recorded with BAPTA containing electrodes [503 ± 162 ms (mean ± SE)] than when recorded with normal TEA electrodes (Fig. 3) (unpaired t-test $P < 0.02$). Thus intracellular Ca$^{2+}$ does influence the apparent inactivation kinetics of $I_{CaF}$.

Inward currents recorded in 0 Na$^+$ saline with 37.5 mM Ba$^{2+}$

To explore the full activation range of Ca$^{2+}$ currents in heart interneurons, we took advantage of two observations: Ba$^{2+}$ is an effective charge carrier for Ca$^{2+}$ channels in heart interneurons and high concentrations of Ba$^{2+}$ block outward currents in leech cells (see, e.g., Thompson and Calabrese 1992). Initial tests were done in 0 Na$^+$, 37.5 mM Ba$^{2+}$ saline with voltage-ramp protocol that took advantage of the relatively low threshold and rapid inactivation of the rapidly inactivating component of the Ba$^{2+}$ current to separate it from other Ba$^{2+}$ currents. From $V_{hold}$ of −70 mV, the membrane potential was ramped to +15 mV during the course of 2.8 s (Fig. 5A). The currents obtained were leak subtracted based on extrapolation of $I_{frac}$ obtained from the first 800 ms of the resulting current, which was linear. The leak subtracted currents were plotted versus membrane voltage to produce an I-V plot (Fig. 5B). This ramp protocol clearly separated the current into two distinct components one that activated at relatively low threshold, reaching its peak amplitude at about −35 mV and inactivated during the ramp, and one that activated over a broad range but predominantly above −20 mV and appeared to inactivate little (if at all) during the ramp. These currents were designated $I_{frac}$ and $I_{fus}$, respectively, and their relationship to $I_{CaF}$ and $I_{CdF}$ will be considered in discussion. $I_{frac}$ had its maximum amplitude at approximately −35 mV on the ramp, whereas $I_{fus}$ reached its maximum amplitude at approximately −5 mV.
FIG. 2. Comparison of low-threshold inward currents obtained from depolarizations of heart interneurons in single-electrode voltage-clamp (SEVC) from a holding potential of -70 mV with Ca\(^{2+}\) and with Ba\(^{2+}\) as charge carrier and tetraethylammonium (TEA; 2 M) in microelectrode. 

A: traces obtained in 0 Na\(^{+}\) saline containing 5 mM Ca\(^{2+}\) elicited by voltage pulses to potentials to right of each record. B: traces obtained in 0 Na\(^{+}\) saline containing 5 mM Ba\(^{2+}\) elicited by voltage pulses to potentials to left of each record. C: I-V plots of currents obtained in 0 Na\(^{+}\) saline containing 5 mM Ca\(^{2+}\). D: I-V plots of currents obtained in 0 Na\(^{+}\) saline containing 5 mM Ba\(^{2+}\) (same cell as in A). In both C and D, values were calculated by averaging measurements from 5 trials at peak amplitude (I\(_{\text{CaF}}\) and I\(_{\text{BaF}}\)) and at 1 s from pulse onset (I\(_{\text{CaS}}\) and I\(_{\text{BaS}}\)). In each case, Ca\(^{2+}\) and Ba\(^{2+}\) current was measured in same heart interneuron. Error bars indicate standard errors of all averaged values.

In 0 Na\(^{+}\), 37.5 mM Ba\(^{2+}\) saline, with TEA microelectrodes, we used a conventional pulse protocol from V\(_{\text{hold}}\) of -70 mV to explore more fully I\(_{\text{BaF}}\) and I\(_{\text{BaS}}\) (Fig. 6A). To minimize the effects of run down that can occur in these experiments, the pulse protocol was performed on six heart interneurons in order of increasing pulse voltage and on five cells in order of decreasing pulse voltage and these data averaged (Fig. 6B). The pulses were 1,500 ms in duration and were produced in 10-mV increments with the smallest pulse to -60 mV and the largest pulse to +10 mV. I-V plots
FIG. 3. Average values of inactivation time constants of rapidly inactivating component of inward currents obtained from depolarizations of heart interneurons in SEVC from a holding potential of $-70$ mV. Cells were bathed in 0 Na$^+$ saline containing either 5 Ca$^{2+}$ or 5 mM Ba$^{2+}$. Recording electrodes all contained TEA (2 M) and in some cases 1,2-bis(2-aminophenoxy)ethane-$N,N',N''$-tetraacetic acid (BAPTA; 0.2 M). Data obtained without BAPTA were from 5 heart interneurons bathed successively in Ca$^{2+}$- and Ba$^{2+}$-saline (same cells as in Fig. 2), and data obtained with BAPTA were from 5 different heart interneurons. Error bars indicate standard errors.

FIG. 4. Effect of BAPTA on low-threshold Ca$^{2+}$ currents in heart interneurons measured in SEVC. Currents, obtained in 0 Na$^+$ saline containing 5 mM Ca$^{2+}$ with TEA (2 M) and BAPTA (0.2 M) in recording microelectrode, were elicited from a holding potential of $-70$ mV by voltage pulses, which started at $-55$ mV and rose to $-35$ mV in 2.5-mV increments.
were constructed for the peak $I_{\text{BaF}}$ and the peak $I_{\text{BaS}}$. These plots show that $I_{\text{BaF}}$ reaches its maximum amplitude at approximately $-20$ mV, whereas $I_{\text{BaS}}$ peaks at $0$ mV. During a depolarizing pulse, $I_{\text{BaS}}$ reaches its peak amplitude after $200–250$ ms, well after $I_{\text{BaF}}$ is inactivated substantially (Fig. 6A).
FIG. 6. Ba\(^{2+}\) currents in heart interneurons obtained from a holding potential of \(-70\) mV by pulse depolarizations ranging from \(-60\) mV to \(+10\) mV (10-mV increments) of heart interneurons in SEVC in 0 Na\(^+\) saline containing 37.5 mM Ba\(^{2+}\). A: currents elicited by increasing voltage pulses to the potentials to left of each record. B: I-V plot of \(I_{\text{BaF}}\) and \(I_{\text{BaS}}\). A total of 11 cells were recorded, 6 in which pulses were applied in increasing order and 5 in which pulses were applied in decreasing order. Values were calculated by averaging measurements from all 11 cells at peak amplitude of rapidly inactivating current \([I_{\text{BaF}(\text{peak})}]\) and at peak amplitude of the slowly inactivating current \([I_{\text{BaS}(\text{peak})}]\). Error bars indicate standard errors.

Sensitivity of \(I_{\text{BaF}}\) to Cd\(^{2+}\)

In 0 Na\(^+\), 37.5 mM Ba\(^{2+}\) saline, using TEA-filled microelectrodes and a pulse protocol, we tested various Ca\(^{2+}\) channel blockers (Table 1) to determine whether any selectively blocked one component of the Ba\(^{2+}\) current. The organic Ca\(^{2+}\) channel blockers nifedipine, nitrendipine (Janis and Triggle 1991), calcicludine (Schweitz et al. 1994), and \(\omega\)-conotoxin GIVA (Kasai et al. 1987) had no detectable effect on \(I_{\text{Ba}}\) (Table 1). The inorganic channel blocker, Zn\(^{2+}\) (1 mM), nonselectively blocked both components of \(I_{\text{Ba}}\). However, in five different heart interneurons, 100 \(\mu\)M Cd\(^{2+}\) effec-
TABLE 1.

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* No effect indicates that a blocker had no detectable and reproducible effect on Ba²⁺ currents. Effect indicates that a blocker had a reproducible and substantial effect on Ba²⁺ currents (see Fig. 8).

Effectively blocked Iₚₚ at all pulse potentials, while hardly interfering with Iₚₚ particularly at pulse potentials below −10 mV (Figs. 7 and 8; Table 1). The current that remains in Cd²⁺ (100 μM) appears to decay smoothly with two time constants, and there is no characteristic slow activation of Iₚₚ that appears to account for the late current (Fig. 7B). We fit two exponentials to this decay for the pulse potential to −10 mV for five cells from five different preparations.

**FIG. 7.** Effect of Cd²⁺ (100 μM) on Ba²⁺ currents obtained from a holding potential of −70 mV by pulse depolarizations ranging from −40 to 0 mV of heart interneurons in SEVC in 0 Na⁺ saline containing 37.5 mM Ba²⁺. A: control current. B: current in presence of Cd²⁺. C: currents in B were subtracted from those in A to reveal current blocked by Cd²⁺. Records in A and B are from same cell.
and found average fast time constants of 56.7 ± 4.7 ms (n = 5), and slow time constants of 1,876 ± 935 ms (n = 3)—for two cells slow time constants could not be fit because the decay was so gradual as to be almost linear), respectively.

The difference current (Fig. 7C) in the presence and absence of Cd²⁺ (100 μM), albeit slightly contaminated with a small amount of \( I_{\text{BaF}} \), apparently blocked by Cd²⁺, particularly at the higher pulse potentials, reveals the characteristic less rapid activation of \( I_{\text{BaS}} \). The effects of Cd²⁺ on \( I_{\text{BaS}} \) were not reversible with persistent washing. Similar results to those obtained with Cd²⁺ (100 μM) were obtained with 200 μM Ni²⁺.

Similarly Cd²⁺ (100 μM) appears to block \( I_{\text{BaS}} \) selectively, while leaving \( I_{\text{BaF}} \) relatively unaltered, when these currents are separated using a voltage ramp protocol (Fig. 9). There is some current that remains in the presence of Cd²⁺ at the more depolarized end of the ramp, which may correspond to the slowly inactivating late current observed with the pulse protocol in the presence of Cd²⁺ (Fig. 7B).

Effects of Cd²⁺ (100 μM) on \( I_{\text{CaF}} \) and \( I_{\text{CaS}} \)

We explored the effect of Cd²⁺ on \( I_{\text{CaF}} \) and \( I_{\text{CaS}} \), so that we might correlate these calcium currents with the barium currents, \( I_{\text{BaF}} \) and \( I_{\text{BaS}} \), described above. We found that Cd²⁺ (100 μM) had very little effect on either \( I_{\text{CaF}} \) or \( I_{\text{CaS}} \) (Fig. 10). We obtained similar results with five heart interneurons from five different preparations.

Effects of Cd²⁺ (100 μM) on graded and spike-mediated synaptic transmission between heart interneurons

We reasoned that because \( I_{\text{CaF}} \) or \( I_{\text{CaS}} \) both contribute to the production of plateau potentials and are associated with graded synaptic transmission between heart interneurons (Angstadt and Calabrese 1991) and that these currents are not sensitive to low concentrations of Cd²⁺ (100 μM), it might be possible to determine whether Ca²⁺ currents corresponding to \( I_{\text{BaS}} \) might underlie spike-mediated synaptic inhibition between these neurons while \( I_{\text{CaF}} \) or \( I_{\text{CaS}} \) underlie graded synaptic inhibition. First we determined whether Cd²⁺ (100 μM) had any effect on plateau potential production in or graded synaptic inhibition between heart interneurons. Pairs of reciprocally inhibitory heart interneurons were recorded in 0 Na⁺, 5 mM Ca²⁺ saline, using DCC; one cell was held at −40 mV (presynaptic) and the other at −50 mV (postsynaptic) with injected current (Fig. 11). Plateau potentials were elicited in the presynaptic cell on termination of a hyperpolarizing current pulse (−0.4 nA), and the graded IPSP recorded in the postsynaptic cell. Cd²⁺ (100 μM) had little effect on either the plateau potential in the presynaptic cell or the graded IPSP recorded in the postsynaptic cell. Next we tested whether Cd²⁺ could block selectively spike-mediated inhibition between heart interneurons while leaving graded inhibition intact. Two reciprocally inhibitory heart interneurons were recorded in normal saline (1.8 mM Ca²⁺) using DCC (Fig. 12). Such pairs oscillated in normal alternation (Fig. 12A1). On termination of a hyperpolarizing current pulse injected into one neuron, a plateau was elicited that caused strong graded inhibition of the other interneuron and normal spike-mediated IPSPs were observed (Fig. 12A2). Addition of Cd²⁺ (150 μM) to the saline severely disrupted normal oscillation, but did not noticeably alter the average ‘resting’ potential of the heart interneurons.
neurons (Fig. 12B1). Robust plateaus and graded synaptic inhibition occurred on termination of hyperpolarizing current pulses, but no spike-mediated IPSPs were recorded in the presence of Cd²⁺ (Fig. 12B2). These results indicate that Cd²⁺-sensitive Ca²⁺ currents underlie spike-mediated inhibition between heart interneurons and that this spike-mediated inhibition is necessary for alternating oscillation in these neurons in normal saline. These results also suggest that graded synaptic inhibition between heart interneurons is not sufficient to sustain alternating oscillation in normal saline.

**DISCUSSION**

*Ca²⁺ currents in heart interneurons*

Our previous experiments implicated low-threshold Ca²⁺ currents in plateau production and graded synaptic transmission in heart interneurons (Angstadt and Calabrese 1991). In those studies, we demonstrated that Ca²⁺ currents with low threshold (below −50 mV) had two kinetic components, one rapidly inactivating and one slowly inactivating. These components were termed \( I_{CaF} \) and \( I_{CaS} \), respectively. These currents were characterized over the range of membrane potential of −60 to −30 mV and both appeared to saturate below −30 mV. Because of contamination with outward currents, we could not explore more depolarized membrane potentials, and thus cannot state definitively that the currents truly saturated. Both currents were implicated in plateau production and graded synaptic transmission (Angstadt and Calabrese 1991): 1) the duration of the plateaus is too long to be accounted for by \( I_{CaF} \) alone. 2) voltage-clamp protocols that simulate the voltage excursions associated with normal plateaus elicit both \( I_{CaF} \) and \( I_{CaS} \). 3) Graded transmission follows plateaus in duration and amplitude. 4) Brief voltage-clamp pulses that elicit predominantly \( I_{CaF} \) elicit correspondingly brief graded IPSP(C)s, whereas longer voltage pulses that elicit both \( I_{CaF} \) and \( I_{CaS} \) elicit correspondingly long graded IPSP(C)s. 5) Graded IPSP(C)s elicited by long voltage-clamp pulses show two decay time constants that correspond to the inactivation time constants of \( I_{CaS} \) and \( I_{CaF} \).

In those studies, no specific blocker for one or the other of these components was found that would allow us to separate these components and assess their different roles in plateau production and graded synaptic transmission (Angstadt and Calabrese 1991).

Our modeling studies (De Schutter et al. 1993) indicated that \( I_{CaF} \) and \( I_{CaS} \)—as characterized in our experiments (Angstadt and Calabrese 1991)—could not account for spike-mediated synaptic transmission between heart interneurons. Moreover, in experiments (Simon et al. 1994) where one heart interneuron was held in voltage clamp at −35 mV—a potential where both \( I_{CaF} \) and \( I_{CaS} \) had been shown to be inactivated completely (Angstadt and Calabrese 1991)—while its postsynaptic partner was clamped at −35 mV to reveal IPSCs, presynaptic voltage pulses to 0 mV elicited robust IPSCs. These results suggested to us a scenario in which heart interneurons possess two types of Ca²⁺ currents with distinctly different functions, one, a low-threshold type consisting of two kinetic components, \( I_{CaF} \) and \( I_{CaS} \), that mediates plateau formation and graded synaptic transmission, and another, with a broad activation range that underlies spike-mediated synaptic transmission. A primary goal of the experiments reported here has been to characterize this other type of Ca²⁺ current and to determine its role in spike-mediated synaptic transmission. A secondary goal was to determine whether \( I_{CaF} \) inactivation is Ca²⁺ mediated.

**Influence of intracellular Ca²⁺ on the apparent inactivation kinetics of \( I_{CaF} \)**

Both our results comparing the rapidly inactivating component of inward current in the same neurons in 0 Na⁺, 5 mM Ca²⁺ saline and 0 Na⁺, 5 mM Ba²⁺ saline and our
FIG. 10. Effect of Cd\(^{2+}\) (100 \(\mu\)M) on low-threshold Ca\(^{2+}\) currents in heart interneurons measured in SEVC in 0 Na\(^{+}\) saline containing 5 mM Ca\(^{2+}\). Currents were elicited from a holding potential of \(-70\) mV by voltage steps, which started at \(-55\) mV and rose to \(-35\) mV in 5-mV increments. A: control currents. B: currents in presence of Cd\(^{2+}\). C: currents in B were subtracted from those in A to reveal low-threshold currents blocked by Cd\(^{2+}\). Records in A and B are from same cell. Similar results were obtained in 5 preparations.

measurements of the inactivation time constant of \(I_{CaF}\) in cells recorded with BAPTA-filled microelectrodes, indicate that the time constant of the inactivation of \(I_{CaF}\) is Ca\(^{2+}\)-sensitive. Several Ca\(^{2+}\) currents in both invertebrates and vertebrates share this characteristic (Byerly and Hagiwara 1988; Eckert and Chad 1984; Hille 1992). Still there is need for caution in interpreting our results, because despite our efforts to block outward currents with internal TEA, the possibility remains that Ca\(^{2+}\)-mediated outward currents may contaminate our measurements. Both using Ba\(^{2+}\) as a charge carrier and BAPTA injection would be expected to block such contaminating currents and influence the apparent inactivation of \(I_{CaF}\).

These considerations emphasize the need for caution in interpreting inactivation kinetics of fast Ca\(^{2+}\) currents in heart interneurons.

\(\text{Ba}^{2+}\) currents in heart interneurons

We were not able to measure directly Ca\(^{2+}\) currents in heart interneurons at more depolarized potentials, primarily because of our failure to block outward currents effectively. External TEA (Simon et al. 1992) and Cs\(^{+}\)-filled microelectrodes (unpublished results) have proved ineffective. Our best efforts, before this study, at blocking outward currents have been with TEA-filled microelectrodes, which provide only a partial block so that no Ca\(^{2+}\) currents can be recorded at pulse potentials above \(-30\) mV. Thus in this study, we were forced to measure Ca\(^{2+}\) currents indirectly by replacing Ca\(^{2+}\) with Ba\(^{2+}\) as a charge carrier. The high concentration (37.5 mM) of Ba\(^{2+}\) in the saline employed, in addition to providing robust Ba\(^{2+}\) currents, had the additional salubrious effect of
blocking outward currents effectively when used in conjunction with TEA-filled microelectrodes. Under these experimental conditions, we were able to record largely uncontaminated Ba\(^{2+}\) currents over the range of membrane potential from \(-70\) to \(+15\) mV. Using a voltage-ramp protocol, we separated the Ba\(^{2+}\) currents into two types—rapidly inactivating and slowly inactivating. During the ramps, these currents had overlapping activation ranges, but clearly \(I_{\text{BaF}}\) reached its peak amplitude (around \(-40\) mV) and appeared fully activated well below \(I_{\text{BaS}}\)'s peak amplitude (around \(-10\) mV) (Fig. 5). Thus \(I_{\text{BaF}}\) appears to be a low-threshold transient current, while \(I_{\text{BaS}}\) can be viewed as broadly activating and persistent. Studies with pulse protocols confirm these impressions but the range of overlap of the activation is shown to be more extensive. Because \(I_{\text{BaF}}\) appears to inactivate below the maximum activation of \(I_{\text{BaS}}\) during the ramp protocol, the ramp protocol provides better separation of the two currents. However, the pulse protocol clearly reveals the delayed activation of \(I_{\text{BaS}}\), which does not reach peak amplitude until \(\sim 500\) ms after the start of the pulse (particularly at pulse potentials \(-20\) and above) during which time \(I_{\text{BaF}}\) is inactivated substantially (Figs. 6 and 7A).

Low concentrations of Cd\(^{2+}\) (100–150 \(\mu\)M) selectively block \(I_{\text{BaS}}\), while leaving \(I_{\text{BaF}}\) relatively unaltered, except at more depolarized pulse potentials (Figs. 7 and 8). The currents, observed with the pulse protocol, that remain in the presence of Cd\(^{2+}\) (100 \(\mu\)M) show little of the delayed activation of \(I_{\text{BaS}}\); they decay relatively smoothly with two time constants (\(\sim 50\) and 1,800 ms, respectively).

**Relation between Ba\(^{2+}\) currents and Ca\(^{2+}\) currents in heart interneurons**

A determination of relation between \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\), previously described (Angstadt and Calabrese 1991), the \(I_{\text{BaS}}\) and \(I_{\text{BaF}}\) described here, and ultimately the types of Ca\(^{2+}\) currents that heart interneurons possess requires careful consideration of the data. The experimental conditions under which Ba\(^{2+}\) currents were recorded were radically different from those under which Ca\(^{2+}\) currents were recorded; the high concentration of Ba\(^{2+}\) (37.5 mM)—a divalent cation which can dramatically alter membrane surface charge (Hille 1992)—necessary to record inward currents, uncontaminated by outward currents at depolarized potentials, undoubtedly shifts the activation characteristics of these currents as well as altering their inactivation characteristics. It is tempting to equate the Ba\(^{2+}\) currents one-for-one with their Ca\(^{2+}\) counterparts, and this possibility is not excluded by our data, but results with Cd\(^{2+}\) block support a more complicated scenario in which \(I_{\text{BaF}}\) equates to \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\), and \(I_{\text{BaS}}\) reflects a qualitatively different Ca\(^{2+}\) current. Thus \(I_{\text{BaF}}\) reflects the low-threshold type and \(I_{\text{BaS}}\) reflects the broadly activating type Ca\(^{2+}\) current mentioned above. In the presence of Cd\(^{2+}\) (100 \(\mu\)M), the less rapidly developing inward current associated with \(I_{\text{BaS}}\) is blocked selectively, but the remaining current decays (inactivates) with two distinct time constants much as the two low-threshold Ca\(^{2+}\) currents, \(I_{\text{CaF}}\) and \(I_{\text{CaS}}\), that we had previously described, decay with two distinct time constants. It is not possible to compare the time constants directly because the fast time constant is Ca\(^{2+}\) sensitive and because high concentrations of Ba\(^{2+}\) employed (37.5 mM) undoubtedly shift the voltage dependence of inactivation.
Regardless of which scenario is correct, it is clear that Cd\textsuperscript{2+} (100 μM) selectively blocks a type of Ca\textsuperscript{2+} current with a relatively broad activation range and slow inactivation characteristics, referred to henceforth as broadly activating Ca\textsuperscript{2+} current, while leaving a low-threshold-type rapidly inactivating current intact. In the presence of Cd\textsuperscript{2+} the remaining slowly inactivating current may represent a low-threshold current or the dregs of the broadly activating current.

It is with considerable reserve that we compare our findings in leech heart interneurons with the vast literature on vertebrate Ca\textsuperscript{2+} currents, particularly because the organic blockers that are so important in defining channels types in vertebrate neurons (Hille 1992; Tsien et al. 1988) do not appear to work on leech Ca\textsuperscript{2+} currents. Although there are exceptions (Trudeau et al. 1993), other invertebrate Ca\textsuperscript{2+} currents likewise do not fit well into the vertebrate scheme (Charlton and Augustine 1990; Tsien et al. 1988). Nevertheless, \( I_{\text{caF}} \) is similar to T-type currents found in vertebrate neurons that are low-threshold and rapidly inactivating. \( I_{\text{caF}} \) is also similar to T-type currents in that Ba\textsuperscript{2+} appears to be a less effective charge carrier than Ca\textsuperscript{2+} for this current. Unlike most T-type currents, \( I_{\text{caF}} \) is relatively insensitive to block by low concentrations of Ni\textsuperscript{2+} (200 μM) and may show Ca\textsuperscript{2+}-mediated inactivation. The broadly activating Ca\textsuperscript{2+} current identified here with \( I_{\text{bas}} \) appears L-like; it activates predominantly above −20 mV (at least in 37.5 mM Ba\textsuperscript{2+} saline), it has less rapid activation and slow inactivation kinetics, and it is sensitive to block by low concentrations of Cd\textsuperscript{2+}. The low-threshold Ca\textsuperscript{2+} current identified here with \( I_{\text{cas}} \) does not fit well into any vertebrate classification scheme. It is interesting in this regard that Grolleau and Lapié (1996) have been able to demonstrate convincingly using selective block with Ni\textsuperscript{2+} and selective inactivation with holding potentials, that DUM neurons of cockroach possess three different types of Ca\textsuperscript{2+} currents two low-threshold—one rapidly and one more slowly inactivating—and a high-threshold current.

**Separation of graded and spike-mediated synaptic transmission between heart interneurons**

Inhibition between reciprocally inhibitory heart interneurons consists of both a spike-mediated component and a graded component associated with the slow wave of oscillation in these neurons and with plateau potential formation (Arbas and Calabrese 1987a,b; Olsen and Calabrese 1996). Synaptic inhibition is necessary for oscillation in these neurons, because when all synaptic inhibition is blocked with bicuculline (10\textsuperscript{−4} M), oscillation stops and the neurons become tonically active (Schmidt and Calabrese 1992). We demonstrated here that plateau potentials in and graded synaptic transmission between heart interneurons is largely unaffected by Cd\textsuperscript{2+} (100−150 μM), indicating that the Ca\textsuperscript{2+} currents that control these processes, previously identified as \( I_{\text{caS}} \) and \( I_{\text{bas}} \), were largely unaffected by these concentrations of Cd\textsuperscript{2+}. This result and our results with Cd\textsuperscript{2+} block of \( I_{\text{bas}} \) allowed us to test the role of broadly activating Ca\textsuperscript{2+} currents in spike-mediated synaptic transmission between heart interneurons. We found that spike-mediated IPSPs were blocked by Cd\textsuperscript{2+} (150 mM), but that plateau production and graded inhibition in the same preparation were
largely unaffected. This result suggests that a broadly activating Ca\textsuperscript{2+} current, different from those that mediate plateau potentials and graded inhibition, underlies spike-mediated synaptic transmission. \( I_{\text{bad}} \) described here may correspond to this current. T-like currents have been associated with neurosecretion in pituitary cells (Cohen et al. 1988; Tsien et al. 1988), and they presumably play an important role in all graded synaptic transmission (see, e.g., Hartline and Graubard 1992). L-like currents are thought to participate in spike-mediated synaptic transmission and neurosecretion in several systems (Artalejo et al. 1994; Hille 1992; Tsien et al. 1988; Wang et al. 1993), while in others they are not associated with synaptic transmission but with activation of K\textsuperscript{+} currents (Fossier et al. 1993).

**Relative roles of spike-mediated and graded synaptic inhibition in sustaining oscillation in reciprocally inhibitory heart interneuron pairs**

Our observation that Cd\textsuperscript{2+} (150 \( \mu \)M) blocks alternating oscillation in reciprocally inhibitory pairs of heart interneurons in otherwise normal saline suggests that spike-mediated inhibition is necessary for and that graded inhibition is insufficient for oscillation in normal saline. This result fulfills the specific predictions of a detailed model of such an oscillatory pair of neurons that has been based on our previously obtained biophysical data on the intrinsic ionic and synaptic currents of heart interneurons (Nadim et al. 1995; Olsen et al. 1995). This model also predicts that spike-mediated synaptic transmission should be sufficient to sustain oscillation in heart interneurons, but unfortunately we have no selective blocker of graded inhibition in this system. In recent experiments designed to measure spike-mediated and graded transmissions, the graded inhibitory synaptic current was found to be relatively small when compared with the spike-mediated synaptic current (Olsen and Calabrese 1996). Graded inhibition was measured using voltage-clamp waveforms, which closely approximate the slow wave oscillation of heart interneurons, to drive the presynaptic interneuron (spikes were blocked by bathing the preparation in 0 Na\textsuperscript{+} saline), while the postsynaptic cell was held in voltage clamp. Spike-mediated inhibition was measured with the presynaptic heart interneuron oscillating normally in association with the greater heart interneuron network, while the postsynaptic cell was held in voltage clamp. Selective block of spike-mediated synaptic transmission may be possible in other pattern generating networks, where spike-mediated and graded synaptic transmission work in conjunction, thus also allowing a direct assessment of their relative importance. In the pyloric network of the stomatogastric ganglion, spike-mediated transmission was blocked by blocking spikes with tetrodotoxin (TTX) and oscillation remained robust but only in the presence of the neuromodulator dopamine (Raper 1979). Such an experiment could not be performed in our system because TTX and similar compounds are not effective in blocking spikes (Kleinhaus and Angstadt 1995). More recent experiments in this system (Johnson et al. 1991, 1995) and in the leech swim system (Mangan et al. 1994) demonstrate the dramatic sensitivity of graded transmission to neuromodulators and other environmental factors. These findings point out the possibility that the proper modulatory environment may permit oscillation based solely on graded transmission in heart interneurons. Indeed in high Ca\textsuperscript{2+} (\( \geq 5 \) mM) low Na\textsuperscript{+} (20 mM) salines, spikes are suppressed and oscillations based solely on graded inhibition occur in heart interneurons (Arbas and Calabrese 1987b; Nadim et al. 1995).

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