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Activity-Dependent Reduction in Voltage-Dependent Calcium Current in a Crayfish Motoneuron

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The effect of increased impulse activity upon voltage-dependent Ca^{2+} currents was studied in the cell body of a crayfish phasic motoneuron using two-electrode voltage-clamp technique. Increased electrical activity in this relatively inactive motoneuron produces a short-term and long-term reduction in the voltage-dependent Ca^{2+} current. Both forms of activity-dependent reduction in Ca^{2+} current are Ca^{2+} dependent. The short-term reduction in Ca^{2+} current appears to involve the Ca^{2+}-dependent inactivation of Ca^{2+} channels, previously described in a variety of neurons. The long-term reduction in Ca^{2+} current is produced by prolonged Ca^{2+} influx and persists for days: *in vivo* stimulation of the phasic motor axon at 5 Hz for 1 hr results in a 30% reduction in Ca^{2+} current density, which persists for at least 3 d. Both the short-term and long-term reductions in Ca^{2+} current appear to result from changes in a single type of high-voltage-activated (HVA) Ca^{2+} channel. Inhibition of protein synthesis attenuates the long-term reduction in Ca^{2+} current and has no effect upon the short-term Ca^{2+} current reduction. During the long-term reduction in Ca^{2+} current, it appears that Ca^{2+} channels located distant to the site of Ca^{2+} influx are affected. The relationship of these results to a previously described Ca^{2+}-dependent reduction in transmitter release is discussed.

[Key words: activity, calcium channel, inactivation, protein synthesis, plasticity, motoneuron, crayfish, voltage clamp]

Calcium influx plays an important role in the short- and long-term regulation of neuronal structure and function. The relationship between electrical activity and Ca^{2+} influx is largely determined by the properties of the neuron's voltage-dependent Ca^{2+} channels, and therefore the development and regulation of voltage-dependent Ca^{2+} channels is of considerable interest. Depolarization and Ca^{2+} influx have been shown to play a role in the regulation of voltage-dependent Ca^{2+} channels. In particular, a short-term Ca^{2+}-dependent inactivation of voltage-dependent Ca^{2+} channels has been described in many vertebrate and invertebrate neurons (Tillotson, 1979; Lux and Brown, 1984; Chad and Eckert, 1986; Kay and Wong, 1987; Gutnick et al., 1989; Williams et al., 1991; Johnson and Byerly, 1993b; Fryer and Zucker, 1993). Although much less is known regarding the long-term effect of Ca^{2+} influx upon voltage-dependent Ca^{2+} channels, recent cell culture studies have shown that prolonged depolarization resulting from high extracellular potassium produces a long-term reduction in Ca^{2+} current density (Franklin et al., 1992; Berdan et al., 1993) and the number of Ca^{2+} channels (DeLorme and McGee, 1986; DeLorme et al., 1988). The reduction in the number of Ca^{2+} channels is Ca^{2+} dependent (DeLorme et al., 1988).

It is not known whether long-term, Ca^{2+}-dependent changes in voltage-dependent Ca^{2+} channels are produced *in situ* by physiological stimulation. To determine this, we examined the effects of elevated impulse activity upon Ca^{2+} currents recorded from the cell body of a crayfish abdominal phasic motoneuron (F3). The normal impulse activity level of F3 is very low, allowing us to characterize Ca^{2+} channels under conditions of low impulse activity, and examine the short- and long-term effects of experimentally increasing impulse activity both *in vitro* and *in vivo*.

The effect of Ca^{2+} influx upon voltage-dependent Ca^{2+} channels in motoneuron F3 is of particular interest since we have recently demonstrated a long-term, Ca^{2+}-dependent decrease in transmitter release from its motor terminals. Tonic stimulation of crustacean phasic motoneurons produces long-term changes in the physiology and morphology of the motor terminals, including a long-lasting decrease in transmitter release (for review see Lnenicka, 1991). This long-term, activity-dependent reduction in transmitter release has been reported for both vertebrate and invertebrate motoneurons (Lnenicka and Atwood, 1985; Hinz and Wernig, 1988; Bradacs et al., 1990; Mercier and Atwood, 1990). We have shown that this reduction in transmitter release is Ca^{2+} dependent; that is, increased Ca^{2+} influx in the proximal region of F3 produces a long-term reduction in transmitter release from its motor terminals (Hong and Lnenicka, 1993a).

We report a Ca^{2+}-dependent reduction in voltage-dependent Ca^{2+} current density that has two phases: a short-term reduction that appears similar to the previously described Ca^{2+}-dependent inactivation of Ca^{2+} channels (Tillotson, 1979) and a long-term reduction that persists for at least 3 d.

A preliminary report of these findings has appeared (Hong and Lnenicka, 1993b).

Materials and Methods

Preparation

Juvenile crayfish, *Procambarus clarkii,* with carapace lengths of 2–3 cm were used in these experiments. Crayfish were obtained from Atchafalaya Biological Supply (Raceland, LA). The third abdominal gan-

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glion was isolated and desheathed in van Harrevald's solution (van Harrevald, 1936) containing 1 mM glucose and 10 mM HEPES (pH 7.4). Further details of the dissection and identification of the F3 soma have been previously reported (Hong and Lnenicka, 1993a). All experiments were performed at 15°C.

Measurement of Ca^{2+} currents

Conventional two-electrode voltage-clamp techniques were used for recording Ca^{2+} currents from the cell body of F3 using an Axoclamp-2A preamplifier (Axon Instruments Inc., Burlingame, CA). To block Na^+ and K^+ currents, 1 μM TTX, 50 mM TEA, and 1 mM 4-AP were added to the saline. Changes in osmolarity were compensated by decreasing the concentration of NaCl. To block K^+ currents further, the cell body was loaded with Cs^+ for 30–60 min using microelectrodes (5–10 MΩ) filled with 3 M CsCl (Tillotson, 1979; Gutnick et al., 1989). After blocking Na^+ and K^+ currents, the remaining inward current is carried by Ca^{2+}. The application of the Ca^{2+} channel blockers, 10 mM Co^{2+} or 6 mM Mn^{2+}, blocks the remaining inward current completely (Hong and Lnenicka, 1993b). Data were acquired (sampling rate 5 kHz) and analyzed using VCAN V3.0 software and a DMA interface (LabMaster, Scientific Solutions Inc., Solon, OH). The holding potential was set to −80 mV in most experiments. For some neurons, it was set to their resting membrane potentials of −70–−75 mV. All of the inward Ca^{2+} currents were corrected for linear leak currents by subtracting current produced by corresponding hyperpolarizing pulses. Calcium current density was determined by dividing the peak amplitude of Ca^{2+} current by the membrane capacitance. Current–voltage (*I–V*) curves were obtained by increasing depolarization in 10 mV steps from the holding potential at 3 min intervals. Unless otherwise indicated, a paired Students *t* test was used in all statistical comparisons.

Stimulation

In vitro *stimulation.* The effect of elevated impulse activity on the Ca^{2+} current was examined by stimulating F3 at 5 Hz for 30–45 min. Stimulation pulses were delivered through a suction electrode placed against the F3 axon, and the impulse activity of F3 was monitored by intracellular recording from the axon (Fig. 1). Calcium currents were measured from the stimulated motoneuron 6–7 hr later and compared to those of the contralateral control motoneuron. In addition, to determine if Ca^{2+} influx is required for the reduction in Ca^{2+} current, Ca^{2+} influx during stimulation was blocked by applying 1 mM Cd^{2+} to the bath. To further minimize the possibility of Ca^{2+} influx during stimulation, the extracellular Ca^{2+} concentration was reduced to 5 mM. (Extracellular Ca^{2+} concentrations less than 5 mM frequently resulted in unstable resting membrane potentials.)

To examine the voltage-dependent Ca^{2+} currents during and immediately after stimulation, the cell was voltage clamped and stimulated with 50 msec voltage steps to 0 mV at 1 or 5 Hz for 10 min. Ca^{2+} currents were measured during stimulation and at regular intervals for 1 hr after stimulation. To examine the effect of Ca^{2+} influx on the reduction in the Ca^{2+} current, the amount of Ca^{2+} influx was altered by changing the extracellular Ca^{2+} concentration or changing the amplitude of the depolarizing pulses. Stimulation was performed in high (2× normal, 27 mM) extracellular Ca^{2+} with voltage steps to 0 mV, or in normal extracellular Ca^{2+} with voltage steps to +35 mV. Changes in osmolarity of high Ca^{2+} (2×) saline were compensated by decreasing the NaCl concentration.

In vivo *stimulation.* To increase F3 impulse activity *in vivo,* crayfish were pinned with the ventral side up in a chamber filled with normal saline. After making a small slit along the midline of the third abdominal segment, a hook electrode was inserted through the slit and applied to the third root. Motoneuron F3 was stimulated at 5 Hz for approximately 1 hr; stimulation was applied for two 20–30 min periods separated by a 10–15 min rest. Activation of F3 was continuously monitored by recording electromyograms with two pin electrodes positioned through the cuticle near muscle A_{iii}VIII (Rayner and Wiersma, 1965). In sham-operated animals, the electrodes were positioned as described but no stimulation was delivered. After stimulation, the animals were kept in an aquarium and Ca^{2+} currents were measured 3 d later.

Double-pulse experiment

The Ca^{2+}-dependent inactivation of Ca^{2+} was examined with paired pulses delivered every 3 min. The amplitude of the prepulse (P1) was increased by 10 mV steps from the holding potential (−80 mV) to +80 mV, whereas the peak amplitude of the subsequent test pulse (P2) was fixed at 0 mV (see Fig. 6*A*). The double-pulse experiment was performed in normal (13.5 mM) Ca^{2+} or 13.5 mM Ba^{2+} (0 mM Ca^{2+}).

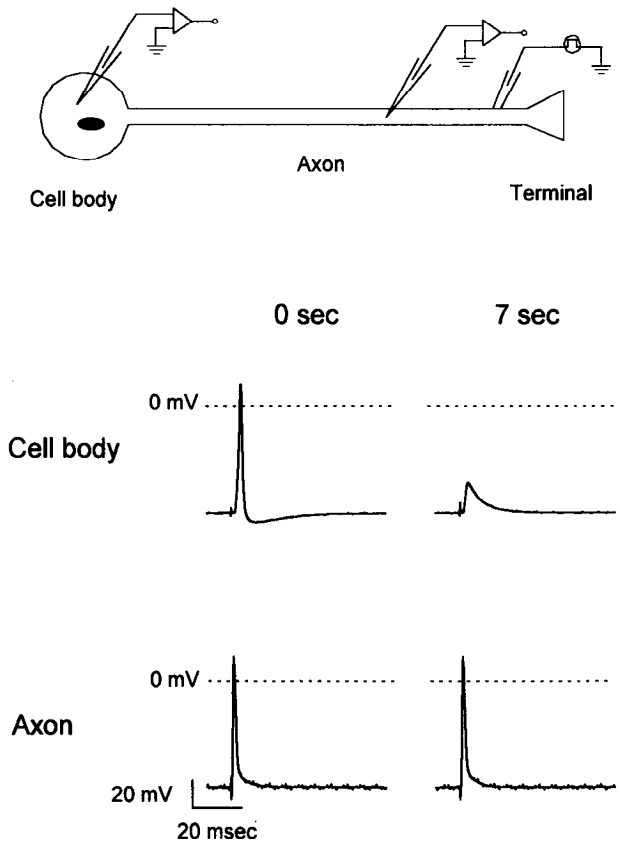

Figure 1. Stimulation of the phasic motoneuron, F3. *Top,* The axon was electrically stimulated with a suction electrode at 5 Hz for 30–45 min. Suprathreshold stimulation of the axon was verified by recording action potentials from the axon with an intracellular electrode. *Bottom,* The *upper panel* shows the electrical activity recorded from the axon and cell body at the onset of stimulation (0 sec) and 7 sec later. Action potentials are generated in the cell body only during the first few seconds of stimulation, and afterward only an electrotonic potential is observed in the cell body.

Inhibition of protein synthesis

The role of protein synthesis in the long-term reduction in voltage-dependent Ca^{2+} currents was examined by applying the reversible protein synthesis inhibitor 0.6 mM cycloheximide (CHX) (Boehringer Mannheim Corp., Indianapolis, IN) to the bath 4–5 hr prior to *in vitro* stimulation. CHX (0.6 mM) has been shown to produce a 90% block of protein synthesis and no immediate effect on neuromuscular transmission in the crayfish (Nguyen and Atwood, 1990). To examine the effect of CHX upon the short-term reduction in Ca^{2+} currents, stimulation (50 msec voltage steps to 0 mV at 1 Hz for 10 min) was performed in the presence of CHX. To examine the effect of CHX upon the long-term reduction in Ca^{2+} current, the axon of F3 was electrically stimulated at 5 Hz for 45 min *in vitro* in the presence of CHX. After stimulation, CHX was washed out and the preparation was maintained in normal saline. The Ca^{2+} currents were measured 6–7 hr after stimulation.

Results

Activity-dependent reduction in voltage-dependent Ca^{2+} current

To examine the effect of increased impulse activity upon the voltage-dependent Ca^{2+} current, the relatively silent motoneuron F3 was electrically stimulated *in vitro* (Fig. 1, top). The third

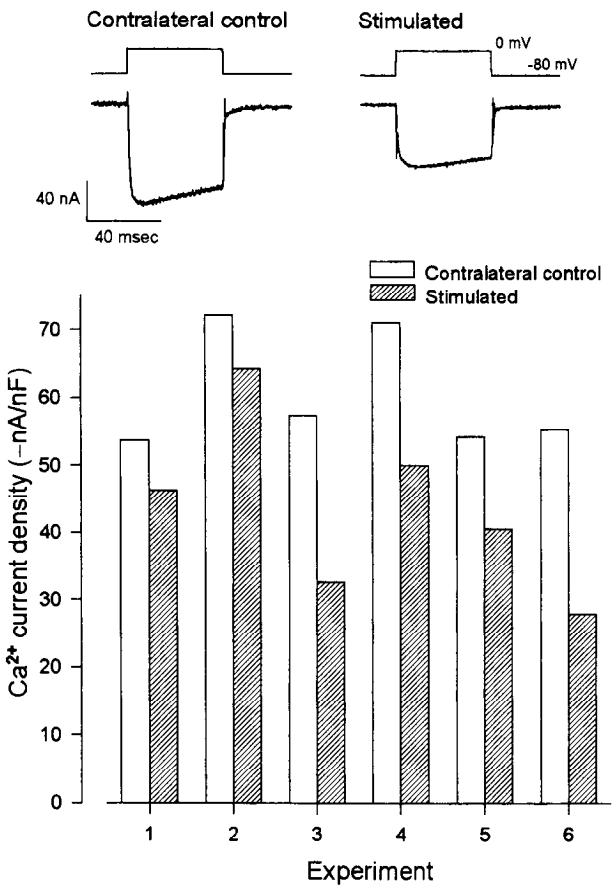

Figure 2. Increased impulse activity produces a long-term reduction in Ca^{2+} current density. The peak Ca^{2+} current was measured in stimulated and contralateral control cell bodies 6–7 hr after stimulation. *Top,* Both stimulated and contralateral control cell bodies were voltage clamped and the peak Ca^{2+} current was recorded during step depolarizations to 0 mV. *Bottom,* In all six experiments, the stimulated cell body had a smaller Ca^{2+} current density than the contralateral control. The Ca^{2+} current density was significantly less in stimulated (-43.6 ± 5.3 nA/nF) compared to contralateral control cells (-60.6 ± 3.5 nA/nF, $n = 6$; $p < 0.01$).

abdominal ganglion was isolated along with the fast flexor musculature. The F3 axon was stimulated at 5 Hz for 30–45 min, producing a substantial increase in normal impulse activity. Figure 1 (bottom) shows the electrical activity in the axon and cell body produced by suprathreshold electrical stimulation. The axon produces action potentials throughout the period of stimulation. However, after 30–50 stimulations the cell body fails to produce action potentials, leaving only an electrotonic depolarization of approximately 20 mV.

The effect of increased impulse activity was examined 6–7 hr after stimulation by measuring the amplitude of the peak Ca^{2+} current during depolarizing voltage steps to 0 mV in stimulated and contralateral control cell bodies (Fig. 2, top). In every experiment, the density of the peak Ca^{2+} current was less in the stimulated cell than the contralateral control cell (Fig. 2, bottom). The mean density of the voltage-dependent Ca^{2+} current was 28.7 ± 6.3% less in the stimulated cell bodies (-43.6 ± 5.3 nA/nF) compared to the contralateral control cell bodies (-60.6 ± 3.5 nA/nF, $n = 6$; $p < 0.01$). These results demonstrate that the increased impulse activity results in a decrease in the peak amplitude of the voltage-dependent Ca^{2+} current recorded from the cell body.

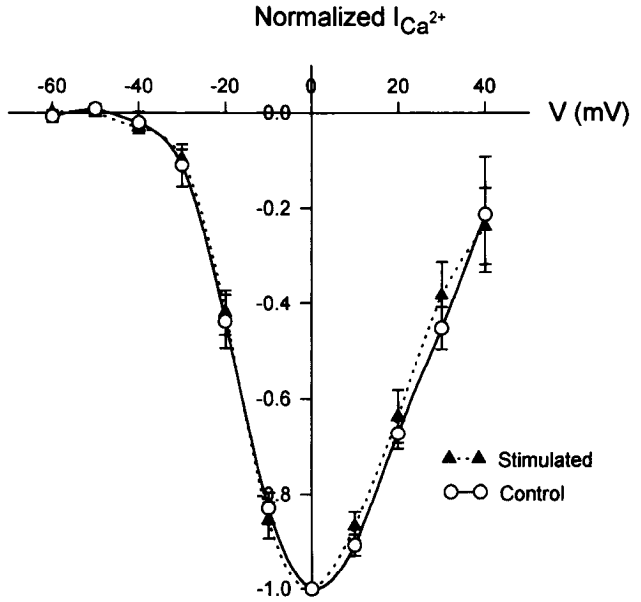

Figure 3. The long-term reduction in the Ca^{2+} current occurs in the absence of changes in the voltage dependence of the Ca^{2+} channels. *I–V* curves for stimulated and control motoneurons were compared. The *I–V* curves were normalized and mean values were obtained for nine stimulated and 12 control motoneurons. The *I–V* curves for the stimulated and control motoneurons are very similar. Thus, while the Ca^{2+} current density decreases as a result of stimulation, there is no change in the voltage dependence of the individual Ca^{2+} channels.

This reduction in Ca^{2+} current does not appear to involve changes in the voltage dependence or kinetics of the Ca^{2+} channels. The voltage-dependent Ca^{2+} conductance in this cell body appears to be dominated by a single class of HVA Ca^{2+} channels (S. J. Hong and G. A. Lnenicka, unpublished observations). The decrease in Ca^{2+} conductance occurs without a change in the voltage dependence of the Ca^{2+} channel since the normalized *I–V* curves from stimulated and contralateral control cells are identical (Fig. 3). The kinetics of the Ca^{2+} current decay during the pulse are not altered by stimulation since the decay follows a single exponential curve with a similar time constant (τ) in both control (215 ± 13 msec, $n = 6$) and stimulated cells (206 ± 10 msec, $n = 6$).

Long-term reduction in Ca^{2+} current persists for days

To examine the duration of this activity-dependent reduction in Ca^{2+} current, the motoneuron was stimulated *in vivo* and the Ca^{2+} current was measured 3 d later. As previously described (see Materials and Methods), the third root was electrically stimulated at 5 Hz for approximately 1 hr with a hook electrode, and activation of F3 was verified by recording electromyograms from muscle A_{iii}VIII. Three days later, the Ca^{2+} current was measured in the cell bodies of stimulated motoneurons and sham-operated control motoneurons (Fig. 4). The mean Ca^{2+} current density of stimulated cells (-40.8 ± 3.5 nA/nF, $n = 7$) was significantly less than that of sham-operated controls (-57.0 ± 2.4 nA/nF, $n = 5$; t test, $p < 0.01$). The procedure used for *in vivo* stimulation had no direct effect upon the Ca^{2+} currents since the Ca^{2+} current density of sham-operated controls was not significantly different from that of normal animals (-59.9 ± 2.0 nA/nF, $n = 32$; t test, $p > 0.1$). The percent reduction in Ca^{2+} current density (31.9 ± 5.8%, $n = 7$) 3 d after *in vivo* stimulation was similar to that observed 6–7 hr after *in vitro*

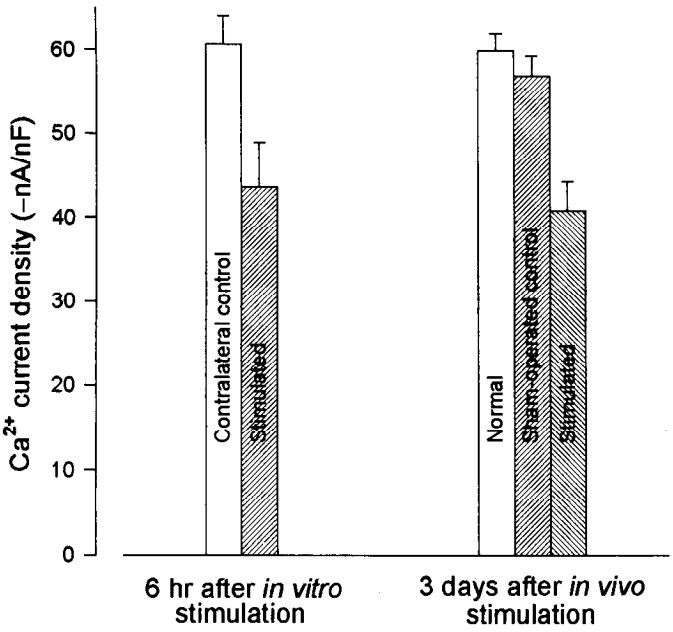

Figure 4. The long-term reduction in Ca^{2+} current lasts for days. The F3 axon was electrically stimulated *in vivo* at 5 Hz for approximately 1 hr. Three days later, the Ca^{2+} current was measured in the stimulated and the sham-operated control cells. The Ca^{2+} current density was significantly less in the stimulated F3 motoneurons (−40.8 ± 3.5 nA/nF, $n = 7$) compared to measurements from the sham-operated control motoneurons (−57.0 ± 2.4 nA/nF, $n = 5$; t test, $p < 0.01$). There was no significant difference between the Ca^{2+} current densities of F3 motoneurons in sham-operated controls and normal animals (−59.9 ± 2.0 nA/nF, $n = 32$; t test, $p > 0.1$). These values are compared to the previous experiment where measurements were performed 6–7 hr after stimulation. The 31.9 ± 5.8% ($n = 7$) reduction in Ca^{2+} current density observed 3 d after *in vivo* stimulation is similar to the 28.7 ± 6.3% ($n = 6$) reduction observed 6–7 hr after *in vitro* stimulation. Thus, the effect of stimulation lasts for 3 d with no decline.

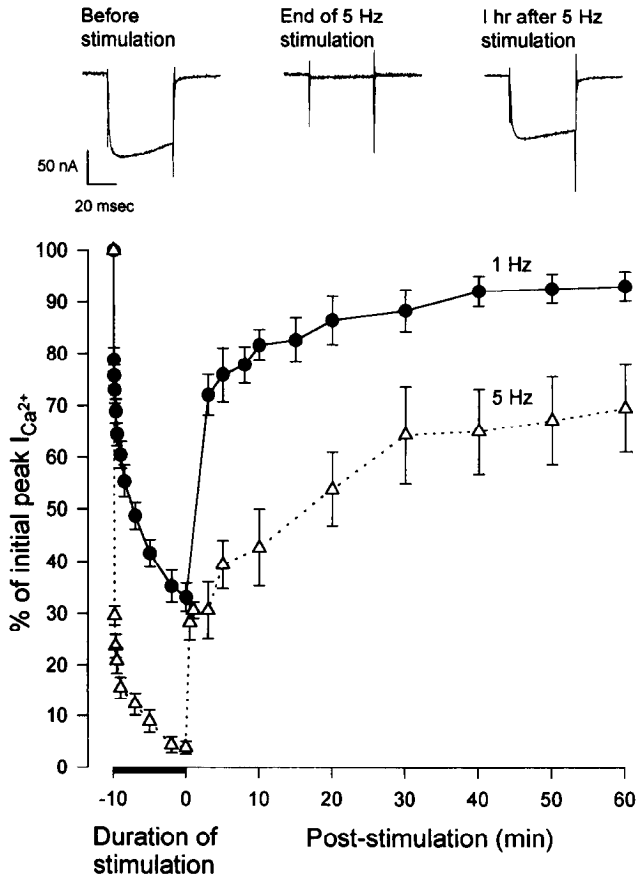

Figure 5. A rapid, short-term reduction in Ca^{2+} current occurs during stimulation. The cell body was voltage clamped and repetitive 50 msec depolarizing pulses to 0 mV were applied. The depolarizing pulses were applied at 1 and 5 Hz and the peak Ca^{2+} currents were measured during stimulation and at regular intervals for 1 hr after stimulation. The Ca^{2+} currents were normalized to the initial Ca^{2+} current measured at the onset of stimulation. The Ca^{2+} current depressed rapidly during stimulation. After stimulation, there was an initial rapid recovery, followed by a more gradual recovery. At both stimulation frequencies, there was a persistent reduction in the Ca^{2+} current that was observed 1 hr after stimulation. *Insert,* Representative Ca^{2+} currents measured before 5 Hz stimulation, at the end of stimulation, and 1 hr after stimulation.

stimulation (28.7 ± 6.3%, $n = 6$). Thus, the effect of stimulation shows no attenuation after 3 d. In addition, there was no change in the shape of the *I–V* curve for the Ca^{2+} current after *in vivo* stimulation.

Reduction in Ca^{2+} current during stimulation

To examine further the activity-dependent reduction in Ca^{2+} current, Ca^{2+} current was measured during stimulation. The cell body was voltage clamped and repetitive 50 msec voltage steps to 0 mV were applied at 1 or 5 Hz for 10 min. Sodium and potassium currents were blocked during stimulation to allow the measurement of the Ca^{2+} current. During repetitive stimulation, there is a rapid, short-term decrease in Ca^{2+} currents: the peak Ca^{2+} current was reduced by approximately 66% at the end of 1 Hz stimulation and by approximately 96% at the end of 5 Hz stimulation (Fig. 5). After stimulation, there is an initial rapid recovery of the Ca^{2+} current, followed by a slower recovery. One hour after 1 Hz stimulation the recovery is almost complete, but there remains a significant 6.7 ± 2.8% reduction in Ca^{2+} current ($n = 4$; $p < 0.05$). This persistent reduction is particularly evident after 5 Hz stimulation: there is a significant 30.4 ± 8.4% ($n = 7$; $p < 0.05$) decrease in the peak Ca^{2+} current 1 hr after stimulation. This reduction in Ca^{2+} current occurs in the absence of any change in the shape of the *I–V* curve.

These results demonstrate that depolarizing pulses applied to the cell body produce a rapid, short-term reduction in Ca^{2+} current that recovers immediately after the end of stimulation along with a more persistent reduction in Ca^{2+} current. It is unclear whether this persistent reduction is the same as the long-term reduction in Ca^{2+} current that lasts for days. Both of these effects are produced in the absence of sodium influx.

Short-term reduction in Ca^{2+} current is Ca^{2+} dependent

A Ca^{2+}-dependent inactivation of Ca^{2+} channels has been described in a number of vertebrate and invertebrate neurons (for review see Eckert and Chad, 1984; Chad, 1989). To determine if the short-term reduction in Ca^{2+} current observed during repetitive stimulation is due to Ca^{2+}-dependent inactivation of Ca^{2+} channels, the relationship between Ca^{2+} entry and Ca^{2+} current inactivation was examined. As in a previous study (Tillotson, 1979), paired pulses were applied under voltage-clamp conditions to determine the effect of prior Ca^{2+} entry upon Ca^{2+} current (Fig. 6*A*). It was found that the inactivation of the Ca^{2+} current was dependent upon the amount of Ca^{2+} entry and not membrane voltage (Fig. 6*B*). There was a strong correlation between the peak amplitude of the Ca^{2+} current during the first

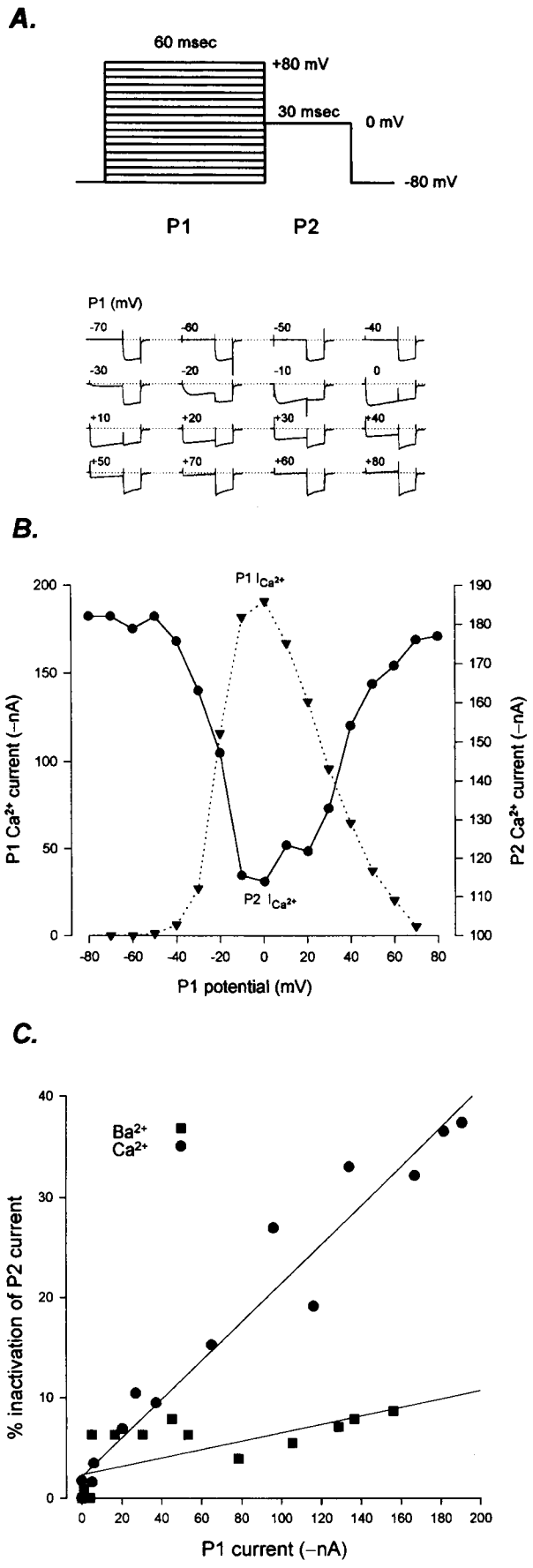

Figure 6. Calcium-dependent inactivation of Ca^{2+} channels. *A*, To determine whether these Ca^{2+} channels show Ca^{2+}-dependent inactivation, paired voltage-clamp pulses were applied to the cell body. The amplitude of a prepulse (*P1*) was varied, while the amplitude of the subsequent test pulse (*P2*) amplitude remained constant. The paired pulses were delivered at 3 min intervals. An increase in amplitude of the Ca^{2+} current during P1 resulted in a decrease in the Ca^{2+} current during P2. *B*, To examine the relationship between P1 membrane potential, P1 Ca^{2+} current, and P2 Ca^{2+} current, the Ca^{2+} currents during P1 (▼) and P2 (●) are plotted against P1 membrane potential. The P2 Ca^{2+} current was minimal when the P1 Ca^{2+} current was maximal. The P2 Ca^{2+} current was not dependent upon the membrane potential during P1. *C*, To examine further the relationship between P1 Ca^{2+} influx and the amount of inactivation of the P2 Ca^{2+} current, the percent inactivation of the P2 Ca^{2+} current [% inactivation = 100 × (P2 I_{Ca} with no P1 pulse − P2 I_{Ca})/P2 I_{Ca} with no P1 pulse] was plotted against the peak amplitude of the P1 Ca^{2+} current. The percent inactivation of the P2 Ca^{2+} current (●) is strongly correlated with the amplitude of the P1 Ca^{2+} current ($r^2 = 0.96$). To confirm that the inactivation of Ca^{2+} current is dependent on Ca^{2+} entry, the effect of substituting Ba^{2+} for Ca^{2+} was examined. The Ba^{2+} currents during P2 (■) showed much less inactivation than the Ca^{2+} currents. The correlation between the P1 current and the inactivation of the P2 current is weaker for Ba^{2+} ($r^2 = 0.45$) than for Ca^{2+}.

pulse and the percent inactivation of the Ca^{2+} current during the second pulse (Fig. 6*C*): there was approximately 40% ($n = 3$) inactivation of P2 Ca^{2+} current when P1 Ca^{2+} current was maximal. This is consistent with previous double-pulse studies of Ca^{2+}-dependent Ca^{2+} channel inactivation (Brehm and Eckert, 1978; Tillotson, 1979; Eckert and Tillotson, 1981; Gutnick et al., 1989; Fryer and Zucker, 1993). In addition, very little Ca^{2+} channel inactivation was seen when Ca^{2+} was replaced with Ba^{2+} (Fig. 6*C*). This is consistent with previous findings that Ba^{2+} is permeable through Ca^{2+} channels, but produces little Ca^{2+} channel inactivation (Brehm and Eckert, 1978; Tillotson, 1979). All these results agree with the criteria of Ca^{2+}-dependent inactivation of Ca^{2+} channels (for review see Chad, 1989).

To determine directly whether the short-term reduction in Ca^{2+} current during repetitive stimulation is Ca^{2+} dependent, Ca^{2+} entry during repetitive stimulation was altered. In the previous experiment, repetitive 50 msec voltage steps to 0 mV were applied at 1 Hz for 10 min (Fig. 5). To alter Ca^{2+} entry, stimulation was performed in 2× normal Ca^{2+} concentration (27 mM) with voltage steps to 0 mV, or in normal Ca^{2+} concentration with voltage steps to +35 mV. Compared to the earlier experiment, the initial Ca^{2+} current before stimulation was increased by 12% in 2× normal Ca^{2+} and was decreased by 72% by applying pulses to +35 mV (Fig. 7*A*). In the previous experiment, 1 Hz stimulation in normal Ca^{2+} concentration resulted in approximately a 66% reduction in Ca^{2+} current at the end of stimulation and a 7% reduction in Ca^{2+} current 1 hr after stimulation. As shown in Figure 7*A*, stimulation in 2× Ca^{2+} resulted in a 70.3 ± 2.7% ($n = 6$) reduction in the Ca^{2+} current at the end of stimulation and a 21.9 ± 2.6% ($n = 6$) reduction 1 hr poststimulation. Stimulation with depolarizing pulses to +35 mV produced a dramatic decrease in the reduction in Ca^{2+} current during stimulation, resulting in only a 34.8 ± 5.7% ($n = 5$) reduction at the end of stimulation, which recovered rapidly. The Ca^{2+} current totally recovered to its original value within 40 min after the end of stimulation.

These results demonstrate that the short-term reduction in Ca^{2+} current observed during repetitive stimulation is Ca^{2+} dependent and appears to result from the previously described Ca^{2+}-dependent inactivation of Ca^{2+} channels. The persistent reduction in Ca^{2+} current observed 1 hr after stimulation is also

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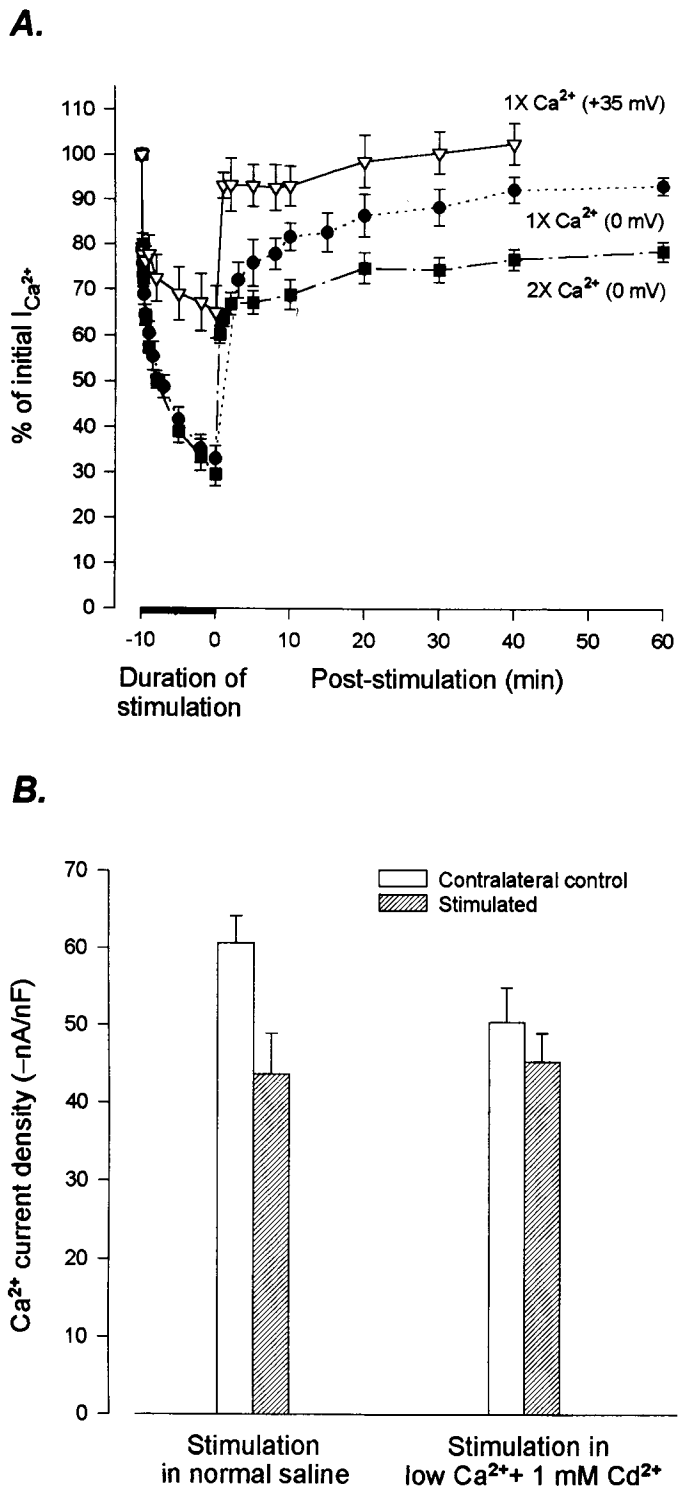

Figure 7. Both the short-term and long-term reductions in Ca^{2+} current are Ca^{2+} dependent. *A*, To determine whether the short-term reduction in Ca^{2+} current was Ca^{2+} dependent, the cell body was stimulated by delivering 50 msec depolarizing voltage steps to 0 mV at 1 Hz for 10 min in 2× normal (27 mM) Ca^{2+} concentration or by delivering 50 msec depolarizing voltage steps to +35 mV in normal Ca^{2+}. These results were compared to the previous experiment in which the cell body was stimulated with depolarizing steps to 0 mV in normal Ca^{2+} (Fig. 5). The peak Ca^{2+} currents were measured during stimulation and at regular intervals for 1 hr after stimulation, and normalized to the initial Ca^{2+} current: −33.6 ± 7.2 nA (n = 5) for 1× Ca^{2+} saline (+35 mV), −119.8 ± 6.3 nA (n = 4) for 1× Ca^{2+} saline (0 mV), and −133.5 ± 8.6 nA (n = 6) for 2× Ca^{2+} saline (0 mV). In 2× Ca^{2+} (0 mV) the Ca^{2+} dependent. It is increased with greater Ca^{2+} influx and is totally eliminated when Ca^{2+} influx is reduced.

Long-term reduction in Ca^{2+} current is Ca^{2+} dependent

To examine the role of Ca^{2+} in the long-term reduction in Ca^{2+} current, the effect of Ca^{2+} influx upon the reduction in Ca^{2+} current observed 6–7 hr after stimulation was examined. In these experiments, Ca^{2+} influx during stimulation was blocked by applying an inorganic Ca^{2+} channel blocker, 1 mM Cd^{2+}, and reducing the Ca^{2+} concentration (see Materials and Methods). As in the previous experiment (Fig. 2), the axon was stimulated at 5 Hz for 30–45 min. After stimulation, the cell was maintained in normal saline for 6–7 hr until Ca^{2+} currents were measured in stimulated and contralateral control motoneurons (Fig. 7*B*). The Ca^{2+} current density of the stimulated neuron (−45.3 ± 3.7 nA/nF) was not significantly different from that of the contralateral control neuron (−50.4 ± 4.5 nA/nF, n = 5; $p > 0.1$). The values for the Ca^{2+} current in the control motoneuron are less (approximately 17%) than in the previous experiments. It is unclear whether this is due to interanimal variability or incomplete removal of the Cd^{2+}. Nonetheless, the results demonstrate that the long-term reduction in Ca^{2+} current is Ca^{2+} dependent since blocking Ca^{2+} influx during stimulation prevented the reduction in Ca^{2+} current.

Effect of inhibition of protein synthesis

In a previous study, it was shown that application of CHX 2–6 hr before stimulation of a crayfish phasic motoneuron attenuated the long-term, activity-dependent reduction in transmitter release from the motor terminals (Nguyen and Atwood, 1990). To determine whether the long-term reduction in Ca^{2+} current has a similar requirement for protein synthesis, 0.6 mM CHX, a reversible translational inhibitor (Wettstein et al., 1964), was added to the bath 4–5 hr before stimulation. We first examined the effect of protein synthesis inhibition upon the short-term reduction in Ca^{2+} current produced during repetitive 50 msec voltage steps to 0 mV delivered at 1 Hz for 10 min. Stimulation was performed in 2× normal Ca^{2+} concentration to produce a greater reduction in Ca^{2+} current. Inhibition of protein synthesis had no

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initial Ca^{2+} current was 12% greater than in the previous experiment, 1× Ca^{2+} (0 mV). The slightly greater Ca^{2+} influx appeared to produce only a small increase in the Ca^{2+} current reduction during stimulation; however, there was a substantial increase in the persistent reduction in Ca^{2+} observed 1 hr after stimulation (21.9 ± 2.6%, n = 6; $p < 0.05$). Stimulation with voltage steps to +35 mV decreased the initial Ca^{2+} current amplitude by 72% and produced a substantial decrease in the Ca^{2+} current reduction during repetitive stimulation. There was a 34.8 ± 5.7% (n = 5) reduction in Ca^{2+} current at the end of 10 min stimulation and the Ca^{2+} current was fully recovered 40 min after stimulation (102.6 ± 4.5%, n = 5; $p > 0.1$). Thus, the short-term reduction in Ca^{2+} current during repetitive stimulation is dependent on the amount of Ca^{2+} influx. *B*, To examine whether the long-term reduction in Ca^{2+} current is Ca^{2+} dependent, the Ca^{2+} influx during stimulation was blocked by applying an inorganic Ca^{2+} channel blocker, 1 mM Cd^{2+}, to low (5 mM) Ca^{2+} saline. The F3 axon was stimulated at 5 Hz for 30–45 min as previously described. After stimulation, Cd^{2+} was washed out and the cell was kept in normal saline for 6–7 hr prior to the measurement of the Ca^{2+} current. The mean Ca^{2+} current density of the stimulated neuron (−45.3 ± 3.7 nA/nF) was not significantly different from that of the contralateral control neuron (−50.4 ± 4.5 nA/nF, n = 5; $p > 0.1$). These values are compared to the previous experiment (Fig. 2) where stimulation in normal saline produced a significant 28.7 ± 6.3% reduction in Ca^{2+} current density (n = 6; $p < 0.01$). Thus, the long-term reduction in Ca^{2+} current is Ca^{2+} dependent.

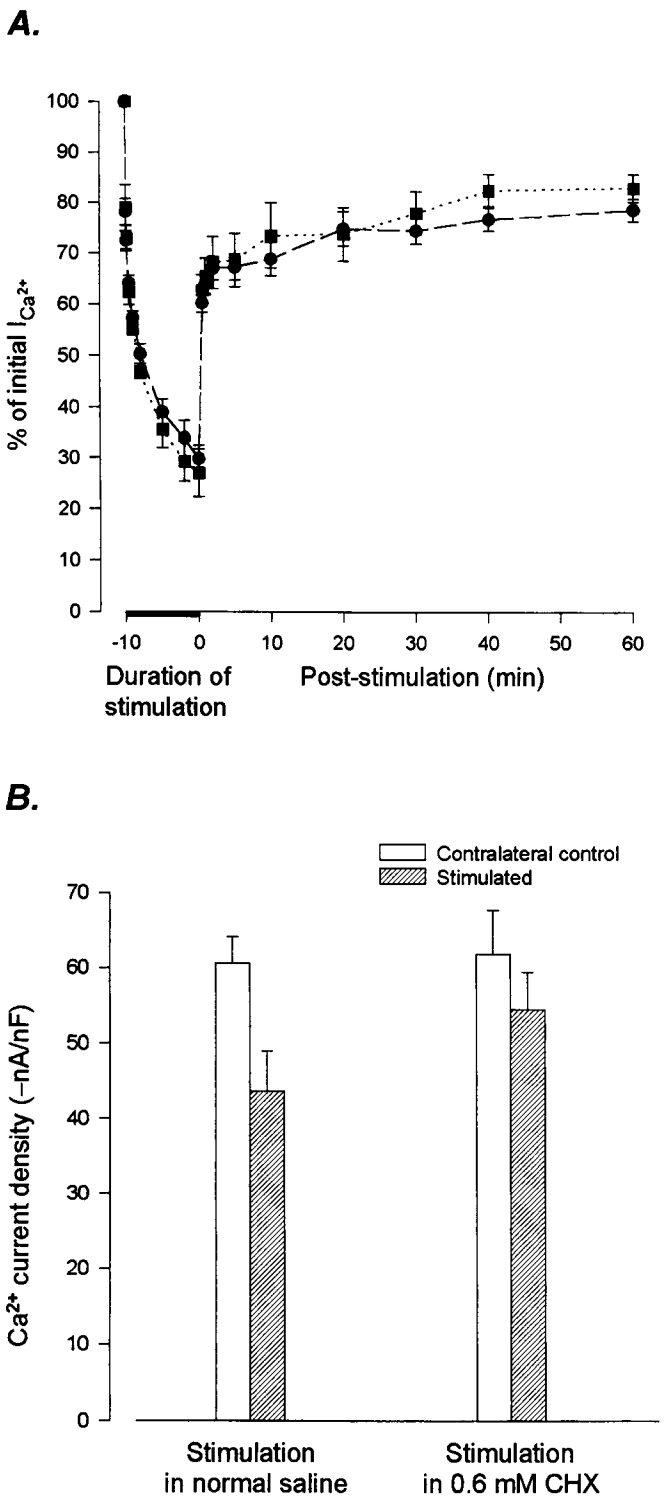

Figure 8. The role of protein synthesis in the short-term and long-term reduction in Ca^{2+} current. *A*, Effect of inhibition of protein synthesis upon the short-term reduction in Ca^{2+} current. To examine the effect of protein synthesis on the short-term reduction in Ca^{2+} current, protein synthesis was blocked by applying 0.6 mM CHX to the bath 4–5 hr before stimulation. The F3 cell body was stimulated with 50 msec depolarizing pulses to 0 mV at 1 Hz for 10 min in 2× normal Ca^{2+} concentration. During stimulation, the short-term reduction in Ca^{2+} was not significantly different in cells stimulated in the presence (■) and in the absence of CHX (●). One hour after stimulation, the decrease in the Ca^{2+} current amplitude in cells stimulated in the presence of the inhibitor (17.0 ± 2.8%, $n = 5$) was not significantly different from cells stimulated without the inhibitor (21.9 ± 2.6%, $n = 6$; $p > 0.1$). *B*, To determine if protein synthesis plays a role in the long-term reduction in Ca^{2+} current observed 6–7 hr after stimulation, protein synthesis was blocked by applying 0.6 mM CHX to the bath 4–5 hr before stimulation. The motoneuron was stimulated with a suction electrode applied to the axon as previously described (Fig. 1). Suprathreshold stimulation of the axon was performed at 5 Hz for 45 min. Six to seven hours after stimulation, the mean density of Ca^{2+} currents in the stimulated cell (−54.5 ± 5.0 nA/nF) was not significantly different from the contralateral control cell (−61.9 ± 5.8 nA/nF, $n = 8$; $p > 0.1$). These values are compared to measurements from the previous experiment performed in normal saline. The reduction in Ca^{2+} current (9.4 ± 5.3%) observed when protein synthesis was blocked is significantly different from that observed in cells stimulated in normal saline (28.7 ± 6.3%, $n = 6$; t test, $p < 0.05$). These results demonstrate that protein synthesis is required for full expression of the long-term, activity-dependent reduction in Ca^{2+} currents.

effect upon the short-term reduction in Ca^{2+} current or the persistent reduction in Ca^{2+} current observed 1 hr after stimulation (Fig. 8*A*). The reduction in Ca current during stimulation and the immediate recovery at the end of stimulation was similar in the absence and presence of the protein synthesis inhibitor. The persistent reduction in the Ca^{2+} current observed 1 hr poststimulation was not significantly different in cells stimulated in the presence (17.0 ± 2.8%, $n = 5$) or absence (21.3 ± 2.2%, $n = 6$; t test, $p > 0.05$) of the protein synthesis inhibitor.

To test the effect of inhibiting protein synthesis upon the long-term reduction in Ca^{2+} current, the axon was stimulated *in vitro* at 5 Hz for 45 min as in the previous experiments (Fig. 1) and the Ca^{2+} currents were measured 6–7 hr later. After stimulation, CHX was washed out and the motoneuron was maintained in normal saline for 6–7 hr. The peak Ca^{2+} current amplitude was then measured in the stimulated and contralateral control cells (Fig. 8*B*). The mean density of the Ca^{2+} current in the stimulated cells (−54.5 ± 5.0 nA/nF) was not significantly different from the contralateral control cells (−61.9 ± 5.8 nA/nF, $n = 8$; $p > 0.1$). In addition, the reduction in the Ca^{2+} current density (9.4 ± 5.3%) observed in the presence of CHX was significantly different from that (28.7 ± 6.3%; t test, $p < 0.05$) observed in cells stimulated in normal saline. These results demonstrate that inhibition of protein synthesis prevents the full expression of the activity-dependent reduction in Ca^{2+} current observed 6–7 hr after stimulation.

Discussion

Short-term reduction in voltage-dependent Ca^{2+} current

We observed a short-term, Ca^{2+}-dependent reduction in Ca^{2+} current during the application of repetitive depolarizing pulses to the cell body of a crayfish motoneuron. There is a progressive reduction in Ca^{2+} current during repetitive stimulation followed by a rapid partial recovery of the Ca^{2+} current at the end of stimulation. Paired-pulse studies show that these Ca^{2+} channels undergo a Ca^{2+}-dependent inactivation similar to that previously described in a broad range of cell types (for review see Eckert and Chad, 1984; Chad, 1989). It appears likely that the progressive reduction in Ca^{2+} current observed during repetitive stimulation is due to accumulation of intracellular Ca^{2+} resulting in increasing Ca^{2+}-dependent Ca^{2+} channel inactivation.

Calcium-dependent inactivation of Ca^{2+} channels results from a decrease in the probability of Ca^{2+} channel opening (Lux and Brown, 1984). The rapid kinetics of Ca^{2+} channel inactivation suggest that Ca^{2+} produces inactivation by binding to a site at or near the mouth of the Ca^{2+} channel (Fryer and Zucker, 1993;

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Johnson and Byerly, 1993b). It has been proposed that the Ca^{2+}-dependent inactivation of Ca^{2+} channels found in *Helix* neurons is produced by activation of a Ca^{2+}-dependent phosphatase such as calcineurin, and subsequent dephosphorylation of the Ca^{2+} channel (Chad and Eckert, 1986). Alternatively, more recent evidence in *Lymnaea* neurons suggests that Ca^{2+}-dependent disruption of the cytoskeleton could play a role in Ca^{2+} channel inactivation (Johnson and Byerly, 1993a).

The initial rapid recovery of the Ca^{2+} current at the end of repetitive stimulation is followed by a slower recovery phase. One hour after the 10 min stimulation period, a persistent reduction in Ca^{2+} current can still be observed. This persistent reduction is Ca^{2+} dependent and can be eliminated under conditions in which Ca^{2+} influx is reduced. It is presently unclear whether the mechanisms of this change are similar to the short-term effect or the long-term change discussed below.

Long-term reduction in voltage-dependent Ca^{2+} current

We have observed a long-term, activity-dependent reduction in Ca^{2+} current 6–7 hr and 3 d after a single stimulation period. It appears likely that this long-term reduction in Ca^{2+} current persists for more than 3 d since the magnitude of the reduction in Ca^{2+} current observed 6–7 hr after stimulation shows little attenuation after 3 d. Similar to the short-term Ca^{2+} channel inactivation, the long-term effect is also Ca^{2+} dependent. The reduction in Ca^{2+} current observed 6–7 hr after stimulation is prevented by blocking Ca^{2+} influx during stimulation. In addition, the short-term and long-term changes appear to involve the same species of Ca^{2+} channel. Application of Ca^{2+} channel blockers indicates that the cell body contains a single type of Ca^{2+} channel, or at least is dominated by a single channel type (Hong and Lnenicka, unpublished observations). The shape of the *I–V* curve and the decay of the Ca^{2+} current remain the same after the reduction in Ca^{2+} current density, providing further evidence that a single species of Ca^{2+} channel is involved.

The mechanisms underlying the short-term and long-term reduction in Ca^{2+} current differ in their sensitivity to inhibitors of protein synthesis. Application of CHX before stimulation attenuates the long-term reduction (6–7 hr poststimulation) in Ca^{2+} current, but has no effect upon the short-term reduction in Ca^{2+} current. Interestingly, the persistent reduction in Ca^{2+} current observed 1 hr after stimulation is also not affected by inhibition of protein synthesis, indicating that it probably results from a different mechanism than the long-term reduction. In addition, it appears that the long-term reduction in Ca^{2+} current is not confined to the site of Ca^{2+} influx, but occurs more globally. During stimulation of the axon, the cell body is no longer excitable after the first few action potentials. Based upon the characteristics of the *I–V* curve for the Ca^{2+} current, the remaining electrotonic depolarization of the cell body is unlikely to produce significant Ca^{2+} influx. Thus, it appears that the Ca^{2+} influx at remote sites produces changes in Ca^{2+} channels at the cell body.

We do not know whether the long-term reduction in Ca^{2+} current density results from a change in the properties of individual channels, that is, decreased unitary conductance or probability of opening, or a decrease in the number of functional channels. The lack of change in Ca^{2+} current kinetics and in the *I–V* curve argues against a change in the properties of individual channels. A reduction in the number of Ca^{2+} channels is supported by earlier findings. Chronic depolarization of PC12 cells produced by elevated potassium concentrations results in a reduction of the number of dihydropyridine-sensitive Ca^{2+} channels, as determined by binding studies (DeLorme and McGee, 1986; DeLorme et al., 1988). This Ca^{2+}-dependent effect has a slow onset: no reduction is observed after 4 hr of constant depolarization, a 30% reduction occurs after 1 d of depolarization, and a maximum 50% reduction is seen after 3 d of constant depolarization (DeLorme et al., 1988).

A reduction in the number of functional Ca^{2+} channels could result from increased degradation or decreased synthesis of Ca^{2+} channel proteins. The latter appears unlikely since a previous study of cultured myenteric neurons found that CHX treatment for 24 hr did not reduce Ca^{2+} channel density (Franklin et al., 1992). This previous study also showed that inhibition of protein synthesis reduced the depolarization-induced decrease in the sustained component of the Ca^{2+} current. Consistent with these results, we found that blocking protein synthesis attenuates the long-term reduction in Ca^{2+} current. The effect of inhibition of protein synthesis suggests that (1) Ca^{2+} influx produces an increase in the production of proteins involved in degrading or modifying Ca^{2+} channels, or (2) Ca^{2+}-dependent pathways that are involved in degrading or modifying Ca^{2+} channels include short-lived proteins, which are rapidly depleted. The latter hypothesis is consistent with the effects of protein synthesis inhibitors upon the long-term, activity-dependent reduction in transmitter release from crustacean motor terminals (Nguyen and Atwood, 1990).

Calcium-activated proteases could be involved in this long-term reduction in Ca^{2+} current. The "washout" of Ca^{2+} currents observed in dialyzed cells includes an irreversible component, which is Ca^{2+} dependent (Chad and Eckert, 1986). Since this component is blocked by the Ca^{2+}-dependent protease inhibitor leupeptin, it has been proposed that the irreversible washout is produced by Ca^{2+}-dependent proteolysis. In addition, the Ca^{2+}-dependent protease calpain has been shown to produce an irreversible reduction in Ca^{2+} currents in guinea pig myocytes (Belles et al., 1988). Ca^{2+}-activated proteases may irreversibly activate phosphatases (Manalan and Klee, 1983; Tallant et al., 1988) that inactivate the Ca^{2+} channel through dephosphorylation (Chad and Eckert, 1986; for review see Armstrong, 1989).

Significance for cellular and synaptic physiology

The Ca^{2+}-dependent reduction in Ca^{2+} current is presumably an important mechanism for adjusting Ca^{2+} influx to prevent excessively high levels of intracellular Ca^{2+}. It is well established that extremely high intracellular Ca^{2+} concentrations can produce cytotoxicity (Choi, 1988). In addition, activity-dependent changes in Ca^{2+} current could modify the intrinsic electrical properties of neurons, as well as directly alter transmitter release. Calcium-dependent changes in ionic conductances may play an important role in determining the firing properties of neurons (LeMasson et al., 1993; Turrigiano et al., 1994).

The activity-dependent reduction in Ca^{2+} current may play a role in changes in transmitter release from crustacean motor terminals. The short-term inactivation of Ca^{2+} currents during repetitive stimulation of the phasic motoneuron could contribute to the prominent synaptic depression observed at the neuromuscular synapses of these motoneurons. In *Aplysia,* it has been proposed that Ca^{2+} channel inactivation is responsible for synaptic depression and the resulting short-term habituation (Klein et al., 1980). At crustacean neuromuscular synapses, a chronic increase in impulse activity produces a long-term reduction in initial transmitter release (Lnenicka and Atwood, 1985; Mercier

and Atwood, 1989; Bradacs et al., 1990), whereas a chronic decrease in impulse activity produces a long-term reduction in initial transmitter release (Pahapill et al., 1985). Similar activity-dependent changes in initial transmitter release have been shown at vertebrate central (Gallego et al., 1979; Gallego and Geijo, 1987; Manabe et al., 1990) and peripheral synapses (Robbins and Fischbach, 1971; Snider and Harris, 1979; Hinz and Wernig, 1988). We have recently shown in the crayfish that the long-term, activity-dependent decrease in transmitter release is triggered by Ca^{2+} influx (Hong and Lnenicka, 1993a).

Since both the long-term, activity-dependent reduction in Ca^{2+} current and transmitter release observed in this crayfish motoneuron are Ca^{2+} dependent, it is reasonable to hypothesize that the reduction in transmitter release is due to a reduction in Ca^{2+} current. Since the Ca^{2+} currents were measured in the cell body, we have no direct evidence that Ca^{2+} currents at the motor terminals undergo an activity-dependent reduction. However, there are parallels between the two effects. The activity-dependent reduction in the voltage-dependent Ca^{2+} current is sensitive to inhibition of protein synthesis in a manner consistent with the activity-dependent reduction in transmitter release (Nguyen and Atwood, 1990). In addition, a reduction in transmitter release from motor terminals can be produced by calcium influx at the cell body (Hong and Lnenicka, 1993a). Similarly, it appears that Ca^{2+} influx in a separate region of the motoneuron can reduce the currents produced by Ca^{2+} channels at the cell body.

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