Characterization of a P-Type Calcium Current in a Crayfish Motoneuron and Its Selective Modulation by Impulse Activity

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Hong, Sungwon J. and Gregory A. Lnenicka. Characterization of a P-type calcium current in a crayfish motoneuron and its selective modulation by impulse activity. J. Neurophysiol. 77: 76–85, 1997. Previous studies have demonstrated that the voltage-dependent Ca\(^{2+}\) current recorded from the cell body of the crayfish abdominal motoneuron, F3, undergoes a long-term reduction as a result of increased impulse activity. The properties of the Ca\(^{2+}\) channels undergoing this long-term change were examined with the use of two-electrode voltage-clamp techniques. The Ca\(^{2+}\) current was activated at −50 to −40 mV and its amplitude was maximal at 0 mV (−135.0 ± 25.8 nA, mean ± SE, n = 14). The current-voltage relationship and the greater sensitivity of the Ca\(^{2+}\) channel to Cd\(^{2+}\) than Ni\(^{2+}\) indicated that Ca\(^{2+}\) influx occurs through high-voltage-activated (HVA) Ca\(^{2+}\) channels. Loose-patch recordings demonstrated that the Ca\(^{2+}\) current was generated by the membrane of the cell body. When Ba\(^{2+}\) was substituted for extracellular Ca\(^{2+}\), there was a 40% increase in the amplitude of the inward current and a negative shift of ~10 mV in the I-V relationship. Application of the P-type Ca\(^{2+}\) channel antagonist ω-agatoxin IVA (ω-AgTX IVA) produced a significant 33% (n = 6) reduction in the peak amplitude of the Ba\(^{2+}\) current, whereas neither the L-type Ca\(^{2+}\) channel antagonist nifedipine nor the N-type channel antagonist ω-conotoxin GVIA produced a reduction in the Ba\(^{2+}\) current. The voltage-dependent activation of this P-type (ω-AgTX-IVA-sensitive) Ca\(^{2+}\) channel was similar to previously identified P-type channels, but different from that of the non-P-type (ω-AgTX-IVA-resistant) Ca\(^{2+}\) channels. When Ca\(^{2+}\) currents were measured 6–7 h after an increase in impulse activity (5-Hz stimulation for 45–60 min), there was a 43% reduction in the amplitude of the P-type current, but no significant changes in the non-P-type current amplitude. These results demonstrate that at least two subtypes of HVA Ca\(^{2+}\) channels contribute to the macroscopic Ca\(^{2+}\) current observed in the cell body of this crayfish phasic motoneuron: one belongs to the previously described P-type Ca\(^{2+}\) channel and the other(s) does not belong to the N-, L-, or P-type Ca\(^{2+}\) channel. The long-term, Ca\(^{2+}\)-dependent reduction in Ca\(^{2+}\) current previously demonstrated in motoneuron F3 is produced by the selective reduction of this P-type Ca\(^{2+}\) current. This activity-dependent reduction in the P-type Ca\(^{2+}\) current is likely involved in the long-term repression of transmitter release observed at the neuromuscular synapses of this motoneuron.

INTRODUCTION

Calcium influx through voltage-dependent Ca\(^{2+}\) channels plays an important role in the regulation of neuronal structure and function (Miller 1987; Kennedy 1989). Neurons often contain multiple types of voltage-dependent Ca\(^{2+}\) channels that are specialized for different cellular functions (for review see Scott et al. 1991). The modulation of specific types of Ca\(^{2+}\) channels could play an important role in shaping the response of the neuron to electrical activity. We have previously shown that the voltage-dependent Ca\(^{2+}\) current recorded from the cell body of a crayfish motoneuron undergoes a long-term reduction as a result of increased impulse activity (Hong and Lnenicka 1995). To characterize this effect further, we examined the biophysical and pharmacological properties of the Ca\(^{2+}\) channels that undergo this long-term modulation.

In vertebrate cells, distinct classes of voltage-sensitive Ca\(^{2+}\) channels have been identified on the basis of electrophysiological and pharmacological characteristics (for review see Carbone and Swandaull 1989; Tsien et al. 1988). The low-voltage-activated (LVA), T-type Ca\(^{2+}\) channels activate at more negative membrane potential than the high-voltage-activated (HVA) Ca\(^{2+}\) channels (Carbone and Lux 1987; Fox et al. 1987a,b; Nowycky et al. 1985). The HVA Ca\(^{2+}\) channels have generally been divided into three subtypes (L, N, and P type) according to their sensitivity to specific organic Ca\(^{2+}\) channel blockers. The L-type Ca\(^{2+}\) channels are sensitive to a variety of 1,4-dihydropyridine antagonists, whereas the N-type Ca\(^{2+}\) channels are specifically blocked by the peptide, ω-conotoxin GVIA (ω-CgTX GVIA), from the venom of the marine fish-hunting cone snail, Conus geographus (Fox et al. 1987a,b; McCleskey et al. 1987; Olivera et al. 1985). P-type Ca\(^{2+}\) channels, insensitive to both ω-CgTX GVIA and dihydropyridines, are specifically sensitive to a polycationic toxin fraction and a peptide antagonist, ω-agatoxin IVA (ω-AgTX IVA), from the venom of the funnel-web spider, Agelenopsis aperta (Llinás et al. 1989; Mintz et al. 1992a,b).

Compared with vertebrates, less is known about the Ca\(^{2+}\) channel types found in invertebrate neurons. Both LVA and HVA Ca\(^{2+}\) currents have been identified in invertebrate neurons (Angstadt and Calabrese 1991; Haydon and Man-Son-Hing 1988). There is pharmacological evidence for L-, N-, and P-type HVA Ca\(^{2+}\) channels in invertebrate neurons. L-type Ca\(^{2+}\) channels have been identified in Aplysia abdominal ganglion sensory and motor neurons (Edmonds et al. 1990), bag cell neurons (Nerbonne and Gurney 1987), and buccal ganglion neurons (Fossier et al. 1994; Trudeau et al. 1993), as well as crayfish swimmeret motoneurons (Chrachi 1995). N-type Ca\(^{2+}\) channels have been observed in Aplysia buccal ganglion neurons (Fossier et al. 1994; Trudeau et al. 1993). There is evidence for P-type Ca\(^{2+}\) channels at the squid giant synapse (Llinás et al. 1989), in Aplysia buccal ganglion neurons (Fossier et al. 1994), and at crayfish motor terminals (Araque et al. 1994; Blundon et al. 1995).
Depolarization can produce long-term changes in Ca\(^{2+}\) channels. Chronic depolarization of cultured PC12 cells, chick retinal cells, rat myenteric neurons, or rat cerebral granule neurons with high-potassium solutions has been shown to produce a reduction in the density of L-type Ca\(^{2+}\) channels and/or current (DeLorme and McGee 1986; DeLorme et al. 1988; Ferrante et al. 1991; Franklin et al. 1992; Liu et al. 1994). We have demonstrated a similar phenomenon produced in situ by an increase in the impulse activity of a relatively inactive neuron, the crayfish abdominal motoneuron F3. Increasing the impulse activity of this phasic motoneuron in vivo produces a reduction in the voltage-dependent Ca\(^{2+}\) current density that persists for days (Hong and Lnenicka 1995). This long-term reduction in Ca\(^{2+}\) current density is triggered by Ca\(^{2+}\) influx.

In this study we characterize the biophysical and pharmacological properties of the Ca\(^{2+}\) channels in the cell body of the crayfish abdominal motoneuron F3. On the basis of electrophysiological and pharmacological characteristics, the cell body contains at least two subtypes of HVA Ca\(^{2+}\) channels: one belongs to the previously described P-type Ca\(^{2+}\) channel and the other(s) do(s) not belong to any of the previously described HVA subtypes. Only the P-type Ca\(^{2+}\) current undergoes a long-term reduction as a result of increased impulse activity. An activity-dependent reduction in the P-type Ca\(^{2+}\) current may be responsible for the long-term depression seen at the neuromuscular synapses of this motoneuron.

**METHODS**

**Preparation**

Experiments were performed on young crayfish, *Procambarus clarkii*, with a carapace length of 2.0–3.2 cm, obtained from Atchafalaya Biological Supply (Raceland, LA). To expose the cell body of the fast flexor motoneuron F3 (Selverston and Remler 1972), the third abdominal ganglion was isolated and desheathed in van Harreveld’s solution (Van Harreveld 1936) containing 1 mM glucose and 10 mM N-2-hydroxyethylpiperazin-N’-2-ethanesulfonic acid (HEPES), pH 7.4. Further details of the dissection and identification of F3 have been previously reported (Hong and Lnenicka 1993). All experiments were performed at 15°C.

**Identification and measurement of Ca\(^{2+}\) currents**

Conventional two-microelectrode voltage-clamp techniques were used for recording Ca\(^{2+}\) currents from the cell body of F3 with the use of an Axoclamp-2A preamplifier (Axon Instruments, Burlingame, CA). To block Na\(^+\) and K\(^+\) currents, 1 μM tetrodotoxin, 50 mM tetraethylammonium, and 1 mM 4-aminopyridine were added to the saline. Changes in osmolarity were compensated for by decreasing the concentration of NaCl. To block K\(^+\) currents more completely, the cell body was loaded with Cs\(^+\) for 30–60 min with microelectrodes (5–10 MΩ) filled with 3 M CsCl (Gutnick et al. 1989; Tillotson 1979). Data were acquired (sampling rate 5–20 kHz) by either DMA (LabMaster, Scientific Solutions, Solon, OH) or Digidata 1200A (Axon Instruments, Foster City, CA) interfaces. The current traces were filtered at 0.5–1 kHz and analyzed with the use of either VCAN V3.0 or pCLAMP 6.0.2 software (Axon Instruments, Foster City, CA). The holding potential was set to −70 mV in most experiments, and in some preparations it was set to the resting membrane potentials that varied between −70 and −80 mV.

To identify the Ca\(^{2+}\) currents, the sensitivity of the inward current to the inorganic Ca\(^{2+}\) channel blockers Cd\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) was examined. Each of these blockers was made up as a concentrated stock (0.1–1 M) in normal saline and added directly to the bath. During voltage steps to 0 mV, the effect of the inorganic blockers on the Ca\(^{2+}\) current was examined 15–20 min after application of the blocker to the bath.

Measurements of the inward Ca\(^{2+}\) current are often contaminated by a nonspecific outward leakage current (Byerly and Hagiwara 1982). The inward Ca\(^{2+}\) and Ba\(^{2+}\) currents were corrected for nonspecific leakage current by subtracting the current produced by corresponding hyperpolarizing pulses on the basis of the current-voltage (I-V) relationship of leakage currents. To examine the I-V relationship of the nonspecific leakage current, Na\(^+\), K\(^+\), and Ca\(^{2+}\) current components were blocked with 1 μM tetrodotoxin, 50 mM tetraethylammonium, 1 mM 4-aminopyridine, 2 mM Cd\(^{2+}\), and Cs\(^+\) loading into the cell. The amplitudes of leakage currents were measured during 50-ms pulses in 10-mV steps from the holding potential to +40 and −180 mV. The mean amplitude of the leak current at 0 mV was +23.8 ± 3.9 nA (n = 12). The I-V relationship of the leakage current was linear at membrane potentials <0 mV; however, at membrane potentials >0 mV, the I-V relation was no longer linear. When the leakage current at hyperpolarizing voltage steps was subtracted from the leakage currents at corresponding depolarizing voltage steps, there was a small residual net outward current at voltages >0 mV. The amplitude of this residual leak current increased at more positive potentials. This residual outward current at high positive potentials may be carried by K\(^+\), resulting from a gradual relief of the tetraethylammonium and 4-aminopyridine block at positive membrane potentials (Hermann and Gorman 1981a,b). However, the amplitude of this residual outward current was <5% of the inward Ca\(^{2+}\) current at voltage steps to 0 mV. For Ca\(^{2+}\) current measurements at 0 mV, the amplitude of the Ca\(^{2+}\) current was not corrected for the residual net outward current unless indicated.

Cell-attached loose-patch techniques (Stühmer et al. 1983) were used to record the Ca\(^{2+}\) current from the dorsal surface of the cell body with the use of a PC-One patch-clamp preamplifier (Dagan, Minneapolis, MN) during voltage clamping of the cell with two microelectrodes as described above. Patch pipettes with tip diameters of 15–30 μm were fire polished and filled with the bath solution. After the pipette was placed against the cell, gentle suction was applied to form the seal. The seal resistance (R\(_{s}\)) generally 2.5 times of the pipette resistance (R\(_{p}\)). To estimate the Ca\(^{2+}\) current density per cell surface area, the Ca\(^{2+}\) current generated by the membrane within the lumen of the electrode was determined by multiplying the measured Ca\(^{2+}\) current by (R\(_{s}\) + R\(_{p}\))/R\(_{p}\) to correct for leakage through the seal (Lnenicka and Mellon 1983). The area of the cell body isolated by the patch pipette was estimated by measuring the inside tip diameter of the patch pipette. However, this is likely an underestimation of the isolated soma area, because the cell is likely to evaporate into the lumen of the pipette when the suction is applied. In addition, some of the Ca\(^{2+}\) current generated by the membrane under the electrode rim will travel into the pipette. We made no attempt to account for these factors, because we were not attempting to measure the precise Ca\(^{2+}\) current density. The total surface area of the soma was estimated from measurements of the soma diameter.

**Characterization of Ca\(^{2+}\) channels with the use of organic Ca\(^{2+}\) channel blockers**

The effects of specific organic Ca\(^{2+}\) channel blockers were examined by directly applying organic blockers to the bath: nifedipine (Sigma, St. Louis, MO) for L-type channels, synthetic ω-CgTX GVIA (RBI, Natick, MA) for N-type channels, and synthetic ω-ApTX IVA (Scientific Marketing Associates, Lake Forest, CA) for P-type channels.
Barnet, UK) for P-type Ca\textsuperscript{2+} channels. A 100 mM stock solution of nifedipine was prepared in 95% ethanol and stored in the dark. The stock solution was diluted just before the experiment. The final concentration of ethanol (<0.01%) did not produce any direct effect on the Ca\textsuperscript{2+} current. Concentrated stock solutions of ω-CgTX GVIA and ω-AgTX IVA were made up by dissolving toxins in distilled H\textsubscript{2}O in 50-μl aliquots, which were stored in the freezer (−20°C).

To examine the effects of specific organic blockers on Ca\textsuperscript{2+} channels, Ba\textsuperscript{2+} was used as a charge carrier instead of Ca\textsuperscript{2+}. The extracellular Ca\textsuperscript{2+} in the recording chamber (volume 0.3–1 ml) was substituted with Ba\textsuperscript{2+} by perfusion with a Ba\textsuperscript{2+} saline at a flow rate of 1 ml/min for >20 min. During the perfusion, the cell body was continuously voltage clamped because the resting membrane potential often became depolarized in Ba\textsuperscript{2+} saline. The perfusion was then stopped and organic blockers were directly applied to the bath. The effects of organic blockers on the Ba\textsuperscript{2+} current were examined 10–15 min after the addition of blockers at depolarizing voltage steps to −10 mV, the voltage at which the amplitude of Ba\textsuperscript{2+} current was maximal.

**Effect of impulse activity on the P-type current**

To determine whether the P-type current undergoes an activity-dependent reduction, impulse activity was increased by stimulating the axon in normal saline at 5 Hz for 45–60 min as previously described (Hong and Lnenicka 1995). Six to seven hours after stimulation, the amplitude of the P-type current was determined by measuring the Ba\textsuperscript{2+} current and then subtracting the Ba\textsuperscript{2+} current remaining after blocking the P-type current with 600 nM ω-AgTX IVA. The P-type current density in the stimulated cells was compared with the P-type current density in separate control cells. Membrane area was determined from measurements of cell capacitance, and Ba\textsuperscript{2+} current density was expressed as nA/μF.

All values are expressed as means ± SE. Mean values were compared with the use of a Student’s t-test.

**RESULTS**

**Identification of the Ca\textsuperscript{2+} current**

Two-electrode voltage clamp was performed on the cell body of the abdominal fast flexor motoneuron F3 to measure Ca\textsuperscript{2+} currents. After voltage-dependent Na\textsuperscript{+} and K\textsuperscript{+} currents were blocked, a voltage-dependent inward current remained. To determine whether this remaining inward current is carried by Ca\textsuperscript{2+}, the influence of inorganic Ca\textsuperscript{2+} channel blockers and changes in extracellular Ca\textsuperscript{2+} concentration was examined. Ca\textsuperscript{2+} currents are normally blocked by the inorganic Ca\textsuperscript{2+} channel blockers Cd\textsuperscript{2+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+}, and Co\textsuperscript{2+} (for review see Hagiswara and Byerley 1981). By adding increasing concentrations of these blockers to the bath, we found that nearly all of the inward current was blocked by 1 mM Cd\textsuperscript{2+}, 6 mM Mn\textsuperscript{2+}, 10 mM Ni\textsuperscript{2+}, or 10 mM Co\textsuperscript{2+} (Fig. 1, top). Furthermore, when the extracellular concentration of Ca\textsuperscript{2+} was increased to 27 mM (2 times normal Ca\textsuperscript{2+}), there was a significant 22.7 ± 6.4% (P < 0.05, n = 4) increase in the amplitude of the peak Ca\textsuperscript{2+} current (−133.8 ± 9.6 nA) with respect to that in normal Ca\textsuperscript{2+} saline (−108.9 ± 6.5 nA) (Fig. 1, bottom). These results demonstrate that Ca\textsuperscript{2+} is the main charge carrier for this remaining inward current.

The greater sensitivity of the Ca\textsuperscript{2+} channels to Cd\textsuperscript{2+} than Ni\textsuperscript{2+} (Fig. 1, top) indicates that they belong to the HVA rather than the LVA class of Ca\textsuperscript{2+} channels (Carbone and Lux 1987; Fox et al. 1987a; Tsien et al. 1988). The relative sensitivity to Cd\textsuperscript{2+} and Ni\textsuperscript{2+} was directly examined by comparing the reduction in Ca\textsuperscript{2+} current produced by 1 mM Cd\textsuperscript{2+} and 1 mM Ni\textsuperscript{2+}. Nearly all of the Ca\textsuperscript{2+} current was blocked by 1 mM Cd\textsuperscript{2+} (99.1 ± 3.5%, n = 5), whereas 1 mM Ni\textsuperscript{2+} produced only a 30.0 ± 9.7% (n = 4) reduction in the Ca\textsuperscript{2+} current.

We used a loose-patch technique to determine whether the Ca\textsuperscript{2+} current originates primarily from the cell body or from a more remote region of the cell (Fig. 2). The Ca\textsuperscript{2+} current was measured along the dorsal surface of the soma with patch pipettes during two-microelectrode voltage clamp of the cell. The estimated density of the Ca\textsuperscript{2+} current measured with the loose-patch electrode was −11.8 ± 1.7 pA/μm² surface area (n = 6) with a range of −4.6 to −16.1 pA/μm². The mean amplitude of the whole-cell Ca\textsuperscript{2+} current density was −4.6 ± 0.4 pA/μm² surface area with a range of −3.1 to −5.6 pA/μm². The greater Ca\textsuperscript{2+} current density measured with the loose-patch technique is likely due to an underestimation of the cell surface area that was isolated by the patch pipette (see METHODS). Nevertheless, these results clearly show that the soma generates a significant Ca\textsuperscript{2+} current.
Gentle suction. The pipette resistance (bottom, Figure 3, resistance (R) is mediated through HVA rather than LVA Ca^2+ channels. The onset of a depolarizing voltage step to 0 mV. The amplitude of the Ca^2+ current was maximal (−135.0 ± 25.8 nA, n = 14) at ~0 mV and decreased at more positive membrane potentials.

The I-V curve provides further evidence that the Ca^2+ current is mediated through HVA rather than LVA Ca^2+ channels. Figure 3, bottom, shows that the Ca^2+ channels begin to activate at ~50 to ~40 mV. The I-V plot has only a single peak and there is no shoulder in the ascending limb of I-V curve of Ca^2+ current, suggesting the absence of LVA Ca^2+ current with a more negative activation range. In addition, there was no change in the shape of the I-V curve when the holding potential was set at a more negative potential of ~90 mV (data not shown).

Substitution of Ba^{2+} for Ca^{2+} as a charge carrier

To examine the effects of organic Ca^{2+} channel blockers, Ba^{2+} was substituted for Ca^{2+} as a charge carrier. Previous studies have demonstrated that high extracellular Ca^{2+} concentrations, such as found in crayfish saline, can block the effects of organic Ca^{2+} channel blockers. The inhibitory action of ω-agatoxin subtypes (I, II, and III) on Ca^{2+} channels in insect motor terminals and neuronal cell bodies is more effective at lower extracellular Ca^{2+} concentrations (Bindokas and Adams 1989; Bindokas et al. 1991). Also, elevation of extracellular Ca^{2+} has been shown to abolish the inhibitory effect of ω-CgTX GVIA on Ca^{2+} currents in chick dorsal root ganglion neurons (McCleskey et al. 1987) and at the neuromuscular junction of the mouse (Protti et al. 1991). It appears that the inhibitory effect of high Ba^{2+} concentration is less than that of high Ca^{2+} concentration (Abe et al. 1986). The substitution of Ba^{2+} for Ca^{2+} provides the additional advantage of a larger inward current, because Ba^{2+} is more permeable through HVA Ca^{2+} channels than Ca^{2+} (Carbone and Lux 1987; Fox et al. 1987a).

The I-V curves for the Ca^{2+} and Ba^{2+} currents were compared. The maximum amplitude of the Ba^{2+} current (−164.6 ± 15.5 nA, n = 5) at ~10 mV was 40% larger than that of the Ca^{2+} current (−115.8 ± 12.4 nA) at 0 mV (Fig. 4, top). Compared with the I-V curve for the Ca^{2+} current, the peak of the I-V curve for Ba^{2+} current was shifted by ~10 to ~15 mV (Fig. 4, bottom). This was also noted in previous studies and attributed to the weaker screening effect of Ba^{2+} than that of Ca^{2+} on the extracellular surface charge of the membrane (Bargas et al. 1994; Hernández-Cruz and Pape 1989).

Voltage-dependent activation of the Ca^{2+} current

To examine the voltage dependence of the Ca^{2+} current activation, the cell body was depolarized with 50-ms voltage steps in 10-mV increments from the holding potential (−70 mV) to +40 mV. The activation rate becomes faster as the depolarizing voltage step is increased (Fig. 3, top). The time to peak was 6–10 ms after the onset of a depolarizing voltage step to 0 mV. The amplitude of the Ca^{2+} current was maximal (−135.0 ± 25.8 nA, n = 14) at ~0 mV and decreased at more positive membrane potentials.

FIG. 2. Ca^{2+} currents generated by the soma membrane are demonstrated by applying a cell-attached loose-patch clamp while voltage clamping the cell body with 2 microelectrodes. Representative experiment in which these 2 techniques were applied simultaneously. Top: depolarizing voltage step to 0 mV from a holding potential of ~70 mV. Middle: whole-cell Ca^{2+} current was measured from the cell body with the use of 2-microelectrode voltage clamping. Bottom: while the cell body was voltage clamped, the Ca^{2+} current was recorded with the use of a loose-patch pipette (tip diameter 24 μm) placed against the cell body (100 μm diam) with gentle suction. The pipette resistance (R_p) was 0.13 MΩ and the seal resistance (R_s) was 0.29 MΩ. The Ca^{2+} current trace was adjusted to account for leakage through the seal as discussed in METHODS.

FIG. 3. Voltage dependence of Ca^{2+} current activation. Top: representative recordings of Ca^{2+} currents during a series of 10-mV depolarizing steps. Bottom: to obtain current-voltage (I-V) curve, the peak amplitude of the Ca^{2+} current (●) was plotted against the membrane potential. Activation begins at ~40 to ~50 mV and the amplitude of the Ca^{2+} current is maximal at ~0 mV.
FIG. 4. Effects of substitution of Ba\(^{2+}\) for Ca\(^{2+}\) as a charge carrier. Extracellular Ca\(^{2+}\) (13.5 mM) was replaced with 13.5 mM Ba\(^{2+}\) (see METHODS). Top: representative experiment comparing Ca\(^{2+}\) and Ba\(^{2+}\) currents produced in the same cell by depolarizing voltage steps to -10 and 0 mV. The maximum Ba\(^{2+}\) current recorded at -10 mV was 40% larger than the maximum Ca\(^{2+}\) current recorded at 0 mV. Bottom: I-V curves for the Ca\(^{2+}\) and Ba\(^{2+}\) currents were normalized to the maximum Ca\(^{2+}\) current. The I-V curve for the Ba\(^{2+}\) current was shifted by -10 to -15 mV compared with the I-V curve for the Ca\(^{2+}\) current. Mean values are from 5 experiments.

Effect of organic Ca\(^{2+}\) channel blockers

To classify the HVA Ca\(^{2+}\) channels into their subtypes, the effects of specific organic blockers on Ba\(^{2+}\) currents were examined. The polypeptide toxin from the funnel-web spider, \(\omega\)-agatoxin IVA, is a specific blocker of P-type Ca\(^{2+}\) channels (Mintz et al. 1992a,b). The peak amplitude of the control Ba\(^{2+}\) current (-164.6 ± 15.5 nA) was reduced to -110.5 ± 19.2 nA (n = 5) after the addition of 600 nM \(\omega\)-agatoxin IVA, a significant 32.9 ± 8.3% (P < 0.01, n = 5) reduction (Fig. 5, top). L-type Ca\(^{2+}\) channels are sensitive to a variety of dihydropyridines (Triggle et al. 1991), whereas N-type Ca\(^{2+}\) channels are specifically blocked by \(\omega\)-conotoxin GVIA (Fox et al. 1987a,b; McCleskey et al. 1987; Olivera et al. 1985). Neither 50 \(\mu\)M nifedipine nor 2 \(\mu\)M synthetic \(\omega\)-conotoxin GVIA produced a significant reduction in the Ba\(^{2+}\) current (n = 6). The P-type Ca\(^{2+}\) channels appear to be responsible for ~33% of the Ba\(^{2+}\) current.

FIG. 5. Effects of 3 organic Ca\(^{2+}\) channel blockers on the Ba\(^{2+}\) current were examined. Only \(\omega\)-agatoxin IVA (\(\omega\)-AgTX IVA) reduces the Ba\(^{2+}\) current. Top: specific blocker for the P-type Ca\(^{2+}\) channel, \(\omega\)-AgTX IVA (600 nM), produced a significant reduction in the peak Ba\(^{2+}\) current. Middle and bottom: neither 50 \(\mu\)M nifedipine, specific for L-type channels, nor 2 \(\mu\)M synthetic \(\omega\)-conotoxin GVIA (\(\omega\)-CgTX GVIA), specific for N-type channels, produced a significant reduction in the Ba\(^{2+}\) current (n = 6). The P-type Ca\(^{2+}\) channels appear to be responsible for ~33% of the Ba\(^{2+}\) current.

The dose-response relationship for inhibition of the Ca\(^{2+}\) current by \(\omega\)-agatoxin IVA was examined. The mean normalized Ba\(^{2+}\) current was plotted against concentrations of \(\omega\)-AgTX IVA and fitted to the Hill equation to obtain the half-maximum block (IC\(_{50}\)) for \(\omega\)-AgTX IVA. The dose-response curve shows that the maximum reduction in the amplitude of the Ba\(^{2+}\) current occurs at concentrations of ~600 nM \(\omega\)-AgTX IVA (Fig. 6). The estimated IC\(_{50}\) of the Ba\(^{2+}\) current occurs at a concentration of 183.5 ± 45.4 nM. These results confirm that a P-type Ca\(^{2+}\) channel is responsible for 33% of the inward Ba\(^{2+}\) current. The remaining 67% of the current occurs through Ca\(^{2+}\) channels that cannot be classified as P, N, or L type.

To characterize the biophysical differences between the P-type Ca\(^{2+}\) channels and the non-P-type (\(\omega\)-AgTX-IVA-resistant) Ca\(^{2+}\) channels, the voltage-dependent activation of these Ca\(^{2+}\) channels was studied by estimating the mem-
brane permeability for the Ba\(^{2+}\) current with the use of the Goldman-Hodgkin-Katz constant current equation

\[
I = P_m [C_e - [C_i] \exp(-z_E F V / RT)] / [1 - \exp(-z_E F V / RT)]
\]  

where \(I\) is the measured transmembrane current (A/cm\(^2\)), \(V\) is the membrane potential (V), \(z\) is the charge valence, \(F\) is the Faraday constant, \(R\) is the gas constant, \(T\) is the absolute temperature (\(^\circ\)K), \([C_e]\) and \([C_i]\) are the extracellular and intracellular concentration, respectively (mM). The intracellular ion concentration of Ba\(^{2+}\) was assumed to be 0 mM. The surface area of the cell body (1.9 \(\times\) 10 3 cm\(^2\)) was estimated by measuring the membrane capacitance (average 1.9 nF, \(n = 6\)), assuming a specific membrane capacitance of 1 \(\mu\)F/cm\(^2\). This estimation of Ca\(^{2+}\) channel permeability has been used in previous studies to compare the activation voltages of different Ca\(^{2+}\) channel types (Bargas et al. 1994; Kay and Wong 1987; Surmeier et al. 1994).

To obtain the voltage-dependent activation curve of the P-type Ba\(^{2+}\) current as a function of membrane potential, the magnitude of the P-type Ba\(^{2+}\) current was estimated by subtracting the \(\omega\)-AgTX-IVA-resistant Ba\(^{2+}\) current from the total Ba\(^{2+}\) current (Fig. 7, bottom). The non-P-type Ba\(^{2+}\) current was further corrected for the residual outward current present after the addition of 2 mM Cd\(^{2+}\). The estimated permeabilities \((P_m)\) of the P-type and the non-P-type Ca\(^{2+}\) channels obtained from Eq. 1 were plotted as a function of membrane potential and fitted to the Boltzmann equation

\[
P_m = \frac{P_{\text{max}}}{1 + \exp\left(-\frac{V - V_h}{V_c}\right)}
\]

where \(V\) is the membrane potential during the test pulse, \(P_{\text{max}}\) is the maximum permeability, \(V_h\) is the potential corresponding to half-activation, and \(V_c\) is the slope factor.

Figure 8 shows the activation curves fit by the Boltzmann equation. The normalized permeability shows the differences in the voltage dependence of these Ca\(^{2+}\) channels. It appears that the non-P-type Ba\(^{2+}\) current \((P_{\text{max}} = 2.7 \times 10^{-5} \, \text{cm/s}, V_h = -9.9 \pm 2.3 \, \text{mV}, V_c = 13.3 \pm 1.8 \, \text{mV}; \, n = 5\) begins to activate at a more negative membrane potential than the P-type Ba\(^{2+}\) current \((P_{\text{max}} = 1.96 \times 10^{-3} \, \text{cm/s}, V_h = 7.2 \pm 4.0 \, \text{mV}, V_c = 16.5 \pm 2.3 \, \text{mV})\). Thus the voltage-dependent activation of the non-P-type Ca\(^{2+}\) channels is different from that of the P-type Ca\(^{2+}\) channels.

P-type Ca\(^{2+}\) channels selectively undergo a long-term, activity-dependent reduction

We have previously shown that an increase in impulse activity produces a Ca\(^{2+}\)-dependent, long-term decrease in the Ca\(^{2+}\) current recorded from the cell body of this motoneuron (Hong and Lnenicka 1995). An increase in impulse activity produced by 5-Hz electrical stimulation of the axon for 0.5–1 h resulted in a reduction of ~28% in Ca\(^{2+}\) current 6–7 h after stimulation. This reduction persisted for ~3 days. To determine whether the P-type and/or the non-P-type Ca\(^{2+}\) current is reduced by increased impulse activity, the axon was stimulated in normal saline at 5 Hz for 45–60 min with a suction electrode. Six to seven hours after stimulation, Ba\(^{2+}\) currents were measured before and after block of the P-type currents with 600 nM \(\omega\)-AgTX IVA. The P-type current was determined by subtracting the Ba\(^{2+}\) current remaining after the addition of \(\omega\)-AgTX IVA from the total Ba\(^{2+}\) current. The
FIG. 9. P-type currents selectively undergo a long-term, activity-dependent reduction. Top: I-V curves for the P-type and the non-P-type currents in unstimulated control cells and cells stimulated 6 ± 7 h earlier at 5 Hz for 45 ± 60 min (n = 4±5). The P-type currents were determined by subtracting the Ba\(^{2+}\) current in the presence of 600 nM \(\omega\)-AgTX IVA from the total Ba\(^{2+}\) current recorded before the addition of the \(\omega\)-AgTX IVA. Bottom: peak Ba\(^{2+}\) current density recorded at 0 mV from control (n = 8) and stimulated cells (n = 9). An increase in impulse activity of the cell produced a significant 43% reduction in the P-type current density, but there was no reduction in the density of the non-P-type current.

P-type and the non-P-type currents remaining after stimulation were compared with those in control neurons (Fig. 9, **top**). Six to seven hours after stimulation, there was a significant 43% reduction in the peak P-type current density (Fig. 9, **bottom**); the current density of the control cell was \(-25.1 \pm 2.7\) nA/nF, n = 8, whereas that of the stimulated cell was \(-14.4 \pm 2.9\) nA/nF, n = 9 (P < 0.05). There was no significant reduction in the density of the non-P-type current; \(-57.1 \pm 4.1\) nA/nF, n = 8 in the control cell and \(-56.1 \pm 6.3\) nA/nF, n = 9 in the stimulated cells (P > 0.10). These results demonstrate that an increase in neuronal impulse activity selectively produces a long-term reduction in the P-type Ca\(^{2+}\) current.

**DISCUSSION**

**HVA Ca\(^{2+}\) channels**

The properties of the voltage-dependent Ca\(^{2+}\) channels found in this crayfish motoneuron are more similar to those of HVA Ca\(^{2+}\) channels than LVA Ca\(^{2+}\) channels. The LVA, T-type Ca\(^{2+}\) channels identified in vertebrate cells have two prominent biophysical characteristics that differentiate them from HVA Ca\(^{2+}\) channels: a low threshold for activation and a rapid voltage-dependent inactivation (for review see Tsien et al. 1988). The activation voltage for the Ca\(^{2+}\) current in motoneuron F3 is \(-40\) to \(-50\) mV and its inactivation is Ca\(^{2+}\) dependent, not voltage dependent (Hong and Lnenicka 1995). In addition, the Ca\(^{2+}\) channels in motoneuron F3 are more sensitive to Cd\(^{2+}\) than Ni\(^{2+}\) and have a greater conductance for Ba\(^{2+}\) than Ca\(^{2+}\). These properties are generally considered to be characteristics of HVA Ca\(^{2+}\) channels (Carbone and Lux 1987; Fox et al. 1987a,b; Haydon and Mon-Son-Hing 1988; Nowycky et al. 1985; for review see Tsien et al. 1988).

**P-type Ca\(^{2+}\) channel**

On the basis of the I-V curve and inactivation properties, the Ca\(^{2+}\) current in the cell body appeared to be produced by a single homogeneous population of HVA Ca\(^{2+}\) channels (Hong and Lnenicka 1995). However, the pharmacological studies presented here have demonstrated at least two subtypes of HVA Ca\(^{2+}\) channels with slight differences in activation voltages. One of these subtypes appears to be a P-type Ca\(^{2+}\) channel, on the basis of its sensitivity to the peptide \(\omega\)-AgTX IVA from the funnel-web spider, *A. aperta* (Mintz et al. 1992a,b). The peptide \(\omega\)-AgTX IVA produced a 33% reduction in the amplitude of the Ba\(^{2+}\) current at a saturating concentration of 600 nM. The P-type channel in this cell appears to have less binding affinity for \(\omega\)-AgTX IVA than the P-type channels in vertebrate neurons. In the previous vertebrate studies, saturating concentrations of \(\omega\)-AgTX IVA were in the range of 50–200 nM (Bargas et al. 1994; Mintz et al. 1992a,b).

This P-type Ca\(^{2+}\) channel in the crayfish has biophysical properties that are similar to those in other species. The P-type Ca\(^{2+}\) current in the motoneuron F3 begins to activate at \(-40\) to \(-50\) mV and is more permeable to Ba\(^{2+}\) than Ca\(^{2+}\). These properties are similar to those of the P-type Ca\(^{2+}\) channel first described in rat Purkinje cells and the
squad giant synapse (Llinás et al. 1989). This range of activation voltage, intermediate to that of the LVA T-type and HVA L- and N-type channels, is also seen in other central and peripheral neurons (Regan et al. 1991).

The P-type Ca\(^{2+}\) channel appears to play a prominent role in transmitter release in both vertebrates and invertebrates. P-type channel blockers inhibit transmitter release from mouse motor terminals (Uchitel et al. 1992); rat cerebellar, spinal, and hippocampal neurons (Takahashi and Momiyama 1993); and rat brain synaptosomes (Turner et al. 1993). In invertebrates, P-type channel antagonists inhibit transmitter release at the squid giant synapse (Llinás et al. 1989), synapses in the buccal ganglion of *Aplysia* (Fossier et al. 1994), and crayfish neuromuscular synapses (Araque et al. 1994; Blundon et al. 1995). P-type Ca\(^{2+}\) channels are involved in transmitter release from excitatory and inhibitory axon terminals in the crayfish claw opener muscle: an 80% reduction in excitatory postsynaptic current amplitude results from the addition of 300 nM \(\omega\)-AgTX IV A (Araque et al. 1994). Presumably the P-type channel identified in the cell body of motoneuron F3 is the same as that found at the motor terminals.

The non-P-type Ca\(^{2+}\) current is resistant to nifedipine and \(\omega\)-CgTX GVIA as well as \(\omega\)-AgTX IV A. A considerable portion (67%) of the Ba\(^{2+}\) current remained at a saturating concentration of 600 nM that was not reduced by either 50 \(\mu\)M nifedipine or 2 \(\mu\)M \(\omega\)-CgTX GVIA. It appears that the cell body contains a type(s) of Ca\(^{2+}\) channel that does not belong to any of the previously identified L-, N-, and P types. In other invertebrate as well as vertebrate neurons, Ca\(^{2+}\) currents insensitive to L-, N-, and P-type antagonists have been observed (Mintz et al. 1992a; Penington and Fox 1995; Regan et al. 1991; Surmeier et al. 1994).

### P-type Ca\(^{2+}\) current selectively undergoes an activity-dependent reduction

We have previously shown that increased electrical activity in the relatively inactive crayfish motoneuron F3 produces a short-term and long-term reduction in the voltage-dependent Ca\(^{2+}\) current (Hong and Lnenicka 1995); a short-term reduction that results from the previously described Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels (Tillotson 1979) and a long-term reduction that persists for \(\geq 3\) days. Both forms of activity-dependent reduction in Ca\(^{2+}\) current are dependent on Ca\(^{2+}\) influx. It appears that both the P-type and the non-P-type channels undergo the short-term Ca\(^{2+}\)-dependent inactivation, because nearly all the Ca\(^{2+}\) current ( \(\sim 96\%\) ) was inactivated during repetitive depolarizing pulses (Hong and Lnenicka 1995). In the present study we demonstrate that only the P-type channel current undergoes the long-term reduction. There was a significant 42% reduction in the P-type current density after stimulation, but no change in the non-P-type current density. Thus an increase in neuronal impulse activity selectively downregulates the P-type Ca\(^{2+}\) channel through a Ca\(^{2+}\)-dependent mechanism.

We do not know whether the reduction in P-type Ca\(^{2+}\) current results from a change in the properties of individual Ca\(^{2+}\) channels or a reduction in the number of functional Ca\(^{2+}\) channels. A number of cell culture studies have demonstrated that prolonged depolarization resulting from high external K\(^+\) produces a long-term Ca\(^{2+}\)-dependent reduction in Ca\(^{2+}\) current density (Franklin et al. 1991), apparently resulting from a reduction in the number of Ca\(^{2+}\) channels (DeLorme and McGee 1986; DeLorme et al. 1988; Ferrante et al. 1991). In some cases, this reduction in Ca\(^{2+}\) channel number was produced by the internalization of Ca\(^{2+}\) channels (Liu et al. 1994). There is evidence for other Ca\(^{2+}\)-dependent mechanisms that can produce a reduction in the number of functional Ca\(^{2+}\) channels. The Ca\(^{2+}\)-activated protease, calpain, has been shown to be involved in the permanent degradation of various membrane proteins (Melloni and Pontremoli 1989). The irreversible ‘‘washout’’ of Ca\(^{2+}\) currents in dialyzed *Helix* neurons is blocked by perfusion with the tripeptide leupeptin, an inhibitor of calpain (Chad and Eckert 1986). In guinea pig myocytes, calpain produced an irreversible reduction in Ca\(^{2+}\) currents, possibly through the proteolytic modification of Ca\(^{2+}\) channels (Belles et al. 1988).

### Possible role of P-type Ca\(^{2+}\) channels in long-term changes in transmitter release

The large initial release of transmitter from the motor terminals of crustacean phasic motoneurons is reduced for days as a result of prior repetitive stimulation (Bradacs et al. 1990; Lnenicka and Atwood 1985; Mercier and Atwood 1990). This long-term depression of transmitter release is likely to play a role in the differentiation of phasic and tonic motor terminals (Lnenicka 1991). We have demonstrated that this long-term synaptic depression is triggered by Ca\(^{2+}\) influx (Hong and Lnenicka 1993). Because P-type Ca\(^{2+}\) currents are involved in evoking transmitter release from crayfish motor terminals (Araque et al. 1994; Blundon et al. 1995), a Ca\(^{2+}\)-dependent reduction in the P-type Ca\(^{2+}\) current at the motor terminals could underlie this synaptic change. This is supported by recent findings that the activity-dependent, seasonal changes in transmitter release from crayfish motor terminals (Lnenicka and Zhao 1991) are accompanied by corresponding seasonal changes in the amplitude of the Ca\(^{2+}\) currents in the cell body (unpublished observations).

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