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Activity-Dependent Changes in Voltage-Dependent Calcium Currents and Transmitter Release

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Abstract

Voltage-dependent Ca^{2+} channels are important in the regulation of neuronal structure and function, and as a result, they have received considerable attention. Recent studies have begun to characterize the diversity of their properties and the relationship of this diversity to their various cellular functions. In particular, Ca^{2+} channels play a prominent role in depolarization-secretion coupling, where the release of neurotransmitter is very sensitive to changes in voltage-dependent Ca^{2+} currents. An important feature of Ca^{2+} channels is their regulation by electrical activity. Depolarization can selectively modulate the properties of Ca^{2+} channel types, thus shaping the response of the neuron to future electrical activity. In this article, we examine the diversity of Ca^{2+} channels found in vertebrate and invertebrate neurons, and their short- and long-term regulation by membrane potential and Ca^{2+} influx. Additionally, we consider the extent to which this activity-dependent regulation of Ca^{2+} currents contributes to the development and plasticity of transmitter releasing properties. In the studies of long-term regulation, we focus on crustacean motoneurons where activity levels, Ca^{2+} channel properties, and transmitter releasing properties can be followed in identified neurons.

Index Entries: Transmitter release; calcium channel; plasticity; long-term depression; short-term depression; calcium channel inactivation; calcium current facilitation; development; growth cone; synapse.

Introduction

Intracellular Ca^{2+} levels are extremely important in the regulation of neuronal structure and function. Intracellular Ca^{2+} levels regulate many cellular processes including gene transcription (Morgan and Curran, 1991), ion channel development and function (Blatz and Magleby, 1987; Partridge and Swandulla, 1988; Linsdell and Moody, 1995), transmitter release (Augustine et al., 1987), and neuronal growth and degeneration (Choi, 1988; Kater and Mills, 1991). A major avenue for the entry of Ca^{2+} into neurons is through voltage-depen-

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dent Ca^{2+} channels, which allow Ca^{2+} entry on membrane depolarization. Neurons contain multiple types of voltage-dependent Ca^{2+} channels that regulate specific neuronal functions through their cellular localization and unique biophysical properties. The selective modulation of these various types of Ca^{2+} channels can play an important role in determining the response of the neuron to subsequent depolarization.

Electrical activity can modulate Ca^{2+} channels through voltage-dependent and Ca^{2+}-dependent mechanisms. Although there is evidence that increased electrical activity can produce both an upregulation and downregulation of Ca^{2+} currents, examples of the latter are more prevalent. Both voltage-dependent and Ca^{2+}-dependent Ca^{2+} channel inactivation operate to reduce Ca^{2+} influx over a period of seconds to minutes. These mechanisms limit the increase in intracellular Ca^{2+} during periods of increased electrical activity, which is important, since high concentrations are often detrimental. Ca^{2+}-dependent inactivation is a particularly effective self-limiting mechanism, since the channel is regulated based on intracellular Ca^{2+} levels.

There is also evidence for a long-term, Ca^{2+}-dependent reduction in Ca^{2+} current that can persist for days. In some cases, this has been shown to result from a decrease in the number of Ca^{2+} channels. It presumably acts to "match" the voltage-dependent calcium conductance to the normal electrical activity levels. This mechanism would presumably prevent an overload of Ca^{2+} influx during future bouts of activity and readjust the range of impulse frequencies at which the neuron can effectively operate.

The Ca^{2+} channels at the axon terminal play a particularly important role in the development and function of the nervous system. During development and regeneration, the terminal region of the axon becomes specialized as a growth cone to provide the motive forces and sensory capabilities for axon navigation. Ca^{2+} channels at the growth cone play an important role in regulating its motility. After target contact and synapse formation, intracellular Ca^{2+} levels control the release of transmitter from the axon's presynaptic terminal. Specific Ca^{2+} channel types localized at the active zone provide the link between depolarization and the fusion of synaptic vesicles to the presynaptic membrane. The modulation of Ca^{2+} channels at axonal endings would be an efficient and effective mechanism for regulating transmitter release and possibly growth cone motility. Accordingly, there has been considerable interest in studying the modulation of Ca^{2+} currents at axon terminals; however, it is difficult to examine these Ca^{2+} currents directly owing to the small size of the endings.

In this article, we briefly summarize the types of Ca^{2+} channels identified in vertebrate and invertebrate neurons, and their involvement in transmitter release. Activity-dependent changes in Ca^{2+} channels produced by depolarization and Ca^{2+} influx are reviewed. We examine the evidence that activity-dependent changes in Ca^{2+} channels are involved in short- and long-term synaptic changes, particularly synaptic depression. Finally, we consider the role of activity in the development of differences in Ca^{2+} channels at growth cones and synapses. Many of our examples are drawn from studies of crustacean motoneurons in which normal impulse activity levels have been characterized and experimentally altered.

Ca^{2+} Channel Types

Vertebrate Neurons

In vertebrate neurons, multiple types of Ca^{2+} channels have been identified based on biophysical and pharmacological criteria (for comprehensive reviews, *see* Scott et al., 1991; Stea et al., 1995). The low-voltage activated (LVA), T-type Ca^{2+} channels have biophysical characteristics that differentiate them from high-voltage activated (HVA, Ca^{2+} channels (N-, L-, P-, and Q-types). The LVA channel has a lower threshold of activation than the HVA

channels and a rapid voltage-dependent inactivation (Llinás and Yarom, 1981; Carbone and Lux, 1984). HVA channels are more permeable to Ba^{2+} than Ca^{2+}, whereas LVA channels are not (Fox et al., 1987a; Carbone and Lux, 1987). Pharmacologically, LVA channels are more sensitive to Ni^{2+} than Cd^{2+} (Carbone and Lux, 1984, 1987); the opposite is true for HVA channels (Nowycky et al., 1985; Fox et al., 1987a,b).

HVA Ca^{2+} channels can be grouped into L-, N-, P-, and Q-types according to their sensitivity to organic Ca^{2+} channel blockers. The L-type Ca^{2+} channels are specifically blocked by a variety of 1,4-dihydropyridine (DHP) antagonists (Fox et al., 1987a; Triggle et al., 1991). The N-type Ca^{2+} channels are sensitive to the peptide ω-conotoxin GVIA (ω-CgTX GVIA from the venom of the marine cone snail, *Conus geographus* (Fox et al., 1987a). P-type Ca^{2+} channels are blocked by either a polyamine toxin fraction (FTX) or the peptide ω-agatoxin IVA (ω-AgTX IVA from the venom of the funnel-web spider, *Agelenopsis aperta* (Llinás et al., 1989; Mintz et al., 1992). The Q-type Ca^{2+} channel, which was recently identified in rat cerebellar granule neurons, is also blocked by ω-AgTX IVA; however, a higher concentration is required for Q-type channels than for P-type channels (Randall and Tsien, 1995).

L- and N-type Ca^{2+} channels in chick dorsal root ganglion (DRG) neurons have characteristic differences in their activation voltage, unitary conductance, and inactivation rate (Nowycky et al., 1985; Fox et al., 1987a,b). In these original studies, the N-type channel had a more negative activation voltage and a faster inactivation rate than the L-type channel. The unitary conductance of the N-type channel was approximately half that of the L-type channel. In other neurons these distinctions in the biophysical properties of N- and L-type channels are less clear, largely because of variability in the properties of the N-type channel (Swandulla et al., 1991). For example, in some mammalian neurons N-type channels have an activation voltage more positive than the L-type channel (Regan et al., 1991) and a unitary conductance similar to the L-type channel (Plummer et al., 1989). Thus, the pharmacological distinctions are often the major criteria used to distinguish these HVA channels.

The biophysical properties of the more recently identified P- and Q-type channels have not been as widely examined as the N- and L-type channels. The P-type channel, originally described in rat Purkinje cells and the squid giant synapse, has a more negative activation voltage than L- and N-type channels and a slow rate of inactivation (Llinás et al., 1989; Mintz et al., 1992). The Q-type current found in rat cerebellar granule cells is similar to the P-type current, except for its more rapid inactivation (Randall and Tsien, 1995).

There are Ca^{2+} channels that are insensitive to all of the organic Ca^{2+} channel blockers. A component of the Ca^{2+} current in rat cerebellar granule neurons does not show the typical sensitivity to any Ca^{2+} channel antagonists and has been designated as a R-type current (Zhang et al., 1993; Ellinor et al., 1993; Randall and Tsien, 1995). It is not known whether single or multiple types of Ca^{2+} channels produce the R-type current. Approximately half the Ca^{2+} current in rat dorsal raphe neurons is insensitive to the organic Ca^{2+} channel blockers and differs from the R-type current (Penington and Fox, 1995).

Cloning of Ca^{2+} channel subunits in mammals has begun to provide a molecular basis for the various Ca^{2+} channel types. The pore-forming α_1-subunit of Ca^{2+} channels appears to be responsible for the voltage-dependent gating and the pharmacological characteristics of the Ca^{2+} channel (Tanabe et al., 1987; Perez-Reyes et al., 1989; Mori et al., 1991). The α_1-subunits have been divided into A, B, C, D, E, and S classes (Snutch et al., 1990, 1992; Zhang et al., 1993). Based on pharmacology and biophysics, these subunits correspond to HVA channel types: the α_{1C}-, α_{1D}-, and α_{1S}-subunits to the L-type channel (Tanabe et al., 1987; Perez-Reyes, 1989; Mikami et al., 1989; Williams et al., 1992a) and the α_{1B}-subunit to the N-type channel (Williams et al., 1992b; Dubel et al., 1992; Fujita et al., 1993). α_{1A}-subunits exhibit biophysical and pharmacological characteristics that are similar to the P- and Q-type channels

(Sather et al., 1993; Stea et al., 1994). The α_{1E}-subunit is not sensitive to any of the organic Ca^{2+} channel antagonists and may represent a R-type channel (Ellinor et al., 1993; Zhang et al., 1993).

Invertebrate Neurons

Beginning with the early studies by Hagiwara et al. (1975), multiple Ca^{2+} channel types have been identified in invertebrate cells (for reviews, *see* Hagiwara and Byerly, 1981; Skeer et al., 1996). Although the interrelationship of these various Ca^{2+} channels has not been established, many neuronal channels have been classified into categories established largely from vertebrate studies.

Most of the Ca^{2+} channels identified in invertebrate neurons are HVA based on their activation and inactivation properties. The cell bodies of neurons examined in crayfish (Czternasty et al., 1989a; Chrachri, 1995; Hong and Lnenicka, 1997), lobster (Tazaki and Cooke, 1986, 1990), crab (Meyers et al., 1992), *Aplysia* (Trudeau et al., 1993), and leech (Bookman and Liu, 1990) appear to contain only HVA channels. The activation voltages for these HVA channels range from approx –40 mV in crustacean motoneurons (Tazaki and Cooke, 1990; Chrachri, 1995; Hong and Lnenicka, 1997) to –5 mV in leech neurons (Johansen et al., 1987). Similar to vertebrate HVA channels, HVA channels in crayfish abdominal motoneurons (Chrachri, 1995; Hong and Lnenicka, 1997) and leech Retzius cells (Bookman and Liu, 1990) have a greater conductance to Ba^{2+} than Ca^{2+}; however, this was not true for lobster cardiac ganglion motoneurons (Tazaki and Cooke, 1990). LVA Ca^{2+} currents have been identified in the cell body of leech heart interneurons (Angstadt and Calabrese, 1991) and the *Helisoma* neuron, B5, where it was accompanied by a HVA current (Haydon and Man-Son-Hing, 1988; Berdan et al., 1993).

Individual neurons can contain multiple types of HVA channels with differences in pharmacology and biophysics (Edmonds et al., 1990; Trudeau et al., 1993; Fossier et al., 1994). Some of these channels have been classified as L-, N-, and P-type based upon their sensitivity to the organic Ca^{2+} channel blockers. DHP-sensitive, L-type channels were found in *Aplysia* abdominal ganglion 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 (Chrachri, 1995). ω-CgTX GVIA-sensitive, N-type channels were shown in *Aplysia* buccal ganglion neurons (Fossier et al., 1994; Trudeau et al., 1995). P-type channels were initially identified at the squid giant synapse (Llinás et al., 1989), and subsequently distinguished in *Aplysia* buccal ganglion neurons (Fossier et al., 1994) of crayfish motoneurons (Araque et al., 1994; Blundon et al., 1995; Hong and Lnenicka, 1997) based on their sensitivity to FTX and ω-AgTX IVA. Some of these P-type channels might belong to the recently identified Q-type, since it is also sensitive to ω-AgTX IVA (Randall and Tsien, 1995). There are Ca^{2+} currents that are insensitive to all of these Ca^{2+} channel blockers and cannot be classified as L, N, or P (Hong and Lnenicka, 1997).

An initial comparison of the molecular structure of Ca^{2+} channel types in vertebrates and invertebrates has been performed based on the recent cloning of Ca^{2+} channels in the fruitfly and housefly. The *Drosophila melanogaster* Ca^{2+} channel α_1-subunit (Dmca1D) cloned from the head belongs to the same supergene family as the mammalian α_1-subunits (Zheng et al., 1995). Dmca1D has been classified as an α_{1D}-subunit based on its 78% sequence similarity to the rat brain α_{1D}-subunit. The Ca^{2+} channel α_1-subunit from the body wall muscle of the larval housefly *Musca domestica* ($Mdl\alpha_1$) also appears to belong to the L-type subfamily based on its 50% sequence identity to vertebrate L-type α_1-subunits (Grabner et al., 1994).

Ca^{2+} Channels Involved in Transmitter Release

Transmitter release from the presynaptic terminal is triggered by Ca^{2+} influx through Ca^{2+} channels concentrated at the active zone (Robitaille et al., 1990). Based on the short

delay between Ca^{2+} entry and transmitter release, the channels triggering release must be located very close to the docked vesicles (Llinás et al., 1981). The close association between the site of Ca^{2+} influx and releasable vesicles may be owing to a direct interaction of the putative vesicle docking protein, syntaxin, with the Ca^{2+} channel (Bennett et al., 1992). At the time of vesicle fusion, theoretical studies predict that the Ca^{2+} concentration along the membrane is not uniform: hemispheric "domains" of high Ca^{2+} concentration are centered over the cytoplasmic mouth of the Ca^{2+} channel, where concentrations may reach several hundred micromolar (Chad and Eckert, 1984; Fogelson and Zucker, 1985; Simon and Llinás, 1985). Experimental studies have shown localized, high Ca^{2+} concentrations produced by Ca^{2+} channel clusters at active zones of the squid giant synapse (Llinás et al., 1992; Sugimori et al., 1994).

The number and distribution of functional Ca^{2+} channels at the active zone are likely to play an important role in regulating transmitter release. Freeze-fracture of the presynaptic membrane reveals large particles at active zones that represent Ca^{2+} channels (Pumplin et al., 1981; Farinas et al., 1993) and possibly other channels, such as Ca^{2+}-activated potassium channels (Roberts, 1994). A greater density and/or number of these intramembranous particles has been correlated with greater transmitter release at neuromuscular junctions of lizard, moth, and lobster (Walrond and Reese, 1985; Rheuben, 1985; Walrond et al., 1993). These results have been interpreted as evidence that the number and spacing of Ca^{2+} channels can influence the amount of transmitter released. Theoretically, an increase in the density or number of Ca^{2+} channels at the active zone could increase the peak Ca^{2+} concentration by producing greater overlap of Ca^{2+} channel domains (Simon and Llinás, 1985; Zucker and Fogelson, 1986).

Recent studies of presynaptic terminals in the chick ciliary ganglion provide evidence that Ca^{2+} entering through a single channel can trigger vesicle fusion (Stanley, 1993). This argues against a requirement for overlapping domains in transmitter release; however, if the Ca^{2+} that enters through a single channel does not saturate the Ca^{2+} sensor, neighboring Ca^{2+} channels could still influence the probability of release. There is considerable potential for this type of interaction, since many Ca^{2+} channels may open during an action potential: estimates of 100 channel openings/active zone have been reported for the squid giant synapse (Sugimori et al., 1994). Alternatively, if vesicles only dock adjacent to a Ca^{2+} channel, then additional Ca^{2+} channels could increase transmitter release by providing more potential release sites.

The properties of the individual Ca^{2+} channels at the active zone will also be important in determining the characteristics of transmitter release. There have been several studies examining the types of Ca^{2+} channels involved in transmitter release. In general, only HVA channels appear to be directly involved in evoked transmitter release. Among the HVA channels, the N- and P-types appear to play the most prominent role in the fast release of transmitter. Since Ca^{2+} currents cannot be directly examined at most terminals, information about Ca^{2+} channel types has come largely from studies examining the effect of organic Ca^{2+} channel blockers on transmitter release.

Specific blockers of N- and P-type Ca^{2+} channels reduce evoked transmitter release from synaptic terminals at both central and peripheral synapses in vertebrates. Inhibition of N-type Ca^{2+} channels by ω-CgTX GVIA reduces transmitter release from synaptic terminals in the mammalian CNS (Kamiya et al., 1988; Takahashi and Momiyama, 1993; Luebke et al., 1993; Regehr and Mintz, 1994) and sympathetic nervous system (Hirning et al., 1988), and from frog motor terminals (Kerr and Yoshikami, 1984). P-type channel blockers also inhibit transmitter release in the mammalian CNS (Takahashi and Momiyama, 1993; Luebke et al., 1984; Regehr and Mintz, 1994; Castillo et al., 1994) as well as from mouse motor terminals (Uchitel et al., 1992). Studies of rat brain synaptosomes provide further evidence that P-type channels are involved in transmitter

release in the mammalian brain (Reynolds et al., 1986; Turner et al., 1992; Tareilus et al., 1993). Since transmitter release is usually not entirely blocked by a N- or P-type antagonist, more than one type of Ca^{2+} channel is often involved in the release of transmitter. Both N- and P-type channels are involved in transmitter release from synaptic terminals in the rat cerebellum, hippocampus, and spinal cord (Luebke et al., 1993; Takahashi and Momiyama, 1993; Tareilus et al., 1993).

In invertebrates, the N- and P-type channels also play an important role in transmitter release. The P-type channel antagonist, FTX, inhibits the release of transmitter at the squid giant synapse (Llinás et al., 1989). The P-type antagonists FTX and ω-AgTX IVA reduce transmitter release from motor terminals in the crayfish opener muscle (Araque et al., 1994; Blundon et al., 1995). Both N- and P-type channels are involved in the release of transmitter from synaptic terminals in the *Aplysia* CNS (Fossier et al., 1994).

The involvement of multiple Ca^{2+} channel types in transmitter release would potentially increase the capacity to regulate synaptic transmission through selective Ca^{2+} channel modulation. The effect of activating or modulating a single class of channels will be dependent on their biophysical properties as well as their distribution in relation to the active zone. For example, it is currently not known whether different types of Ca^{2+} channels intermix at active zones or segregate to separate active zones. There is evidence in the *Aplysia* CNS that transmitter release is regulated by the selective modulation of a single Ca^{2+} channel type . The neuromodulators, FMRF-amide, histamine, and buccalin influence transmitter release by their modulation of the N-type, but not the P-type, channels found at the synaptic terminals (Fossier et al., 1994). In addition, at *Aplysia* sensory neuron–motoneuron synapses in culture, L-type channels do not directly participate in evoked transmitter release; however, they are responsible for enhanced release produced by tonic depolarization (Edmonds et al., 1990). Presumably, these channels are distant from release sites and only influence Ca^{2+} levels at release sites during prolonged depolarization.

Short-Term Changes in Ca^{2+} Currents

Ca^{2+} current inactivation represents the most prevalent form of short-term activity-dependent change in Ca^{2+} channels. Ca^{2+} channels undergo voltage-dependent and Ca^{2+}-dependent inactivation. Ca^{2+} channel inactivation appears as a reduction in the Ca^{2+} current during a prolonged depolarizing pulse, or a decrease in Ca^{2+} current resulting from a conditioning pre-pulse. Classically, Ca^{2+}- and voltage-dependence are distinguished by examining the relationship between the amount of Ca^{2+} influx during the pre-pulse and the inactivation of Ca^{2+} current during the test-pulse (Tillotson, 1979).

Ca^{2+}-Dependent Ca^{2+} Channel Inactivation

Ca^{2+}-dependent inactivation of Ca^{2+} channels, first demonstrated in Paramecium and Aplysia (Brehm and Eckert, 1978; Tillotson, 1979), has been described in a number of neurons in both invertebrates and vertebrates. It appears that a variety of HVA channel types can show Ca^{2+}-dependent inactivation. In invertebrates, Ca^{2+} influx produces inactivation of HVA Ca^{2+} currents in neurons from *Helix* (Plant and Standen, 1981; Plant et al., 1983; Chad and Eckert, 1986) *Lymnaea* (Johnson and Byerly, 1993a), *Aplysia* (Tillotson, 1979; Eckert and Tillotson, 1981; Fryer and Zucker, 1993), lobster (Tazaki and Cooke, 1990), and crayfish (Hong and Lnenicka, 1995). Ca^{2+}-dependent inactivation has been reported for P-type currents in crayfish and squid (Augustine and Eckert, 1984; Hong and Lnenicka, 1997). In vertebrate neurons, Ca^{2+}-dependent inactivation of HVA currents includes: N-type currents in chick DRG neurons (Kasai and Aosaki, 1988), mammalian neuroblastoma-glioma cells (Kasai and Neher, 1992), and bull-

frog sympathetic neurons (Jones and Marks, 1989); L-type currents in goldfish retinal bipolar neurons (Von Gersdorff and Matthews, 1996) and mammalian neuroblastoma-glioma cells (Kasai and Neher, 1992); and P-type currents in rat cerebrocortical synaptosomes (Tareilus et al., 1994).

Patch-clamp studies in *Helix* neurons and cardiac myocytes show that the Ca^{2+}-dependent inactivation of Ca^{2+} current results from a decrease in the probability of Ca^{2+} channel openings (Lux and Brown, 1984; Imredy and Yue, 1994). Ca^{2+} influx through a single L-type channel can influence its own inactivation and that of adjacent channels without producing a general increase in cytoplasmic Ca^{2+} (Yue et al., 1990; Mazzanti et al., 1991; Imredy and Yue, 1992) suggesting that Ca^{2+} acts near the channel to produce inactivation. The photolytic release of caged Ca^{2+} in *Aplysia* and *Lymnaea* neurons produces a rapid reduction in the Ca^{2+} current (Johnson and Byerly, 1993a; Fryer and Zucker, 1993). This rapid action of Ca^{2+} provides further evidence that it binds at, or near, the channel; however, the effect of exogenous Ca^{2+} chelators on inactivation indicates that the Ca^{2+} binding site is not within the channel pore. Injection of the Ca^{2+} chelators EGTA and BAPTA serves to increase Ca^{2+} current, and reduce the inactivation that occurs during paired pulses or long pulses (Brown et al., 1981; Eckert and Ewald, 1981; Eckert and Tillotson, 1981; Plant et al., 1983; Gutnick et al., 1989). Since these chelators should not influence Ca^{2+} levels within tens of nanometers of the pore, the Ca^{2+} binding site is unlikely to be near the inner mouth of the channel, unless diffusion to the binding site is restricted (Gutnick et al., 1989).

A Ca^{2+} binding site located near the channel will be influenced by the high Ca^{2+} concentration domains that exist during channel openings. The Ca^{2+} concentration required for inactivation has been examined using Ca^{2+}-buffered intracellular solutions. These studies have produced a variety of results with values ranging from <100 n*M* to a few micromolar (Hagiwara and Nakajima, 1966; Kostyuk and Krishtal, 1977; Plant et al., 1983; Dupont et al., 1986; Ohya et al., 1988; Gutnick et al., 1989). This range of concentrations suggests differences in binding affinities, or it may reflect the difficulty in accurately altering local Ca^{2+} concentrations owing to endogenous Ca^{2+} buffers. The apparent high binding affinity found in some cells is paradoxical considering the high Ca^{2+} concentrations (hundreds of micromolar) predicted to exist near the channel.

Molecular studies have provided evidence that Ca^{2+} produces inactivation of L-type channels from cardiac ventricular myocytes by binding directly to the channel. An EF-hand motif from the COOH-terminus of the cardiac L-type α_{1C}-subunit may act as a Ca^{2+} binding site for Ca^{2+}-dependent inactivation (de Leon et al., 1995). L-type channels containing α_{1C}-subunits undergo Ca^{2+}-dependent inactivation; homologous Ca^{2+} channels containing α_{1E}-subunits do not. When the EF-hand region of the α_{1C}-subunit was replaced with the corresponding region of the α_{1E}-subunit, the chimeric α_{1C}-subunit no longer showed Ca^{2+}-dependent inactivation. Conversely, substitution of the EF-hand region of α_{1C}- into the α_{1E}-subunit conferred Ca^{2+}-dependent inactivation (de Leon et al., 1995). These results demonstrate that the Ca^{2+} binding EF-hand of the α_{1C}-subunit is essential for Ca^{2+}-dependent inactivation in cardiac L-type channels.

Other mechanisms for Ca^{2+}-dependent inactivation have been proposed. Early studies in *Helix* neurons suggested that activation of a Ca^{2+}-dependent phosphatase, such as calcineurin, produced inactivation through dephosphorylation of the Ca^{2+} channel (Chad and Eckert, 1986). Subsequent studies of cardiac L-type channels provided further evidence that phosphorylation regulates Ca^{2+} channel function (Trautwein and Hescheler, 1990). However, recent studies do not support dephosphorylation as a mechanism for inactivation; blocking phosphatase activity in *Aplysia* neurons and mammalian cardiac myocytes failed to reduce Ca^{2+} channel inactivation (Fryer and Zucker, 1993; Imredy and Yue, 1994). In addition, the rapid inactivation

reported for some cells appears inconsistent with an enzymatic mechanism, unless the enzyme is directly associated with the channel.

An alternative mechanism, which has not been widely tested, involves an interaction of Ca^{2+} with the cytoskeleton. The application of cytoskeletal stabilizers reduces Ca^{2+}-dependent inactivation in *Lymnaea* neurons (Johnson and Byerly, 1993b). This suggests that Ca^{2+}-dependent disruption of the cytoskeleton could play a role in inactivation. The authors proposed that the gating properties of the Ca^{2+} channel are influenced by its interaction with the cytoskeleton, particularly the microtubules. An attractive feature of this mechanism is that it might allow Ca^{2+} to influence the channel rapidly, even though it does not bind directly to the channel.

These mechanisms might not be mutually exclusive. Rather, there may be multiple mechanisms for inactivation. This could account for the variability in the time-course of Ca^{2+}-dependent inactivation. Ca^{2+}-dependent inactivation often begins immediately at the onset of a depolarizing pulse, develops over a period of tens of milliseconds, and recovers within hundreds of milliseconds. However, slower forms of inactivation, induced by very high Ca^{2+} concentrations, develop over a period of seconds and recover over an interval spanning tens of seconds (Von Gersdorff and Matthews, 1996).

Repetitive depolarizing pulses can result in the buildup of substantial Ca^{2+} current inactivation and a persistent decrease in Ca^{2+} current. Repetitive depolarizing pulses applied to a crayfish phasic abdominal motoneuron, which normally has low levels of impulse activity, produces a dramatic reduction in the Ca^{2+} current amplitude (Fig. 1). The recovery from inactivation has an initial rapid phase followed by a slower phase: 1 h after 5 Hz stimulation for 10 min, the Ca^{2+} current remains reduced. The initial buildup of inactivation and the persistent reduction in Ca^{2+} current are dependent on the amount of Ca^{2+} influx during stimulation (Hong and Lnenicka, 1995). Other neurons show a similar buildup of Ca^{2+}-

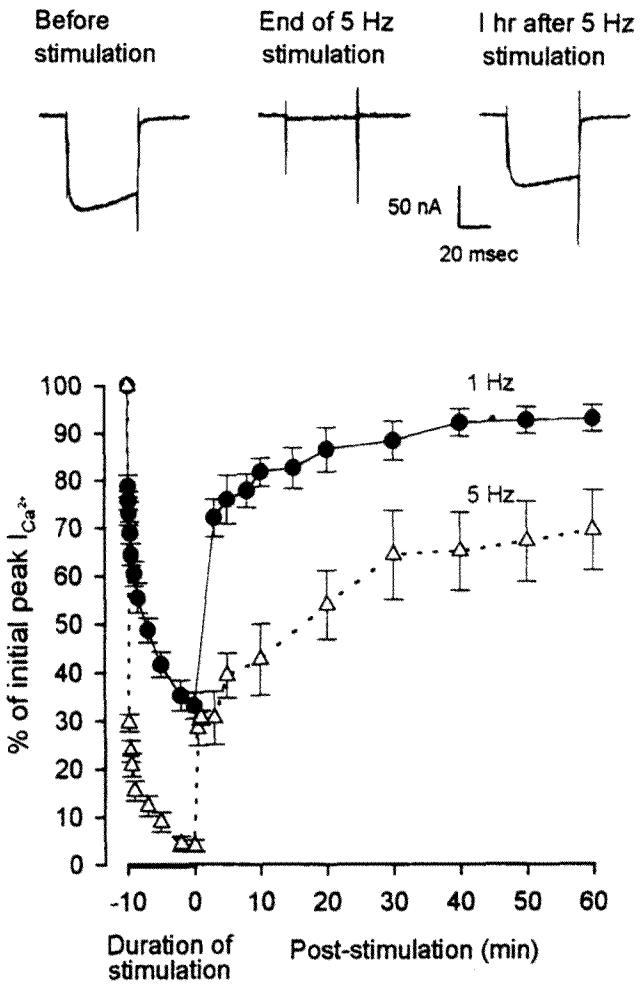

Fig. 1. Ca^{2+}-dependent inactivation of HVA Ca^{2+} current during repetitive stimulation. The cell body of a crayfish phasic motoneuron F3 was voltage-clamped, and repetitive 50-ms depolarizing pulses to 0 mV were applied. The depolarizing pulses were applied at 1 and 5 Hz, and the peak Ca^{2+} currents were measured during stimulation and at regular intervals for 1 h after stimulation. **(Top)** Representative Ca^{2+} currents measured before stimulation, at the end of 5 Hz stimulation, and 1 h after stimulation. **(Bottom)** A rapid, short-term reduction in Ca^{2+} current occurs during stimulation at 1 and 5 Hz. The Ca^{2+} current amplitude was normalized to that 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. The magnitude of the short-term and persistent decrease in Ca^{2+} current was dependent on the stimulation frequency and the amount of the Ca^{2+} influx. (From Hong and Lnenicka, 1995).

dependent Ca^{2+} channel inactivation (Jia and Nelson, 1986; Tazaki and Cooke, 1990; Von Gersdorff and Matthews, 1996). Presumably, the buildup of inactivation results from increasing levels of cytoplasmic Ca^{2+}. The persistent reduction in Ca^{2+} current could result directly from a maintained elevation of intracellular Ca^{2+}. For example, intracellular Ca^{2+} levels in dorsal root ganglion neurons remain elevated for minutes after a large Ca^{2+} load (Thayer and Miller, 1990; Werth and Thayer, 1994).

Alternatively, this persistent reduction in Ca^{2+} current could have features in common with the "rundown" of Ca^{2+} current observed in dialyzed cells (Byerly and Hagiwara, 1988). Chad and Eckert (1986) found that rundown in *Helix* neurons consisted of a reversible and an irreversible component. They proposed that the reversible component resulted from dephosphorylation of the Ca^{2+} channel by a Ca^{2+}-activated phosphatase and that the irreversible phase was produced by Ca^{2+}-dependent proteolysis, since it was blocked by the Ca^{2+}-dependent protease inhibitor, leupeptin (Chad and Eckert, 1986). 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 could act directly on the Ca^{2+} channel or act indirectly by irreversibly activating phosphatases (Manalan and Klee, 1983; Tallant et al., 1988).

Neuron-specific differences in Ca^{2+} buffering capacity are likely to affect Ca^{2+}-dependent inactivation, since exogenous Ca^{2+} buffers influence the rate of inactivation. Ca^{2+} buffering in neurons involves several processes (Miller, 1991). The cytosolic Ca^{2+} binding proteins have a limited capacity, but can rapidly reduce Ca^{2+} to resting levels and could influence inactivation during a single pulse or paired pulses. The buildup of inactivation seen during repetitive stimulation is likely to be influenced by the low-affinity/high-capacity buffering systems, which include mitochondria. Although the role of mitochondria in buffering Ca^{2+} in the physiological range has been questioned, recent studies of DRG cell bodies have shown that mitochondria are involved in buffering Ca^{2+} after trains of action potentials, and can reduce the intracellular Ca^{2+} concentration down to 0.4–0.5 μM (Thayer and Miller, 1990; Werth and Thayer, 1994).

Previous activity levels could influence Ca^{2+} channel inactivation if Ca^{2+} buffering systems adapt to changes in electrical activity. In the rat cerebral cortex, the Ca^{2+} binding protein, parvalbumin, is concentrated in GABAergic neurons that fire at high frequencies, suggesting that its levels might be determined by impulse activity (Celio, 1986). However, experiments that have examined the effect of activity (kindling) on soluble Ca^{2+} buffering proteins have provided conflicting results. As a result of kindling, dentate gyrus cells became depleted of calbindin (Miller and Baimbridge, 1983; Baimbridge et al., 1985) and GABAergic interneurons in the hippocampus increased their levels of parvalbumin (Kamphuis et al., 1989). The mitochondrial content of crustacean axons and terminals is greater in tonically active motoneurons than in phasically active motoneurons (Atwood and Johnston, 1968; Hill and Govind, 1981; Lnenicka et al., 1986). These differences are activity-dependent: the density and oxidative activity of mitochondria can be increased by increases in impulse activity (Lnenicka et al., 1986; Case and Lnenicka, 1992; Nguyen and Atwood, 1994). This suggests that buffering by mitochondria is greater in more active neurons and that increased activity can increase mitochondrial buffering capacity. The dramatic buildup of inactivation observed during repetitive stimulation of a crayfish phasic motoneuron is consistent with a low Ca^{2+}-buffering capacity (Fig. 1).

Voltage-Dependent Ca^{2+} Channel Inactivation

In vertebrate neurons, voltage-dependent inactivation has been observed for all types of HVA channels as well as LVA T-type channels. Inactivation of T-type Ca^{2+} currents appears to be strictly voltage-dependent in a number of vertebrate neurons (Fox et al., 1987a; Carbone and Lux, 1987; Hernández-Cruz and Pape, 1989;

Akaike et al., 1989; Mynlieff and Beam, 1992; Hay et al., 1996). Voltage-dependent inactivation has been demonstrated for N- and L-type currents (Fox et al., 1987a; Mynlieff and Beam, 1992; Cox and Dunlap, 1992; McCarthy and TanPiengco, 1992; Mendelowitz and Kunze, 1992; Bargas et al., 1994; Randall and Tsien, 1995), P-type currents (Bargas et al., 1994; Randall and Tsien, 1995) and Q-type currents (Randall and Tsien, 1995) in vertebrate neurons. In some neurons, both voltage-dependent inactivation and Ca^{2+}-dependent inactivation have been demonstrated for a single channel type (Bossu and Feltz, 1986; Hernández-Cruz and Pape, 1989; Cox and Dunlap, 1994). Voltage-dependent inactivation of HVA Ca^{2+} currents has been observed in several invertebrate neurons (Johansen et al., 1987; Hayashi and Levine, 1992; Prysiezniak and Spencer, 1992).

Molecular studies have identified a region of the α_1-subunit where amino acid substitutions influence the kinetics of voltage-dependent inactivation (Zhang et al., 1994); however, voltage-dependent inactivation is also dependent on the auxiliary Ca^{2+} channel subunits (Tanabe et al., 1987; Singer et al., 1991). Voltage-dependent inactivation appears to operate by affecting the voltage-sensing region of the channel, whereas Ca^{2+} inactivates the channel through a separate mechanism. Voltage-dependent inactivation of the cardiac L-type channel produced a reduction in the gating current, but Ca^{2+}-dependent inactivation did not (Hadley and Lederer, 1991).

Voltage-Dependent Ca^{2+} Current Facilitation

There are fewer examples of Ca^{2+} current facilitation compared to Ca^{2+} current inactivation. Voltage-dependent facilitation was first clearly demonstrated in bovine adrenal chromaffin cells (Fenwick et al., 1982) and has been most thoroughly examined in this system. A strong depolarizing pulse produced a maximum 25–35% facilitation of Ca^{2+} current that decayed over a few hundred milliseconds (Hoshi et al., 1984). This Ca^{2+} current facilitation is independent of the species of permeant ion: Facilitation occurs with either Ba^{2+} or Sr^{2+} used as a charge carrier, and is dependent on the voltage of the prepulse (Hoshi et al., 1984). In chromaffin cells, depolarizing prepulses increased the frequency of long Ca^{2+} channel openings (Hoshi and Smith, 1987), apparently owing to the recruitment of a L-type channel (Artalejo et al., 1991). It has been proposed that this recruitment involves voltage-dependent phosphorylation (Artalejo et al., 1992).

Voltage-dependent facilitation of Ca^{2+} currents has also been demonstrated in rat DRG neurons (Scott and Dolphin, 1990), sympathetic neurons (Ikeda, 1991), and hippocampal neurons (Kavalali and Plummer, 1996). In neurons, there is evidence for facilitation of both L-type (Bourinet et al., 1994; Kavalali and Plummer, 1996) and N-type currents (Ikeda, 1991). The facilitation of the L-type current results from an increase in both the frequency and duration of channel openings (Bourinet et al., 1994; Kavalali and Plummer, 1996). There may be multiple mechanisms for producing voltage-dependent facilitation. In some neurons, facilitation appears to result from a voltage-dependent relief of G-protein-mediated inhibition (Grassi and Lux, 1989; Scott and Dolphin, 1990; Ikeda, 1991); however, in other cases, G-proteins are not involved in this facilitation (Bourinet et al., 1994).

Long-Term Changes in Ca^{2+} Currents

Increased electrical activity can produce a reduction in voltage-dependent Ca^{2+} current that is longer lasting than that produced by Ca^{2+} channel inactivation. Cell-culture and in vivo studies demonstrate that depolarization or increased impulse activity can produce a reduction in Ca^{2+} current density lasting from hours to days. Like Ca^{2+}-dependent inactivation, this reduction in Ca^{2+} current is triggered by an increase in intracellular Ca^{2+}. Although originally studied by high K^+ depolarization of cultured cells, it has recently been produced by increased impulse activity in vivo.

Reduction in Ca^{2+} Current Produced by Chronic Depolarization of Cultured Neurons

Chronic depolarization of PC12 cells with high K^+ solutions produces a reduction in the density of L-type channels, measured by nitrendipine and isradipine binding (DeLorme and McGee, 1986; DeLorme et al., 1988; Feron and Godfraind, 1995). This reduction has a slow onset: There is no decrease in Ca^{2+} channel number after 4 h of constant depolarization, a 30% reduction after 1 d of depolarization, and a maximum 50% reduction after 3 d of constant depolarization (DeLorme et al., 1988). This reduction in Ca^{2+} channel number results in a decrease in the depolarization-induced Ca^{2+} influx. After 4 d of chronic depolarization, there was a 40% reduction in the depolarization-induced $^{45}Ca^{2+}$ uptake (DeLorme et al., 1988). The depolarization-induced reduction in Ca^{2+} channel density appears to be triggered by increased intracellular Ca^{2+} since it is mimicked by the application of the Ca^{2+} ionophore, ionomycin (DeLorme et al., 1988).

A similar, but more rapid decrease in L-type channel number was observed during high K^+ depolarization of cultured chick retinal neurons and rat cerebellar granule neurons. In chick retinal cells, the reduction in channel number and $^{45}Ca^{2+}$ uptake was observed as early as 4 h after the beginning of depolarization (Ferrante et al., 1991). This reduction in channel number was also Ca^{2+}-dependent: It was blocked by the Ca^{2+} channel antagonist, D600 and mimicked by addition of the Ca^{2+} ionophore, A23187. Chronic depolarization of rat cerebellar granule cells produced an even more rapid reduction in Ca^{2+} channel number: There was approximately a 90% reduction in L-type channels after only 2 h of depolarization (Liu et al., 1994).

The direct measurement of Ca^{2+} currents in cultured rat myenteric neurons and *Helisoma* neurons has provided further evidence for the downregulation of Ca^{2+} channels by depolarization (Franklin et al., 1992). The amplitude of the L-type current in rat myenteric neurons reached its maximum reduction after 24 h of high K^+ depolarization and showed complete recovery after 24 h of repolarization. This effect is also dependent on Ca^{2+} influx, since the addition of nitrendipine during depolarization blocked the reduction in the L-type current density. A depolarization-induced reduction in Ca^{2+} current has also been observed in an invertebrate neuron in cell culture. The *Helisoma* neuron, B5 shows a large reduction in both its HVA and LVA Ca^{2+} currents after 4 d of high K^+ depolarization (Berdan et al., 1993).

In all of the above cases, the reduction in L-type channel number and/or Ca^{2+} current density is apparently triggered by a rise in intracellular Ca^{2+}. During chronic depolarization of PC12 cells, the initial increase in intracellular Ca^{2+} is followed by restoration of normal resting Ca^{2+} levels after 15 h. This presumably results from the decrease in Ca^{2+} channel number. However, in spite of the decrease in intracellular Ca^{2+}, the Ca^{2+} channel density remains downregulated during maintained depolarization (DeLorme et al., 1988). Thus, it appears that the decrease in Ca^{2+} channel number can outlast the original Ca^{2+} signal. On repolarization, Ca^{2+} channel number increases back to normal levels. It may be that repolarization results in a further decrease in Ca^{2+} levels, which triggers the increase in Ca^{2+} channels.

Internalization of L-type channels is responsible for the initial reduction in channel number in rat pituitary cells (Liu et al., 1994). Internalization occurs largely within the first hour of depolarization and involves Ca^{2+}-dependent activation of calmodulin. Recovery on repolarization is almost complete within 2 h and is not sensitive to protein synthesis inhibitors, suggesting that it results from recruitment of internalized channels. Regulation of Ca^{2+} channel number via internalization and recruitment may be particularly relevant to the studies of chick retinal cells and cerebellar granule cells, which show a rapid reduction and recovery of Ca^{2+} channel number. The recruitment of internalized Ca^{2+} channels has been demonstrated in other cultured neurons.

For example, human neuroblastoma cells have been shown to contain an intracellular pool of N-type channels that can be rapidly recruited to the plasma membrane after the addition of Cd^{2+} or ω-CgTX GVIA (Passafaro et al., 1994).

This regulation of Ca^{2+} channel number involves other mechanisms in addition to internalization and recruitment of channels. In rat myenteric neurons, the initial reduction in Ca^{2+} current density is blocked by inhibitors of translation and transcription, suggesting that the initial modification or degradation of channels requires the production of new proteins (Franklin et al., 1992). The long-term maintenance of Ca^{2+} channel reduction is likely to involve a decrease in channel synthesis. The reduced Ca^{2+} channel number in PC12 cells is accompanied by a corresponding decrease in the level of the α_1-subunit mRNA for the L-type channel (Franklin et al., 1992). The recovery of Ca^{2+} current density in myenteric neurons appears to require channel synthesis, rather than recruitment, since it is blocked by inhibitors of transcription or translation (Franklin et al., 1992).

The chronic application of Ca^{2+} channel agonists and antagonists produce changes in Ca^{2+} channels that are consistent with those produced by depolarization. Drugs that directly activate the L-type channel, such as S Bay K 8644, downregulate Ca^{2+} channels, and inhibitors, such as nifedipine, upregulate Ca^{2+} channels in cultured PC12 cells and myenteric neurons (Skattebol et al., 1989). The effect of nifedipine is likely to result from changes in intracellular Ca^{2+}, since application of nifedipine to rat myenteric neurons results in a reduction in intracellular Ca^{2+} levels (Franklin et al., 1992). More direct effects of antagonists are also possible, since application of ω-conotoxin or Cd^{2+} to neuroblastoma cells produces an increase in the number of Ca^{2+} channels that does not appear to be due to a decrease in intracellular Ca^{2+} (Passafaro et al., 1994).

In general, electrical activity appears to play an important role in the regulation of ion channel number. In cultured rat muscle cells, blocking spontaneous impulse activity results in an increase in the number of voltage-dependent Na^+ channels (Sherman and Catterall, 1984). In cultured chick muscle fibers, increased activation of voltage-dependent Na^+ channels produces a decrease in the number of Na+ channels (Bar-Sagi and Prives, 1985). These effects appear to be Ca^{2+}-dependent: increased Ca^{2+} influx produces a reduction in the level of Na^+ channel α-subunit mRNA and the number of Na^+ channels (Sherman et al., 1983; Offord and Catterall, 1989).

Reduction in Ca^{2+} Current Produced by Increased Impulse Activity In Situ

Long-term changes in Ca^{2+} current density that are similar to those observed in culture can be produced by increasing the impulse activity of an identified neuron in vivo. These in vivo studies demonstrate that the changes in Ca^{2+} channels can be produced under physiological conditions in an intact, adult nervous system. The long-term effects of increased impulse activity on Ca^{2+} currents were studied in the crayfish abdominal phasic motoneuron, F-3. The F-3 motor axon was stimulated at 5 Hz for 45 min to 1 h either in vivo or in the isolated abdomen. Action potentials were generated in the axon for the duration of the stimulation; however, the cell body failed to fire action potentials after the first few seconds (Hong and Lnenicka, 1995). Ca^{2+} current was measured from the cell body 6–7 h after in vitro stimulation and 3 d after in vivo stimulation. A 30% reduction in Ca^{2+} current density was seen 6–7 h after stimulation. This long-term reduction in Ca^{2+} current persists for many days, since the full effect was still seen 3 d after stimulation (Fig. 2). The reduction in Ca^{2+} current density is Ca^{2+}-dependent: It is prevented by blocking Ca^{2+} influx during stimulation (Hong and Lnenicka, 1995).

The increase in impulse activity selectively modulates a single type of Ca^{2+} channel. At least two types of HVA channels contribute to the Ca^{2+} current recorded from the cell body of F-3 (Hong and Lnenicka, 1997): an ω-AgTX

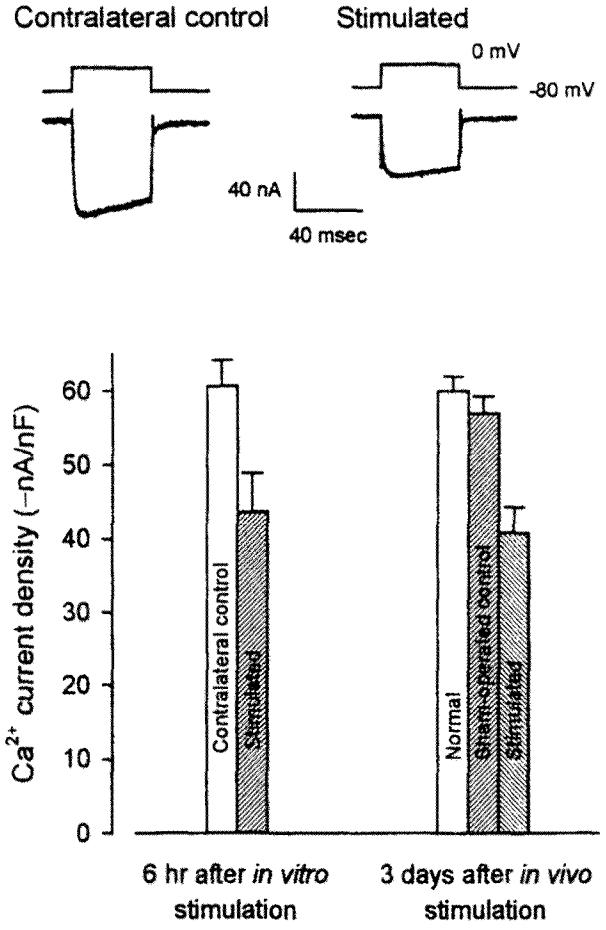

Fig. 2. Long-term, activity-dependent reduction in HVA Ca^{2+} current produced by increased impulse activity. The axon of the crayfish phasic motoneuron F3 was electrically stimulated in vitro or in vivo at 5 Hz for approx 1 h. **(Top)** Three days after in vivo stimulation, the Ca^{2+} currents were measured in the stimulated and the unstimulated contralateral control cells. **(Bottom)** 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 experiment where measurements were performed 6–7 h after in vitro 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 h after in vitro stimulation. Thus, the reduction in Ca^{2+} current density produced by increased impulse activity lasts for 3 d with no decline. (From Hong and Lnenicka, 1995).

IVA-sensitive, P-type channel, as well as unknown type(s) that are not sensitive to N-, L-, or P-type channel blockers (non-P-type). Stimulation produces a 43% reduction in the amplitude of the P-type current, but no change in the non-P-type current amplitude (Fig. 3).

This long-term reduction in Ca^{2+} current is distinct from the Ca^{2+}-dependent Ca^{2+} channel inactivation seen in this motoneuron. A number of features in addition to its longer duration distinguish it from Ca^{2+} channel inactivation. During Ca^{2+} channel inactivation, only channels close to the site of Ca^{2+} influx are affected, but the long-term reduction in Ca^{2+} current apparently involves channels distant to the site of Ca^{2+} influx: The cell body is no longer excitable after the first few action potentials, and based on the characteristics of the I-V curve for the Ca^{2+} current, electrotonic depolarization of the cell body is unlikely to produce significant Ca^{2+} influx. In addition, all of the HVA channels in the cell body undergo Ca^{2+}-dependent inactivation, since the Ca^{2+} current is almost totally eliminated by repetitive depolarizing pulses (Fig. 1); however, only the P-type channels are involved in the long-term change. Finally, protein synthesis inhibition attenuates the long-term change in Ca^{2+} current *(see below)*, but has no effect on Ca^{2+} channel inactivation (Hong and Lnenicka, 1995). The reduction in Ca^{2+} current observed for the first hour after repetitive depolarizing pulses (Fig. 1) is also not influenced by inhibition of protein synthesis, suggesting that it operates by a different mechanism than this long-term change.

The long-term reduction in Ca^{2+} current density could result from a change in the properties of individual channels (decreased unitary conductance or probability of opening or a decrease in the number of functional channels. The voltage dependence and kinetics of the Ca^{2+} channels are not hanged by stimulation, suggesting that the reduction in current does not result from a change in channel properties (Hong and Lnenicka, 1997). It seems probable that the reduced current results from fewer functional channels, possibly because of a

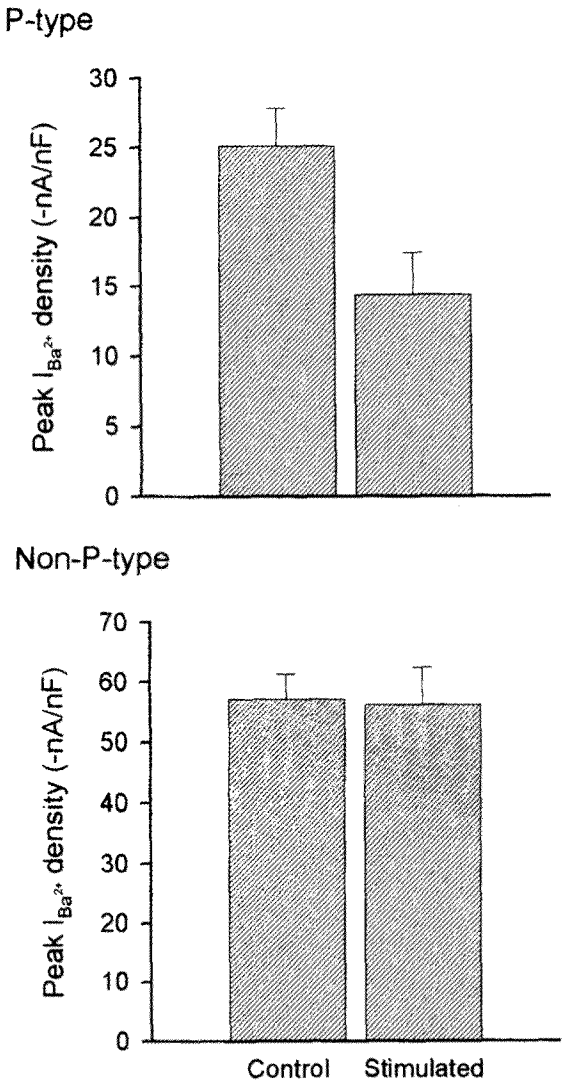

Fig. 3. The P-type currents selectively undergo a long-term, activity-dependent reduction. The axon of crayfish motoneuron F3 was stimulated at 5 Hz for 45–60 min, and Ba^{2+} currents at the cell body were measured 6–7 h after stimulation and compared to Ba^{2+} current from control neurons (n = 4–5). The P-type currents were determined by subtracting the Ba^{2+} current in the presence of 600 n*M* ω-AgTX IVA from the total Ba^{2+} current before adding the ω-AgTX IVA. The increase in impulse activity of the cell produced a significant 43% (n = 8; t-test, $p < 0.05$) reduction in the peak amplitude of the P-type current density (control cells, –25.1 ± 2.7 nA/nF; stimulated cells, –14.4 ± 2.9 nA/nF), but there was no reduction in the density of the non-P-type current (control cells, –57.1 ± 4.1 nA/nF; stimulated cells, –56.1 ± 6.3 nA/nF). (Adapted from Hong and Lnenicka, 1997).

decrease in channel number as seen in the previous cell-culture studies. As in the cell-culture studies, the long-term reduction in Ca^{2+} current does not directly result from a decrease in channel synthesis since the application of protein synthesis inhibitors for 6–7 h does not reduce the Ca^{2+} current (Hong and Lnenicka, 1995). The initial decrease in the number of functional Ca^{2+} channels could result from the Ca^{2+}-dependent modification, internalization, and/or degradation of the channel by a mechanism capable of specifically targeting the P-type channel. Similar to studies of cultured myenteric neurons (Franklin et al., 1995), the application of protein synthesis inhibitors before stimulation actually attenuates this long-term reduction in Ca^{2+} current (Hong and Lnenicka, 1995). These results suggest that the mechanism involves the Ca^{2+}-dependent production of proteins that are involved in downregulating the Ca^{2+} channels, or that the Ca^{2+}-activated pathways include short-lived proteins that are rapidly depleted.

Activity-Dependent Changes in Transmitter Release

The inability to measure Ca^{2+} current directly at most synaptic terminals has made it difficult to examine the relationship between Ca^{2+} influx and changes in transmitter release. There is evidence that increased secretion of catecholamines from chromaffin cells during periods of increased activity involves voltage-dependent facilitation of Ca^{2+} currents (Artalejo et al., 1994), but there is currently no evidence that a similar mechanism is involved in the facilitation of transmitter release. The only evidence that changes in transmitter release are produced by these activity-dependent changes in Ca^{2+} currents involves forms of synaptic depression.

Short-Term Homosynaptic Depression

Ca^{2+} channel inactivation has been considered as a possible mechanism for short-term homosynaptic depression. Short-term homo-

synaptic depression, a decrease in synaptic effectiveness confined to the stimulated pathway, was first described at the neuromuscular junction (Lloyd, 1949). Although it has been most extensively studied at the neuromuscular junction, it also has been demonstrated at a number of central synapses, where in some cases it appears to underlie behavioral habituation (Castellucci et al., 1970; Zucker, 1972). In most cases, it appears to result from a decrease in transmitter release (Del Castillo and Katz, 1954; Zucker, 1972; Castellucci and Kandel, 1974).

The dominant model for short-term synaptic depression is the depletion hypothesis, which proposes that decreased transmitter release during repetitive activation results from a depletion of the releasable store of transmitter (for review, *see* Zucker, 1989). This model is best supported by studies of the vertebrate neuromuscular junction, where the kinetics of depression and subsequent recovery are generally consistent with the depletion and refilling of a releasable store of transmitter (Liley and North, 1953). However, the kinetics of synaptic depression at some neuromuscular and central synapses in crayfish and *Aplysia* cannot be explained by the depletion model (Zucker and Bruner, 1977; Byrne, 1982). Other models include a decrease in Ca^{2+} influx either resulting from changes in the presynaptic action potential or a reduction in voltage-dependent Ca^{2+} conductance.

The proposed involvement of Ca^{2+} channel inactivation in synaptic depression is largely based on studies where Ca^{2+} currents were examined in the cell bodies of neurons whose terminals show synaptic depression. A relationship between Ca^{2+} channel inactivation and synaptic depression was demonstrated in sensory neurons of *Aplysia*. Repetitive stimulation of the sensory neurons at interpulse intervals ranging from milliseconds to tens of minutes results in a decrease in the amount of transmitter released at the sensory-motoneuron synapses (Castellucci et al., 1970; Castellucci and Kandel, 1974). The reduction in transmitter was correlated with Ca^{2+} current inactivation in the cell body, and therefore, it was proposed to result from the inactivation of Ca^{2+} channels at the synaptic terminals (Klein et al., 1980). The Ca^{2+}-channel inactivation has not been thoroughly studied, although it appears to be predominantly voltage-dependent, since inactivation still occurs with Ba^{2+} as the charge carrier (Klein et al., 1980).

Subsequent modeling studies indicated that depletion of transmitter was involved in the reduction of transmitter release from the sensory neuron terminals (Gingrich and Byrne, 1985). This is supported by ultrastructural studies of the depressed sensory neuron-motoneuron synapses, which provide evidence for vesicle depletion (Bailey and Chen, 1988a). Recent cell-culture studies have examined the availability of releasable transmitter by monitoring the frequency of spontaneous miniature potentials at the sensory neuron-motoneuron synapses (Eliot et al., 1994). These studies indicate that depletion is important immediately following stimulation, but another process, such as Ca^{2+} channel inactivation, may predominate at later periods.

Studies of cultured neurons from mouse spinal cord and dorsal root ganglion have also demonstrated a correlation between Ca^{2+} channel inactivation and synaptic depression. In this study, neurons with more prominent inactivation of Ca^{2+} currents, recorded at the cell body showed greater synaptic depression (Jia and Nelson, 1986). The Ca^{2+} channel inactivation was both voltage- and Ca^{2+}-dependent. A similar correlation can be seen in crustacean motoneurons *in situ*. The crayfish phasic motoneuron F-3 has prominent Ca^{2+}-dependent Ca^{2+} channel inactivation (Fig. 1) and also shows dramatic neuromuscular synaptic depression.

It seems likely that Ca^{2+} channels at presynaptic terminals show some degree of inactivation during repetitive, high-frequency stimulation. Ca^{2+}-dependent inactivation seems probable given that the cytoplasmic Ca^{2+} concentrations in synaptic terminals can reach micromolar levels during repetitive stimulation (Delaney et al., 1989; Delaney and Tank, 1994), and some Ca^{2+}

channels studied at the cell body begin to inactivate at Ca^{2+} concentrations of 1 μM or less. Of course, this assumes that Ca^{2+} channels at the terminal show Ca^{2+}-dependent inactivation similar to that studied in cell bodies.

In a few cases, Ca^{2+}-dependent inactivation of Ca^{2+} channels has been examined at synaptic terminals. The P-type channel, studied in rat cerebral cortical synaptosomes, shows a rapid Ca^{2+}-dependent inactivation within milliseconds of the beginning of a depolarizing pulse (Tareilus et al., 1994b). The P-type current at squid synapse and the N-type at the chick ciliary ganglion both show Ca^{2+} dependent inactivation, but it appears to develop slowly (Augustine and Eckert, 1984; Stanley and Goping, 1991; Yawo and Momiyama, 1993). The inactivation of L-type channels at goldfish retinal bipolar terminals requires high Ca^{2+} levels (20–50 μM and has a very slow onset, requiring seconds (Von Gersdorff and Matthews, 1996). In this case, the inactivation properties are well matched to the normal pattern of activity, since these bipolar terminals must release transmitter during prolonged depolarizations.

In the above studies, it is not clear whether Ca^{2+} channel inactivation produces synaptic depression. Prominent Ca^{2+} channel inactivation might be correlated with a reduced capacity to "mobilize" synaptic vesicles for release. Both characteristics could develop in neurons with normally low impulse activity levels. This is consistent with findings in crustacean motoneurons where "inactive" phasic motoneurons show prominent Ca^{2+}-dependent inactivation during repetitive stimulation (Fig. 1) and have motor terminals with few synaptic vesicles (Atwood and Johnston, 1968; Hill and Govind, 1981; Lnenicka et al., 1986). One or both effects could depress transmitter release during periods of unusually high impulse activity. Nonetheless, it is clear that synaptic depression can occur in the absence of Ca^{2+} channel inactivation: During synaptic depression at the squid giant synapse, the amplitude of the Ca^{2+} current remains constant (Charlton et al., 1982). Depletion of transmitter may be particularly prominent at this synapse because of its large release of transmitter, approx 5000 quanta/impulse (Llinás et al., 1992).

Long-Term Homosynaptic Depression

There appear to be multiple mechanisms for producing long-term synaptic depression, LTD (for review, *see* Linden and Connor, 1995). For example, forms of homosynaptic LTD induced by coincident pre- and postsynaptic activity (inverse Hebbian), as well as uncorrelated pre- and postsynaptic activity (converse Hebbian) have been identified in the mammalian CNS (Stanton and Sejnowski, 1989; Artola et al., 1990; Christie and Abraham, 1992; Dudek and Bear, 1992). A form of heterosynaptic LTD found in the mammalian brain is also converse-Hebbian: LTD can be induced in an inactive input by postsynaptic depolarization resulting from activation of converging inputs (Lynch et al., 1977; Pockett et al., 1990). Postsynaptic Ca^{2+} levels appear to play a central role in the induction of these forms of LTD (Artola and Singer, 1993). The expression of LTD also appears to involve multiple mechanisms. In the mammalian brain, LTD can result from a decrease in transmitter release (Bolshakov and Siegelbaum, 1994) or a decrease in the sensitivity of the postsynaptic membrane (Ito et al., 1982). In the CNS of *Aplysia*, LTD lasting for days to weeks (Castellucci et al., 1978) appears to result from a reduction in transmitter release owing to a decrease in the number of synaptic varicosities, active zones, and synaptic vesicles in the presynaptic neuron (Bailey and Chen, 1988b). As discussed below, recent findings demonstrate that homosynaptic LTD can be produced at the crayfish neuromuscular junction by a rise in presynaptic Ca^{2+} (Hong and Lnenicka, 1993). This LTD appears to result from a Ca^{2+}-dependent reduction in Ca^{2+} current.

Neuromuscular LTD can be produced by electrical stimulation of crustacean phasic motoneurons that normally have low-impulse activity levels. Initial experiments demonstrated that prolonged stimulation produced a very long-lasting synaptic depression: in vivo stimulation applied over 2 wk produces LTD that persists for more than 2 wk after the final

stimulation trial (Lnenicka and Atwood, 1985). Briefer periods of stimulation can also produce LTD: In vivo stimulation of a crayfish phasic motoneuron at 5 Hz for 2 h can produce a 30–40% reduction in EPSP amplitude, which persists for at least 3 d (Lnenicka and Atwood, 1989). It seems likely that LTD can be induced at phasic motor terminals by considerably shorter periods of stimulation (Bruner and Kennedy, 1970); however, the minimum stimulation requirements for producing LTD and the maximum duration of LTD have not been established. A direct measurement of the quantal content of release has shown that the reduction in EPSP amplitude results from a decrease in transmitter release from the motor terminals (Mercier and Atwood, 1990). This neuromuscular LTD has been observed at phasic motoneurons in both the lobster and crayfish (Lnenicka and Atwood, 1985; Mercier and Atwood, 1990; Bradacs et al., 1990; Hong and Lnenicka, 1993). A similar effect has been reported for the vertebrate neuromuscular junction. Stimulation of the nerve to the cutaneous pectoris muscle in the frog over a period of 5–8 d produced a 50% reduction in transmitter release from the motor terminals 1–3 d following the final stimulation trial (Hinz and Wernig, 1988).

Homosynaptic LTD at the crustacean neuromuscular junction is strictly presynaptic: it can be produced by increased electrical activity in the motoneuron without activation of the muscle fiber. LTD has been produced by evoking action potentials in the motor axon proximal to a TTX block in vivo (Lnenicka and Atwood, 1989) and by locally depolarizing the cell body or motor axon in vitro (Fig. 4). In both cases, no transmitter is released from the motor terminals. Therefore, LTD does not result from the depletion of transmitter or involve activation of the postsynaptic cell.

LTD is triggered by the prolonged influx of Ca^{2+} into the presynaptic cell. When Ca^{2+} influx is prevented by removing extracellular Ca^{2+} and adding Ca^{2+} channel blockers, presynaptic depolarization does not produce LTD (Fig. 4). It is not necessary for the Ca^{2+} influx to occur at the synaptic terminals to produce LTD

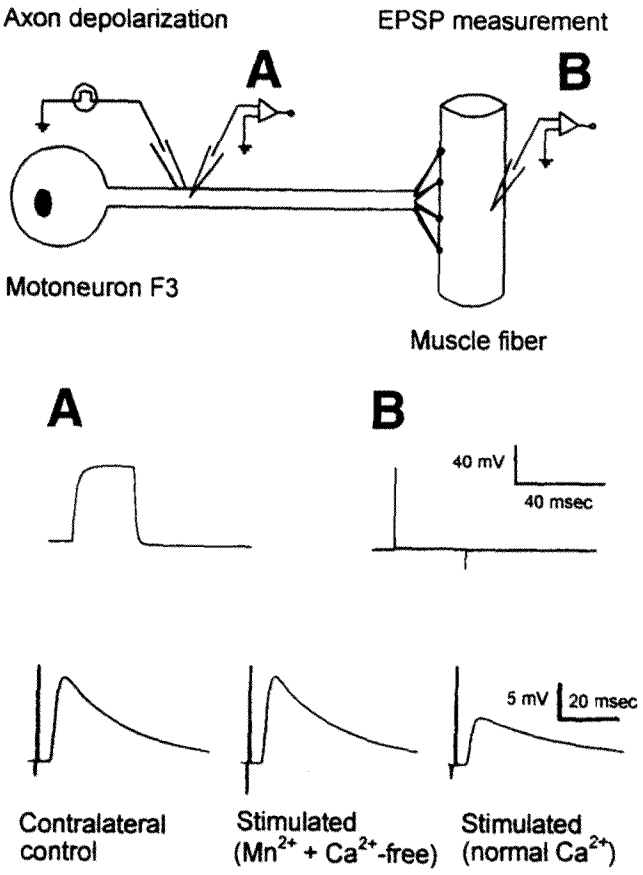

Fig. 4. Local axonal depolarization produces Ca^{2+}-dependent LTD in the crayfish motoneuron, F3. **(Top)** The proximal region of the axon was depolarized with 60–80 mV pulses (30-ms duration delivered at 5 Hz for 1 h. Depolarization was performed in normal saline or Ca^{2+}-free saline containing a Ca^{2+} channel blocker, Mn^{2+}. **(Middle)** The axon was depolarized with an extracellular electrode, and membrane potential was monitored with an intracellular electrode positioned nearby **(A)**. TTX (50 n*M*) was added during depolarization to prevent the generation of action potentials. The axon depolarization did not evoke transmitter release from the motor terminals **(B)**. **(Bottom)** Five hours after axon depolarization, the TTX was washed out, and EPSPs were recorded from muscle fibers during axon stimulation. Depolarization of the axon in Ca^{2+}-free saline containing 6 m*M* Mn^{2+} (Mn^{2+} + Ca^{2+}-free) did not produce LTD. In contrast, depolarizing the axon in normal saline produced LTD of the EPSP. Thus, presynaptic Ca^{2+} influx in the absence of transmitter release and muscle fiber depolarization is sufficient to produce LTD. (Adapted from Hong and Lnenicka, 1993).

(Hong and Lnenicka, 1993); however, under physiological conditions the large Ca^{2+} influx at the terminals is likely to play a major role in the production of LTD.

The LTD probably results from a Ca^{2+}-depen-dent reduction in Ca^{2+} current at the motor terminals. This is supported by the following findings:

1. Both the reduction in Ca^{2+} current and LTD in motoneuron F-3 persist for days and can be triggered by prolonged Ca^{2+} influx at remote Ca^{2+} channels;
2. The reduction in Ca^{2+} current involves the selective modulation of P-type channels and P-type channels are involved in transmitter release from crayfish motor terminals;
3. Both LTD and the reduction in Ca^{2+} current are attenuated by inhibitors of protein synthesis (Nguyen and Atwood, 1990; Hong and Lnenicka, 1995);
4. LTD can not be accounted for by other factors, such as ultrastructural changes in the motor terminals (Lnenicka et al., 1986); and
5. Seasonal changes in transmitter release from the motor terminals, assumed to be activity-dependent, are paralleled by seasonal changes in the magnitude of the Ca^{2+} current recorded from the cell body (Fig. 5). In the summer, the initial release of transmitter from phasic motor terminals is less than in the winter (Lnenicka and Zhao, 1991). The higher activity levels in the summer apparently produce LTD of transmitter release (Lnenicka, 1993). The Ca^{2+} current density recorded from the motoneuron's cell body shows corresponding seasonal changes: the Ca^{2+} current density is approx 20% greater in the winter than in the summer (Fig. 5).

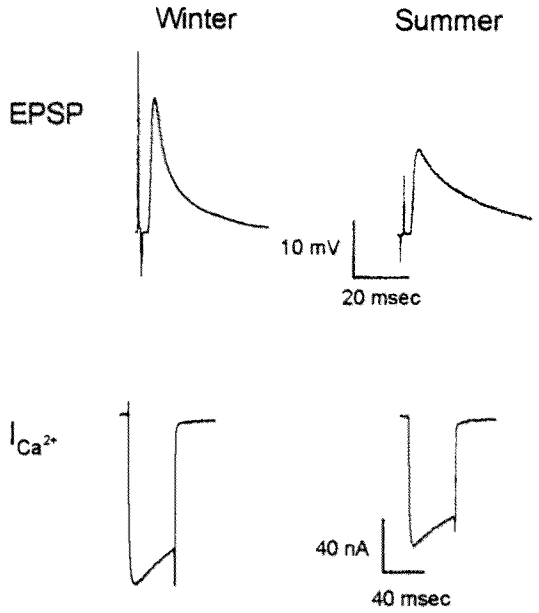

Fig. 5. Parallel seasonal changes in Ca^{2+} current amplitude and transmitter release in the crayfish motoneuron, F3. **(Top)** Representative EPSPs produced by stimulation of F-3 in winter and summer animals. The amplitudes of evoked EPSPs were measured from the lateral, abdominal fast flexor muscle fibers in high Mg^{2+} (10× normal saline to block muscle contraction. The average EPSP amplitude of winter animals (28.9 ± 2.3 mV, n = 41) examined during December 21–March 19 was 59% larger (t-test, $p < 0.05$) than that of summer animals (18.1 ± 1.6 mV, n = 57) examined during June 21–September 21. These seasonal changes in EPSP amplitude are owing to changes in transmitter release (Lnenicka and Zhao, 1991). **(Bottom)** Representative Ca^{2+} currents measured from the cell body of motoneuron F3 in winter and summer animals. The Ca^{2+} current density of winter animals was approx 20% greater than that of summer animals: winter, 58.1 ± 1.7 nA/nF (n = 82); summer, 49.0 ± 1.7 nA/nF (t-test, $p < 0.05$; n = 34).

It is interesting to compare long-term facilitation (LTF) and LTD at these crustacean neuromuscular synapses. LTF, an increase in transmitter release that can persist for at least 24 h, is produced by high-frequency stimulation of the motor terminals over a period of minutes (Atwood and Wojtowicz, 1986). The induction of the long-lasting phase of LTF is voltage-dependent: LTF can be produced by direct depolarization of the motor terminal in the absence of increases in intracellular Ca^{2+} or Na^{+} (Wojtowicz and Atwood, 1988). The expression of LTF appears to involve an increase in the number of active zones per synapse (Wojtowicz et al., 1994). Both LTF and LTD can be produced by stimulation of phasic motor terminals: LTF is seen immediately after stimulation of a phasic motoneuron and subsequently declines revealing the more persistent LTD (Lnenicka and Atwood, 1989).

Stimulation of tonic motoneurons appears to produce only LTF. Since increases in intracellular Ca^{2+} trigger LTD, repetitive stimulation may produce higher Ca^{2+} levels in phasic motoneurons than tonic motoneurons, resulting in greater expression of LTD at the phasic motor terminals.

The corollary of LTD, a disuse-induced enhancement of transmitter release, is also observed at the neuromuscular junction. A reduction in electrical activity of a crayfish phasic motoneuron produced by claw immobilization for 3–4 wk resulted in an increase in EPSP amplitude (Pahapill et al., 1985). Although it has not been demonstrated that a reduction in motoneuron electrical activity produces an increase in Ca^{2+} current, this result seems likely based on previous studies. A disuse-induced enhancement of transmitter release can also be produced at rat motor terminals by chronically decreasing motor activity through limb immobilization or the application of TTX to the motor nerve (Robbins and Fischbach, 1971; Snider and Harris, 1979). Although the mechanisms of this change are not known, it does not appear to be produced by motor terminal sprouting, which often accompanies neuromuscular inactivity (Tsujimoto and Kuno, 1988). A disuse-induced enhancement, possibly related to that observed at the neuromuscular junction, has been reported for the mammalian spinal cord (Gallego et al., 1979; Manabe et al., 1990; Webb and Cope, 1992) and stellate ganglion (Gallego and Geijo, 1987).

Development of Ca^{2+} Currents and Transmitter Release

Activity-Dependent Differentiation of Ca^{2+} Channel Density and Transmitter Release

Differences in impulse activity appear to be involved in the differentiation of transmitter-releasing properties at the neuromuscular junction (Lnenicka, 1991). In crustaceans, tonic stimulation of phasically active motoneurons over a period of days produces physiological and morphological changes that transform phasic motor terminals into terminals that are more similar to tonic motor terminals (Lnenicka and Atwood, 1985; Lnenicka, 1991). These changes include the previously described LTD, as well as an increased resistance to synaptic fatigue during prolonged high-frequency stimulation. The increased fatigue resistance occurs independently of LTD and is produced by a separate mechanism that involves changes in motor terminal morphology (Lnenicka, 1991). This constellation of activity-dependent changes has been previously termed long-term adaptation (LTA) (Lnenicka and Atwood, 1985). LTA presumably acts to reset the frequency response of the synapse, making it less effective during low-frequency activation, but more effective during high-frequency activation.

Does the differentiation of the transmitter releasing properties at phasic and tonic neuromuscular synapses involve activity-dependent regulation of Ca^{2+} channels? Differences in Ca^{2+} channel number could be responsible for the greater transmitter release from phasic motor terminals. Freeze-fracture studies in the moth and lizard demonstrate that phasic motor terminals have a greater number and/or density of intramembranous particles at their active zones than do tonic motor terminals (Rheuben, 1985; Walrond and Reese, 1985). As previously discussed, this likely represents a difference in Ca^{2+} channel distribution that could result in greater release of transmitter from phasic terminals than tonic terminals. This is supported by observations that the differences in transmitter release from crustacean phasic and tonic motor terminals can not be fully attributed to differences in the size or number of active zones (Lnenicka et al., 1986; King et al., 1996). These differences in transmitter release could arise from a Ca^{2+}-dependent reduction in Ca^{2+} channels at tonic motor terminals owing to their high-impulse activity levels.

If impulse activity plays a role in determining the overall Ca^{2+} current density in neurons,

then neurons with low activity levels would be expected to have a higher Ca^{2+} current density than those with high activity. It is difficult to find examples where Ca^{2+} current density has been measured in neurons of similar type, but with known differences in impulse activity. However, it does appear that the Ca^{2+} current density in the inactive phasic motoneurons in the crayfish (Czternasty et al., 1989; Hong and Lnenicka, 1995) are relatively high compared to other crustacean neurons (Meyers et al., 1992; Chrachri, 1995).

Impulse Activity and Ca^{2+} Currents at Growth Cones

The development of activity-dependent differences in Ca^{2+} channels could influence growth cone motility. Like the presynaptic terminal, the function of the growth cone is dependent on intracellular Ca^{2+} levels. Cell-culture studies have demonstrated that depolarization produced by impulse activity, neurotransmitters, or high K^+ can enhance or inhibit neurite outgrowth (Mattson et al., 1988; McCobb et al., 1988; Cohan, 1990; Fields et al., 1990; Neely, 1993). In many cases, the effects of depolarization on axon growth have been shown to result from the entry of Ca^{2+} through voltage-dependent Ca^{2+} channels. There appears to be a range of Ca^{2+} concentrations that is permissive for neurite outgrowth: Ca^{2+} concentrations outside of this permissive range result in inhibition of growth (Kater et al., 1988; Kater and Mills, 1991).

Activity-dependent regulation of Ca^{2+} channels during growth could influence axon growth, as well as the transmitter-releasing properties of the developing synapse. It appears that the growth cone can be viewed as a nascent presynaptic terminal based on its ability to release transmitter in a Ca^{2+}-dependent manner and its rapid transformation into a presynaptic terminal on target contact (Xie and Poo, 1986; Sun and Poo, 1987). It would not be surprising if forms of activity-dependent synaptic plasticity, involving changes in Ca^{2+} regulation, have their counterpart at the growth cone.

The Ca^{2+} currents measured along growing neurites are greatest at the growth cone (Anglister et al., 1982; Streit and Lux, 1989). HVA Ca^{2+} channels, including both L- and N-types have been identified at the growth cones of snail neurons (Haydon and Man-Son-Hing, 1988), PC12 cells (Streit and Lux, 1987), frog sympathetic neurons (Lipscombe et al., 1988), and chick dorsal root ganglion neurons (Gottman and Lux, 1990). Like the presynaptic terminal, Ca^{2+} channels at the growth cone are clustered (Lipscombe et al., 1988). Ca^{2+} influx at these Ca^{2+} channel clusters is important in regulating growth cone function (Silver et al., 1990).

Growth produced by axons with different activity levels may have differences in Ca^{2+} regulation. Studies of regenerating crayfish axons in cell culture demonstrate that phasic motor axons are more sensitive to depolarization than tonic motor axons (Arcaro and Lnenicka, 1997). Although high K^+ depolarization inhibits the advance of growing phasic motor axons, tonic motor axons often continue to grow. During chronic depolarization, phasic axon growth degenerates, whereas tonic axon growth continues to survive and often continues to advance. The inhibitory effects of depolarization are Ca^{2+} dependent. A greater Ca^{2+} current density at the phasic axon growth cone, similar to that thought to exist at the phasic axon presynaptic terminals, could contribute to their greater sensitivity to depolarization. The differential effect of depolarization could also result from less Ca^{2+}-buffering capacity and/or a greater sensitivity of phasic growth cones to intracellular Ca^{2+}.

One study has directly examined the effects of a chronic change in impulse activity on neurite growth. It has demonstrated activity-dependent changes growth cones that include a reduction in voltage-dependent Ca^{2+} currents. Evoking action potentials in cultured dorsal root ganglion neurons initially results in inhibition of outgrowth and neurite retraction; however, during prolonged stimulation (24 h), the neurites resume growth (Fields et al., 1990). This decreased sensitivity to stimulation

involved a decrease in the Ca^{2+} current and a reduced sensitivity of the growth cone to intracellular Ca^{2+} (Fields et al., 1993).

Conclusions

Vertebrate and invertebrate neurons contain numerous types of voltage-dependent Ca^{2+} channels, which can be selectively modulated by voltage and Ca^{2+} influx. Short-term, voltage- and Ca^{2+}-dependent Ca^{2+} channel inactivations are well documented. Although Ca^{2+} channel inactivation is widespread, its magnitude varies among channel types. Ca^{2+} dependent inactivation is likely to be influenced by neuron-specific differences in Ca^{2+} buffering. Voltage-dependent facilitation has also been reported for neuronal Ca^{2+} channels; however, its occurrence is not as extensive as inactivation.

Long-term, activity-dependent changes in Ca^{2+} channels have been demonstrated both in vitro and in vivo. A long-term, Ca^{2+}-dependent reduction in Ca^{2+} current has been observed in both vertebrate and invertebrate neurons. High K^+ depolarization or increased impulse activity can produce a reduction in Ca^{2+} current lasting for days. The reduction in Ca^{2+} current apparently results from a reduction in the number of Ca^{2+} channels and can involve the targeting of specific channel types. The mechanism of this change may include Ca^{2+} channel internalization as well as decreased Ca^{2+} channel synthesis.

Activity-dependent changes in Ca^{2+} channels are likely to affect a number of neuronal processes, including transmitter release. Specific Ca^{2+} channel types localized at the active zone control the evoked release of transmitter. There is indirect evidence for the involvement of Ca^{2+} channel inactivation in short-term depression. In addition, there is evidence that LTD at crustacean neuromuscular Ca^{2+}-dependent Ca^{2+} current reduction. A more direct examination of Ca^{2+} currents at synaptic terminals will be necessary to confirm these findings. The activity-dependent regulation of Ca^{2+} current could play a role in the development of Ca^{2+} current density at synaptic terminals and growth cones.

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