Activity-Dependent Development of Calcium Regulation in Growing Motor Axons

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In cultured nerve cord explants from the crayfish (Procambarus clarkii), the normal impulse activity levels of growing motor axons determine their response to Ca$^{2+}$ influx. During depolarization or Ca$^{2+}$ ionophore application, normally active tonic motor axons continue to grow, whereas inactive phasic motor axons retract and often degenerate. To determine the role of Ca$^{2+}$ regulation in this difference, we measured the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) with fura-2. Growth cones from tonic axons normally had a higher [Ca$^{2+}$]$_i$ than those from phasic axons. When depolarized with 60 mM K$^+$, growth cones and neurites from phasic axons had a [Ca$^{2+}$]$_i$ three to four times higher than did those from tonic axons. This difference in Ca$^{2+}$ regulation includes greater Ca$^{2+}$-handling capacity for growing tonic axons; the increase in [Ca$^{2+}$]$_i$ produced by the Ca$^{2+}$ ionophore 4-bromo-A23187 (0.25 µM) is four to five times greater in phasic than in tonic axons, and the decline in [Ca$^{2+}$]$_i$ at the end of a depolarizing pulse is three to four times faster in tonic axons than phasic ones. Blocking impulses in growing tonic axons for 2–3 d with tetrodotoxin reduces their capacity to regulate [Ca$^{2+}$]. Thus, growing tonic and phasic axons have differences in Ca$^{2+}$ regulation that develop as a result of their different activity levels. These activity-dependent differences in Ca$^{2+}$ regulation influence axon growth and degeneration and probably influence other neuronal processes that are mediated by changes in [Ca$^{2+}$].

Key words: calcium regulation; activity-dependent; fura-2; cell culture; growth cones; crayfish
**MATERIALS AND METHODS**

**Preparation of cultures.** Nerve cord explant cultures were prepared from crayfish (*Procambarus clarkii*), which were obtained from Atchafalaya Biological Supply (Raceland, LA), and maintained at 20°C in shallow, aerated medium. Axon and nerve cords were removed from crayfish with carapace lengths of 1.5–2.5 cm and plated in defined culture medium, as described previously (Arcaro and Lnenicka, 1995). Briefly, the nerve cord was plated on a coverslip with the deep and superficial third roots arranged such that growth from the phasic and tonic motor axons was easily distinguished. The culture medium consisted of L-15 Medium Leibovitz (L-4386; Sigma, St. Louis, MO) that was diluted 1:1 and contained (in mM): 13.5 CaCl$_2$, 2.6 MgCl$_2$, 5.4 KCl, 206.0 NaCl, 5.6 d-glucose, and 10 Na-HEPES, pH 7.4. In experiments in which we blocked impulse activity during growth, 1 μM TTX was added to the medium when the nerve cords were plated (Arcaro and Lnenicka, 1995). All measurements of [Ca$^{2+}$]$_i$ were performed on cultures that were 2–3 d old.

**High-K$^+$ depolarization and ionophore application.** Motoneurons were depolarized with medium containing 60 mM K$^+$. Correct osmolarity was maintained by compensating for the increased KCl with an equal reduction in NaCl. Recordings of resting membrane potentials from the motor giant, the largest of the phasic motor neurons, showed that the addition of 60 mM K$^+$ reduced the resting membrane potential from approximately −65 mV (Arcaro and Lnenicka, 1997). The Ca$^{2+}$ ionophore 4-bromo-A23187 (Br-A23187) was prepared in DMSO at a concentration of 0.1 mM. This solution was diluted (1:400) in normal medium to give a final Br-A23187 concentration of 0.25 μM and was sonicated before application. To eliminate the effect of impulse activity on [Ca$^{2+}$]$_i$, TTX was added initially during incubation in fura-2 AM (see below) and included in all perfusion solutions for both the high-K$^+$ and Br-A23187 experiments. Solutions were exchanged by gravity flow perfusion at a rate of 3 ml/min for the chronic depolarization studies and 13 ml/min for the ionophore and brief depolarization studies (chamber volume, <0.5 ml).

**Measurement of [Ca$^{2+}$]$_i$.** Growing axons were loaded with fura-2 by incubating the cultures in medium containing 2 μM fura-2 AM (Molecular Probes, Eugene, OR) for 50–60 min. Fura-2 AM (1 mM) in DMSO was added to culture medium at a 1:500 dilution. After loading with fura-2, growing axons were imaged with a 40× objective (Nikon Fluor; numerical aperture, 1.3) on an inverted microscope (Nikon Diaphot 200). An A Lambda-1 optical filter changer (Sutter Instrument Co., Novato, CA) equipped with an intensified CCD camera (VS-2525 intensifier and 200E CCD camera; Video Scope International, Herndon, VA). Fura-2 was excited by passing light from a 75 W xenon arc lamp through bandpass filters of 340 ± 7 or 380 ± 8 nm (Chroma Technology, Brattleboro, VT). A Lambda-10 optical filter changer (Sutter Instrument Co., Novato, CA) was used to switch between excitation wavelengths. Typically, illumination intensity was attenuated with an ND4 filter. The excitation and emission wavelengths were separated with a 410 nm dichroic mirror, and excitation intensity was attenuated with an ND4 filter. The excitation and emission wavelengths were separated with a 410 nm barrier filter. Metafluor software (Universal Imaging, West Chester, PA) was used for controlling the shutter, filter wheel, and image acquisition, as well as subsequent analysis.

The fura-2 fluorescence ratio (340:380) was used to estimate [Ca$^{2+}$]$_i$ using standard techniques (Gryniewicz et al., 1985). Ratio pairs were acquired from 16 frame averages, and background values from a blank region of the slide were subsequently subtracted. We used 865 nm as the dissipation constant for fura-2 in crayfish axoplasm (Delaney et al., 1991; Mulkey and Zucker, 1992) and a viscosity correction factor of 0.7 (Pocie´n et al., 1986). As in previous studies of crayfish axons (Delaney et al., 1989), we measured ratios at zero Ca$^{2+}$ (R$_{min}$) and saturating Ca$^{2+}$ (R$_{max}$) in vitro using solutions similar to crayfish cytoplasm (Wallin, 1967). Typical calibration values were R$_{min}$ = 0.32, R$_{max}$ = 10.62, and F$_{0}$/F = 16.00. Note that small errors in estimating the absolute Ca$^{2+}$ concentrations should not affect the major findings of this study, which are based on relative Ca$^{2+}$ concentrations.

In many types of cells (e.g., mammalian) fura-2 is not a reliable indicator of Ca$^{2+}$ concentrations higher than a couple of micromolar because of its high affinity for Ca$^{2+}$ (Tsien, 1988). Fura-2 has a lower affinity for Ca$^{2+}$ in crayfish neurons compared with mammalian cells (K$_{d}$, 865 vs 220 nm), and thus higher levels of Ca$^{2+}$ can be measured. However, in some cases the Ca$^{2+}$ concentrations in crayfish phasic axons were still higher than what could be reliably measured with fura-2. To eliminate these unreliable measurements, we did not use data in which the ratio exceeded 4 (Ca$^{2+}$ concentration of ~12 μM).

The average Ca$^{2+}$ concentration in neurites or growth cones was determined for individual neurons. These average values were used to determine the overall mean and for statistical analysis using a two-tailed Student’s t test. All values were mean ± SE.

**RESULTS**

**[Ca$^{2+}$]$^+$, in growing phasic and tonic axons**

Growing neurites extend from the cut ends of the cultured phasic and tonic axons. Growth cones of phasic and tonic neurites advance at similar rates despite greater impulse activity in the tonic axons (Egid and Lnenicka, 1993; Arcaro and Lnenicka, 1997). Either tonic axons grow with higher [Ca$^{2+}$]$_i$, than phasic axons, or the impulse activity does not produce elevations in [Ca$^{2+}$]$_i$ in the tonic neurites. This was examined by comparing [Ca$^{2+}$]$_i$ in growing phasic and tonic axons.

[Ca$^{2+}$]$_i$ were measured during days 2–3 of growth, which is a period when the neurites are generally elongating (Egid and Lnenicka, 1993). The [Ca$^{2+}$]$_i$ in tonic growth cones (192 ± 36 nm; n = 17 neurons; n = 31 growth cones) and neurites (189 ± 32 nm; n = 15 neurons; n = 16 neurites; n = 8 animals) was significantly higher than in phasic growth cones (110 ± 11 nm; n = 16 neurons; n = 36 growth cones; p < 0.05) and neurites (111 ± 18 nm; n = 14 neurons; n = 16 neurites; n = 10 animals; p < 0.05). In some cases, we verified whether the growth cones were advancing at the time the Ca$^{2+}$ measurements were performed. These results were similar to the previous ones; [Ca$^{2+}$]$_i$ was significantly higher in tonic growth cones (163 ± 31 nm; n = 9 neurons; n = 12 growth cones) than in phasic growth cones (90 ± 14 nm; n = 9 neurons; n = 14 growth cones; p < 0.05). These results show that the active tonic axons normally grow with a higher [Ca$^{2+}$]$_i$, than the silent phasic axons.

**Depolarization produces a larger increase in [Ca$^{2+}$]$_i$, in growing phasic axons than in tonic ones**

When advancing phasic growth cones are depolarized with 60 mM K$^+$ for 40 min, they are initially inhibited and often retract (Arcaro and Lnenicka, 1997). During this depolarization, most tonic growth cones continue to advance. To determine the role of intracellular Ca$^{2+}$, the [Ca$^{2+}$]$_i$ was compared in growing phasic and tonic axons during depolarization.

Intracellular Ca$^{2+}$ was measured in phasic and tonic growth cones during depolarization produced by 60 mM K$^+$ (Fig. 1, top). During the first 10 min of depolarization, [Ca$^{2+}$]$_i$, reached higher levels in phasic than in tonic growth cones (Fig. 2). Subsequently, [Ca$^{2+}$]$_i$, in phasic growth cones gradually increased, whereas [Ca$^{2+}$]$_i$, in the tonic growth cones decreased and plateaued at a lower level. The [Ca$^{2+}$]$_i$, in the neurites was similar to that in the growth cones during this period of depolarization. A number of growth cones and neurites were examined 40–60 min after the beginning of the depolarization. The [Ca$^{2+}$]$_i$, in phasic growth cones (4.61 ± 0.37 μM; n = 37 growth cones; n = 70 growth cones) and neurites (5.54 ± 0.33 μM; n = 33 neurons; n = 43 neurites; n = 16 animals) was significantly higher than in tonic growth cones (1.38 ± 0.21 μM; n = 24 neurons; n = 71 growth cones; p < 0.0001) and neurites (1.56 ± 0.33 μM; n = 21 neurons; n = 32 neurites; n = 14 animals; p < 0.0001).

Thus, the greater depolarization-induced inhibition and degeneration of growing phasic neurites compared with tonic neurites is correlated with higher [Ca$^{2+}$]$_i$. The fact that [Ca$^{2+}$]$_i$, reaches higher levels in phasic axon growth suggests that the growing phasic axons have greater Ca$^{2+}$ influx and/or a lower rate of Ca$^{2+}$ removal than the growing tonic axons.
Ca\textsuperscript{2+} levels go higher in growing phasic axons than tonic axons after addition of the Ca\textsuperscript{2+} ionophore Br-A23187

To determine whether the differences in Ca\textsuperscript{2+} regulation involved differences in Ca\textsuperscript{2+} removal, we measured [Ca\textsuperscript{2+}]\textsubscript{i} during perfusion of the Ca\textsuperscript{2+} ionophore Br-A23187. Although Br-A23187 produced a rapid increase in [Ca\textsuperscript{2+}]\textsubscript{i}, in both phasic and tonic growth cones, the [Ca\textsuperscript{2+}]\textsubscript{i} reached higher levels in phasic growth cones. During the first 5 min of Br-A23187 application, [Ca\textsuperscript{2+}]\textsubscript{i} increased in both phasic and tonic growth cones; however, [Ca\textsuperscript{2+}]\textsubscript{i} decreased in the tonic growth cone. The TTX-tonic growth cone retracts and the tonic growth cone advances slightly. Thus, the previous history of impulse activity determines the ability of the growing axon to regulate intracellular Ca\textsuperscript{2+}.

Ca\textsuperscript{2+} removal after brief depolarizing pulses

To compare Ca\textsuperscript{2+} regulation in phasic and tonic growth cones further, we examined Ca\textsuperscript{2+} removal after brief depolarizing...
After addition of Br-A23187, the pulse,
than in phasic ones (Fig. 4). Because the decline in
values were obtained from 19 phasic growth cones from eight animals and
mean width of 5 μm resulted in a mean width of 5 μm and a time to one-half decay of 187.3 ± 18.3 sec (n = 3 neurons; n = 7 neurites), which was also significantly greater than the tonic neurite values (p < 0.01). These results support faster Ca\(^{2+}\) removal by growing tonic axons compared with growing phasic axons.

**DISCUSSION**

**Depolarization produced higher Ca\(^{2+}\) levels in the phasic axons and growth cones than in tonic ones**

During maintained depolarization with 60 mM K\(^+\), [Ca\(^{2+}\)]\(_i\) reaches higher levels in phasic neurites and growth cones than in tonic ones; [Ca\(^{2+}\)]\(_i\) in phasic axon growth is three to four times greater than in tonic axon growth at the end of 40 min of depolarization. The [Ca\(^{2+}\)]\(_i\) in phasic growth cones and axons during depolarization or ionophore application was very high and presumably was responsible for their retraction and eventual degeneration (Arcaro and Lnenicka, 1997). It is unclear what [Ca\(^{2+}\)]\(_i\) is necessary to produce degeneration or the extent to which increases in [Ca\(^{2+}\)]\(_i\) actually reflect the early stages of
tonic axons to depolarization involve differences in \( \text{Ca}^{2+} \) handling capacity. Thus, the different responses of the growing phasic and tonic axons are compared. Top: Representative results for phasic and tonic growth cones. Phasic and tonic growth cones with similar peak \( [\text{Ca}^{2+}] \), are compared. Bottom: Representative results from phasic and tonic neurites. Phasic and tonic neurites with similar widths and peak \( [\text{Ca}^{2+}] \), are compared. On the left, the phasic and tonic neurites are 7 and 6 \( \mu \text{m} \) wide, respectively. On the right, phasic and tonic neurites are 5 and 6 \( \mu \text{m} \) wide, respectively. Note that at the end of the pulse, \( [\text{Ca}^{2+}] \), consistently declined more rapidly in tonic growth cones and neurites than in phasic ones.

Figure 4. Changes in \( [\text{Ca}^{2+}] \), in phasic and tonic growth cones and neurites produced by brief depolarizations. Pulses of 60 mM K\(^+\) (bars) were applied to growing phasic and tonic axons in six animals, and changes in \( [\text{Ca}^{2+}] \), were measured. Top: Representative results for phasic and tonic growth cones. Phasic and tonic growth cones with similar peak \( [\text{Ca}^{2+}] \), are compared. Bottom: Representative results from phasic and tonic neurites. Phasic and tonic neurites with similar widths and peak \( [\text{Ca}^{2+}] \), are compared. On the left, the phasic and tonic neurites are 7 and 6 \( \mu \text{m} \) wide, respectively. On the right, phasic and tonic neurites are 5 and 6 \( \mu \text{m} \) wide, respectively. Note that at the end of the pulse, \( [\text{Ca}^{2+}] \), consistently declined more rapidly in tonic growth cones and neurites than in phasic ones.

Figure 5. Differences in \( \text{Ca}^{2+} \) regulation for tonic axons grown with or without impulse activity. The \( \text{Ca}^{2+} \)-handling capacity was compared in tonic axons grown in the presence of TTX with tonic axons grown in normal medium. The \( [\text{Ca}^{2+}] \), was measured in inactive tonic growth cones during 40 min of Br-A23187 application. These values are compared with those obtained from tonic growth cones grown in normal medium (Fig. 3). Note that the \( [\text{Ca}^{2+}] \), continued to increase in the inactive tonic growth cones in contrast to control tonic growth cones in which \( [\text{Ca}^{2+}] \), declined after the initial increase.

Although they advance at a similar rate (Arcaro and Lnenicka, 1997). The greater \( [\text{Ca}^{2+}] \), in growing tonic axons apparently results from differences in impulse activity (Egid and Lnenicka, 1993), because the differences in \( [\text{Ca}^{2+}] \), are not seen when TTX is added to the cultures before perfusing high-K\(^+\) or Br-A23187 solutions. Tonic growth cones may have a low sensitivity to intracellular \( \text{Ca}^{2+} \) because when depolarized they continue to advance, although the increase in \( [\text{Ca}^{2+}] \), appears sufficient to inhibit growth cone advance in many other neurons (Cohan et al., 1987; Silver et al., 1989; Lankford and Letourneau, 1991; Fields et al., 1993). It appears that not all growth cones are equally sensitive to intracellular \( \text{Ca}^{2+} \); neurites from rat superior cervical ganglion neurons continue to elongate with elevated \( [\text{Ca}^{2+}] \), (Garyantes and Regehr, 1992), and identified neurons in Heliosoma show differences in their sensitivity to intracellular \( \text{Ca}^{2+} \) during growth (Torreano and Cohan, 1997). In fact, a prolonged increase in impulse activity can produce a reduction in the sensitivity of the growth cone to intracellular \( \text{Ca}^{2+} \) (Fields et al., 1993). Further studies are required to determine whether \( \text{Ca}^{2+} \) sensitivity is different in growing phasic and tonic axons.

Tonic neurites and growth cones have a greater \( \text{Ca}^{2+} \)-handling capacity than phasic ones

\( \text{Ca}^{2+} \) regulation was compared in phasic and tonic growth by adding the \( \text{Ca}^{2+} \)-ionophore Br-A23187 and monitoring changes in \( [\text{Ca}^{2+}] \). After Br-A23187 application, \( [\text{Ca}^{2+}] \), in phasic growth cones plateaued at levels four to five times greater than that observed in tonic growth cones. Thus, under conditions in which the density of \( \text{Ca}^{2+} \) influx was presumably similar, the resultant \( [\text{Ca}^{2+}] \), is dramatically different. Of course this assumes that the ionophore is incorporated into growing phasic and tonic axons at equal densities, which seems likely. Further evidence for stronger \( \text{Ca}^{2+} \) regulation by tonic axon growth was provided by examining \( \text{Ca}^{2+} \) removal at the end of a brief depolarizing pulse. After a 60–90 sec depolarizing pulse, the \( [\text{Ca}^{2+}] \), decreases more rapidly in tonic growth cones and neurites than in phasic ones. Thus, the differences in \( \text{Ca}^{2+} \) regulation include differences in \( \text{Ca}^{2+} \) removal; however, we do not know whether there are also differences in \( \text{Ca}^{2+} \) entry during depolarization.

The removal of intracellular free \( \text{Ca}^{2+} \) involves chelation by degeneration. Very high levels of intracellular \( \text{Ca}^{2+} \) (>5 \( \mu \text{M} \)) appear to be required to trigger excitotoxic death of mouse cortical neurons (Hyrc et al., 1997). During depolarization of growing tonic axons, \( [\text{Ca}^{2+}] \), stabilizes at values that are not high enough to trigger retraction or degeneration. In fact, although there is a loss of filopodia, growth cones usually continue to advance. Thus, the different responses of the growing phasic and tonic axons to depolarization involve differences in \( \text{Ca}^{2+} \) regulation.

There may also be differences in the sensitivity of phasic and tonic growth to \( \text{Ca}^{2+} \) because normally the \( [\text{Ca}^{2+}] \), in the tonic growth cones is \( \sim 70\% \) greater than in phasic growth cones, which \( \text{Ca}^{2+} \), is added to the cultures before perfusing high-K\(^+\) or Br-A23187 solutions. Tonic growth cones may have a low sensitivity to intracellular \( \text{Ca}^{2+} \) because when depolarized they continue to advance, although the increase in \( [\text{Ca}^{2+}] \), appears sufficient to inhibit growth cone advance in many other neurons (Cohan et al., 1987; Silver et al., 1989; Lankford and Letourneau, 1991; Fields et al., 1993). It appears that not all growth cones are equally sensitive to intracellular \( \text{Ca}^{2+} \); neurites from rat superior cervical ganglion neurons continue to elongate with elevated \( [\text{Ca}^{2+}] \), (Garyantes and Regehr, 1992), and identified neurons in Heliosoma show differences in their sensitivity to intracellular \( \text{Ca}^{2+} \) during growth (Torreano and Cohan, 1997). In fact, a prolonged increase in impulse activity can produce a reduction in the sensitivity of the growth cone to intracellular \( \text{Ca}^{2+} \) (Fields et al., 1993). Further studies are required to determine whether \( \text{Ca}^{2+} \) sensitivity is different in growing phasic and tonic axons.

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The removal of intracellular free \( \text{Ca}^{2+} \) involves chelation by
Ca\textsuperscript{2+}-binding proteins, sequestration by intracellular organelles, and extrusion across the plasma membrane by the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange and Ca\textsuperscript{2+}ATPase (for review, see Miller, 1991). Generally, Ca\textsuperscript{2+}-binding proteins have a low capacity for buffering Ca\textsuperscript{2+}; therefore, they probably contribute little to buffering the large Ca\textsuperscript{2+} loads imposed in these experiments. In addition, greater cytoplasmic buffering should slow the removal of Ca\textsuperscript{2+}, whereas Ca\textsuperscript{2+} is removed more rapidly from tonic than phasic growth cones. Mitochondria are likely to be more effective than the endoplasmic reticulum at sequestering these large Ca\textsuperscript{2+} loads because of their greater capacity. Mitochondrial Ca\textsuperscript{2+} uptake can reduce the peak [Ca\textsuperscript{2+}], during a depolarizing pulse and accelerate the initial decay of [Ca\textsuperscript{2+}], at the end of the pulse; however, mitochondria then release Ca\textsuperscript{2+}, which causes the [Ca\textsuperscript{2+}] to plateau before returning to resting levels (Friel and Tsien, 1994; Werth and Thayer, 1994; Herrington et al., 1996). Again, because Ca\textsuperscript{2+} transients decay more rapidly in tonic growth cones than phasic ones, the greater Ca\textsuperscript{2+}-handling capacity of tonic growth cones is unlikely to be attributable simply to greater mitochondrial Ca\textsuperscript{2+} uptake.

A higher rate of Ca\textsuperscript{2+} extrusion from growing tonic axons than from phasic ones could be responsible for their lower [Ca\textsuperscript{2+}] during ionophore application and more rapid decay of [Ca\textsuperscript{2+}], at the end of depolarizing pulses. According to a model describing the dynamics of residual Ca\textsuperscript{2+} in crayfish motor terminals, an increase in the rate of Ca\textsuperscript{2+} extrusion will decrease the [Ca\textsuperscript{2+}], plateau during a train of impulses and increase the rate of decay of [Ca\textsuperscript{2+}], at the end of the train (Tank et al., 1995). In crayfish motor terminals, the rate of Ca\textsuperscript{2+} extrusion appears to be a linear function of [Ca\textsuperscript{2+}], and can be modeled using a first-order Michaelis–Menten reaction (Tank et al., 1995). This formulation does not distinguish between the plasma membrane Ca\textsuperscript{2+}ATPase or Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange (Sala and Hernandez-Cruz, 1990; Lagnado et al., 1992; Tank et al., 1995). Based primarily on studies of squid axons, the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange has been classified as having a low affinity but high capacity for Ca\textsuperscript{2+} transport, whereas the Ca\textsuperscript{2+}ATPase has a high affinity but low capacity (Baker and DiPolo, 1984).

It appears reasonable that the plasma membrane Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger could play a major role in Ca\textsuperscript{2+} extrusion during the large Ca\textsuperscript{2+} loads imposed in our experiments. The Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger was important in removing intracellular Ca\textsuperscript{2+} from *Helisoma* neurons during a large Ca\textsuperscript{2+} load produced by BrA23187 (Mills and Kater, 1990). There is evidence that Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange extrudes Ca\textsuperscript{2+} from rat brain synaptosomes (Sanchez-Armass and Blaustein, 1987), and based on immunocytochemistry the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger is concentrated at presynaptic terminals, as well as in growing neurites and growth cones (Luther et al., 1992). Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange, as well as mitochondrial Ca\textsuperscript{2+} uptake, play a role in removing cytoplasmic free Ca\textsuperscript{2+} from crayfish motor terminals (Mulkey and Zucker, 1992; Tang and Zucker, 1997). Differences in Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange in growing phasic and tonic axons could result from differences in the exchanger and/or their ability to extrude Na\textsuperscript{+} (Blaustein, 1988).

**Differences in Ca\textsuperscript{2+} regulation are activity-dependent**

The strong Ca\textsuperscript{2+} regulation seen in tonic axons appears to result from its high-impulse activity levels during growth. Tonic axons growing in TTX showed weaker Ca\textsuperscript{2+} regulation than control ones during the application of BrA23187. The strong Ca\textsuperscript{2+} regulation produced by high-impulse activity persists for at least 1 hr, and probably much longer, because impulse activity in the tonic axons was blocked for >1 hr before examining their Ca\textsuperscript{2+} handling (see Materials and Methods).

The activity-dependent strengthening of Ca\textsuperscript{2+} regulation could involve changes in protein synthesis. For example, the synthesis of Ca\textsuperscript{2+}-binding proteins can be upregulated by increased electrical activity (Lowenstein et al., 1991). In addition, there are activity-dependent differences in the density and activity of mitochondria in crayfish motor axons and terminals (Lnenicka et al., 1986, 1997; Nguyen and Atwood, 1994). Mitochondrial changes could play a role in modulating Ca\textsuperscript{2+} regulation, if not through direct Ca\textsuperscript{2+} uptake, then indirectly through the production of ATP.

**Relevance to neuronal physiology**

Because [Ca\textsuperscript{2+}]\textsubscript{i} is so important to neuronal function, the activity-dependent development of Ca\textsuperscript{2+} regulation could affect a number of neuronal properties. For the developing axon, Ca\textsuperscript{2+} regulation can affect how impulse activity and environmental cues shape the pattern of growth. In the adult, greater Ca\textsuperscript{2+}-handling capacity could make neurons more resistant to Ca\textsuperscript{2+} neurotoxicity produced by insults such as excitotoxicity (Choi, 1988). Activity-dependent differences in Ca\textsuperscript{2+} regulation could influence cell excitability, e.g., intracellular Ca\textsuperscript{2+} levels influence the firing properties of lobster neurons (Turrigiano et al., 1994).

Activity-dependent differences in Ca\textsuperscript{2+} regulation at motor terminals are likely to influence transmitter release. For example, Ca\textsuperscript{2+} removal is likely to influence the production of post-tetanic potentiation (PTP), because PTP requires the buildup of residual Ca\textsuperscript{2+} (Delaney et al., 1989; Delaney and Tank, 1994). Because chronic *in vivo* stimulation of crayfish phasic motor terminals reduces their capacity to produce PTP (Pahapill et al., 1986), it may be that increased impulse activity strengthens Ca\textsuperscript{2+} regulation in mature motor terminals, as well as in growing axons.

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