Ca\textsuperscript{2+} Clearance at Growth Cones Produced by Crayfish Motor Axons in an Explant Culture

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Rumpal, Nidhi and Gregory A. Lnenicka. Ca\textsuperscript{2+} clearance at growth cones produced by crayfish motor axons in an explant culture. J Neurophysiol 89: 3225–3234, 2003; 10.1152/jn.00952.2002. Intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) plays an important role in the regulation of growth cone (GC) motility; however, the mechanisms responsible for clearing Ca\textsuperscript{2+} from GCs have not been examined. We studied the Ca\textsuperscript{2+}-clearance mechanisms in GCs produced by crayfish tonic and phasic motor axons by measuring the decay of [Ca\textsuperscript{2+}]\textsubscript{i} after a high [K\textsuperscript{+}] depolarizing pulse using fura-2AM. Tonic motor axons regenerating in explant cultures develop GCs with more rapid Ca\textsuperscript{2+} clearance than GCs from phasic axons. When Na/Ca exchange was blocked by replacing external Na\textsuperscript{+} with N-methyl-D-glucamine (NMG), [Ca\textsuperscript{2+}]\textsubscript{i}, decay was delayed in both tonic and phasic GCs. Tonic GCs appear to have higher Na/Ca exchange activity than phasic ones since reversal of Na/Ca exchange by lowering external Na\textsuperscript{+} caused a greater increase in [Ca\textsuperscript{2+}]\textsubscript{i}, for tonic than phasic GCs. Application of the mitochondrial inhibitors, Antimycin A1 (1 \textmu M) and CCCP (10 \textmu M), demonstrated that mitochondrial Ca\textsuperscript{2+} uptake/release was more prominent in phasic than tonic GCs. When both Na/Ca exchange and mitochondria were inhibited, the plasma membrane Ca\textsuperscript{2+} ATPase was effective in extruding Ca\textsuperscript{2+} from tonic, but not phasic GCs. We conclude that Na/Ca exchange plays a prominent role in extruding large Ca\textsuperscript{2+} loads from both tonic and phasic GCs. High Na/Ca exchange activity in tonic GCs contributes to the rapid decay of [Ca\textsuperscript{2+}]\textsubscript{i} in these GCs; low rates of Ca\textsuperscript{2+} extrusion plus the release of Ca\textsuperscript{2+} from mitochondria prolongs the decay of [Ca\textsuperscript{2+}]\textsubscript{i} in the phasic GCs.

INTRODUCTION

Intracellular Ca\textsuperscript{2+} signals control many neuronal processes, including transmitter release (Katz 1969), membrane excitability (Turrigiano et al. 1994), gene transcription (Finkbeiner and Greenberg 1998), neuron growth (Kater and Mills 1991), and survival (Nishi and Berg 1981). Thus the development and regulation of processes that control the intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) are of great consequence. Increases in [Ca\textsuperscript{2+}]\textsubscript{i} are produced by Ca\textsuperscript{2+} influx through membrane channels and Ca\textsuperscript{2+} release from intracellular stores. The clearance of intracellular free Ca\textsuperscript{2+} results from chelation by Ca\textsuperscript{2+}-binding proteins, Ca\textsuperscript{2+} sequestration by intracellular organelles, and extrusion across the plasma membrane. The mechanisms responsible for clearing Ca\textsuperscript{2+} can determine the temporal and spatial pattern of the intracellular Ca\textsuperscript{2+} signal. For example, mechanisms that extrude Ca\textsuperscript{2+} restrict the Ca\textsuperscript{2+} signal; however, Ca\textsuperscript{2+} uptake by mitochondria can extend the Ca\textsuperscript{2+} signal since mitochondria subsequently release Ca\textsuperscript{2+} into the cytoplasm.

Intracellular Ca\textsuperscript{2+} regulation is particularly important at axonal endings, both growth cones (GCs), and synaptic terminals. During axon growth, [Ca\textsuperscript{2+}]\textsubscript{i}, at the growth cone influences the rate and direction of axon elongation (Kater and Mills 1991; Zheng 2000), and after synapse formation, [Ca\textsuperscript{2+}]\textsubscript{i} controls transmitter release and plasticity (Zucker 1996). Physiological studies have demonstrated that both Na/Ca exchange and mitochondria can play an important role in Ca\textsuperscript{2+} clearance from axons and synaptic terminals (David et al. 1998; Gleason et al. 1994; Lee et al. 2002; Mulkey and Zucker 1992; Regh et al. 1997; Reuter and Porzig 1995; Tang and Zucker 1997; Verbny et al. 2002; Zhong et al. 2001). Although immunocytochemical studies have shown that the Na/Ca exchanger is expressed at GCs (Luther et al. 1992), the mechanism(s) of Ca\textsuperscript{2+} clearance from GCs have not been identified.

The Na/Ca exchanger and mitochondria are important for clearing large Ca\textsuperscript{2+} loads (Blaustein and Lederer 1999); however, their relative importance appears to vary with cell type. For example, Na/Ca exchange is largely responsible for Ca\textsuperscript{2+} removal from the cell bodies of rat nucleus basalis neurons (Tatsumi and Katayama 1993), retinal amacrine cells (Gleason et al. 1995), and some cell bodies of Helisoma neurons (Mills and Kater 1990). Mitochondria have been shown to play an important role in clearance of cytosolic Ca\textsuperscript{2+} from cell bodies of rat dorsal root ganglia (DRG) neurons (Werth and Thayer 1994), adrenal chromaffin cells (Herrington et al. 1996), and cortical neurons (White and Reynolds 1995).

We have examined the mechanism(s) responsible for clearing large Ca\textsuperscript{2+} loads from GCs produced by crayfish phasic and tonic motor axons. These growth cones are particularly interesting since the tonic GCs, which have high impulse activity, show more rapid Ca\textsuperscript{2+} clearance than inactive phasic ones (Lnenicka et al. 1998a). This difference in Ca\textsuperscript{2+} clearance appears to continue after synapse formation: tonic motor terminals show more rapid Ca\textsuperscript{2+} clearance than phasic ones (Msghina et al. 1999). We found that Na/Ca exchange is important for Ca\textsuperscript{2+} clearance from tonic GCs, and they appear to develop higher Na/Ca exchange activity than phasic GCs. On the other hand, both Na/Ca exchange and mitochondria contribute to Ca\textsuperscript{2+} clearance from phasic GCs. Thus the relative importance of Ca\textsuperscript{2+}-clearance mechanisms can vary with...
growth cone type and appears to correlate with the normal impulse activity of the neuron.

**METHODS**

**Preparation of cultures**

Nerve-cord explant cultures were prepared from crayfish (*Procambarus clarkii*), obtained from Atchafalaya Biological Supply (Raceland, LA), and maintained at 20°C in shallow aerated tanks. Abdominal nerve cords were plated on coverslips and arranged such that growth from the phasic and tonic motor axons was easily distinguished (Egid and Lnenicka 1993). The cultures were grown in a humidified chamber at room temperature (21–25°C). In previous studies, we used L15 culture media; however, we have found that the axons grow equally well in normal crayfish saline. Therefore we used culture medium containing (in mM) 13.5 CaCl$_2$, 2.5 MgCl$_2$, 5.4 KCl, 206 NaCl, 1.0 glucose, 10 Na-HEPES (pH 7.4), and 11 mg/l phenol red. All measurements of [Ca$^{2+}$], were performed on cultures that were 2–4 days old.

**High [K$^+$] depolarization**

Motor axons were depolarized with saline containing 60 mM [K$^+$], which reduces the membrane potential in the motoneuron’s cell body to approximately ~30 mV (Arcaro and Lnenicka 1997). Correct osmolarity was maintained by compensating for the increased KCl with an equal reduction in NaCl. To prevent impulse activity, 0.2 μM TTX (Alomone Laboratories, Jerusalem, Israel) was included in all perfusion solutions. Cultures were mounted in a closed chamber (model RC-21B, Warner Instruments, Hamden, CT) with a volume of 0.26 ml, and the solutions were exchanged by gravity-flow perfusion at approximately 0.4 ml/s. To switch solutions, we used a solenoid manifold (Parker Hannifin, General Valve Division, Fairfield, NJ) attached to the microscope stage. For low external Na$^+$ concentrations ([Na$^+$]$_e$), Na$^+$ was substituted with N-methyl-$eta$-glucamine (NMG). In some cases, mitochondrial inhibitors Antimycin A1 (1 μM) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 10 μM), thapsigargin (1 μM), La$^{3+}$ (0.1 and 1 mM), and KB-R7943 (10 and 50 μM) were included in the perfusion solutions. NMG, Antimycin A1, CCCP, and thapsigargin were obtained from Sigma (St. Louis, MO) and KB-R7943 was from Tocris (Ballwin, MO).

**Measurement of [Ca$^{2+}$]$_i$**

The measurement of [Ca$^{2+}$]$_i$ was previously described in detail (Lnenicka et al. 1998a). Briefly, growing axons were loaded with fura-2 (1 mM stock in DMSO) by incubating the cultures in media containing 2 μM fura-2 AM (Molecular Probes, Eugene, OR) for 50–60 min. The GCs were imaged with a 40× objective (Nikon Fluor, N.A. 1.3) on an inverted microscope (Nikon Diaphot 200). Fura-2 was excited at 340 and 380 nm, and the fluorescence at 510 nm was captured with an intensified CCD camera (VS-2525 intensifier, 200E CCD camera, Video Scope International, Herndon, VA). Metaflour software (Universal Imaging, West Chester, PA) was used for controlling the shutter, filter wheel, and image acquisition, as well as subsequent analysis. Images produced by 340- and 380-nm excitation were acquired from 8-frame averages, and background values were subtracted. The ratio of fluorescence intensity (F340/380) was measured in the body of the growth cone and used to calculate [Ca$^{2+}$], using the standard formula (Grynkiewicz et al. 1985). We have used a value of 0.7 for the viscosity correction factor and a fura-2 $K_d$ of 865 nM, which is the $K_d$ determined initially (Delaney et al. 1991) and used in many subsequent studies (Delaney and Tank 1994; Lnenicka et al. 1998a; Msghina et al. 1999; Mulkey and Zucker 1992; Tang and Zucker 1997). Ravin et al. (1997) re-measured the fura-2 $K_d$ for crayfish and obtained a similar value of 850 nM; however, Tang et al. (2000) have reported a fura-2 $K_d$ of 360 nM. We obtained a fura-2 $K_d$ of 861 nM, which was similar to the initial value, using a calibration solution (in mM: 17 NaCl, 265 KCl, 10 KHEPES, pH 7.02) similar in composition to crayfish axoplasm (Wallin 1967) with Ca$^{2+}$ set to 0, 10 mM, or 500 nM using EGTA (Bers et al. 1994). Nonetheless, the conclusions from this study do not depend critically on the fura-2 $K_d$ rather than the absolute values. All [Ca$^{2+}$]$_i$ values are reported as mean ± SE, error bars represent SE, and $n =$ number of GCs, number of culture dishes. Statistical comparisons were performed with two-tailed t-tests.

**RESULTS**

[Ca$^{2+}$]$_i$ clearance from tonic and phasic GCs

We examined GCs produced by the tonic and phasic motor axons that exit the abdominal nerve cord through separate branches of the third root. The phasic and tonic nerve branches contain 10 and 6 axons, respectively (Kennedy and Takeda 1965a,b). In culture, most tonic axons show spontaneous impulse activity, whereas the phasic axons are silent (Egid and Lnenicka 1993). Tonic axon GCs show strong Ca$^{2+}$ clearance and continue to advance on prolonged application of 60 mM [K$^+$] saline or Ca$^{2+}$ ionophore 4-bromo-A23187. In contrast, the phasic axons accumulate high [Ca$^{2+}$]$_i$ under these conditions and show considerable retraction of the GCs (Arcaro and Lnenicka 1997; Lnenicka et al. 1998a).

In this study, we investigated the mechanisms for Ca$^{2+}$ clearance from tonic and phasic GCs. Decay of [Ca$^{2+}$]$_i$ was measured after depolarization with a 60 mM [K$^+$] pulse (60 s) using fura-2AM (Fig. 1). The increase in [Ca$^{2+}$]$_i$ during depolarization resulted from entry of extracellular Ca$^{2+}$ into GCs, since Ca$^{2+}$-free high [K$^+$] saline did not produce an increase in [Ca$^{2+}$]$_i$. The resting [Ca$^{2+}$]$_i$ in tonic GCs (0.18 ± 0.01 μM; $n = 102, 59$) was not significantly different from phasic GCs (0.16 ± 0.01 μM; $n = 84, 31$; $P = 0.23$), but the peak increase in [Ca$^{2+}$]$_i$ was significantly higher in phasic GCs (2.78 ± 0.29 μM; $n = 84, 31$) than tonic GCs (2.12 ± 0.10 μM; $n = 102, 59$; $P = 0.02$). As previously reported (Lnenicka...
et al. 1998a), the time to decaying of [Ca$^{2+}$]$_i$ ($T_{1/2}$) was considerably smaller in tonic GCs than in phasic ones; the average $T_{1/2}$ for tonic (16.10 ± 1.37 s; $n$ = 102, 59) was significantly smaller than for phasic (148.18 ± 16.27 s; $n$ = 84, 31; $P < 10^{-10}$) GCs (Fig. 2). There was modest variability in the time taken for decay of [Ca$^{2+}$]$_i$ in tonic GCs (88% of GCs had $T_{1/2}$ values in the 5- to 30-s range). On the other hand, a wider range of $T_{1/2}$ values were observed in phasic GCs: phasic GCs (74%) with a prominent shoulder in the [Ca$^{2+}$]$_i$ decay curve (Fig. 2) had a much longer $T_{1/2}$ than those without it. A similar shoulder was observed during Ca$^{2+}$ clearance from rat DRG neurons and has been attributed to Ca$^{2+}$ release from mitochondria (Thayer and Miller 1990).

**Role of Na/Ca exchange in Ca$^{2+}$ clearance from tonic and phasic GCs**

We determined whether Na/Ca exchange was involved in Ca$^{2+}$ clearance from tonic and phasic GCs. Ca$^{2+}$ efflux by the Na/Ca exchanger was inhibited by reducing external Na$^+$ ([Na$^+$]o) to 0 mM, which eliminates the Na$^+$ electrochemical gradient driving Ca$^{2+}$ extrusion (Blaustein and Lederer 1999). This is the standard technique for blocking Na/Ca exchange (Bleakman et al. 1993; Gleason et al. 1995; Herrington et al. 1996; Sanchez-Armass and Blaustein 1987; White and Reynolds 1995) since specific inhibitors of the Na/Ca exchanger are not available. As in a previous study (White and Reynolds 1995), we reduced [Na$^+$]o to 0 mM only during the wash so it did not affect the Ca$^{2+}$ load produced by high [K$^+$] depolarization. The $T_{1/2}$ was monitored in paired-pulse experiments: the first 60 mM [K$^+$] pulse was followed by a normal saline wash (NS wash) and the second pulse was followed by 0 mM [Na$^+$]o wash (0 Na$^+$ wash). The 0 Na$^+$ wash prolonged the decay of [Ca$^{2+}$]$_i$ in both tonic and phasic GCs (Fig. 3). For tonic GCs ($n$ = 15, 7), there was a fivefold increase in $T_{1/2}$ in 0 Na$^+$ wash (from 20.23 ± 2.85 to 103.92 ± 23.90 s; $P = 1.76 \times 10^{-3}$). All phasic GCs ($n$ = 18, 6) also showed an increase in $T_{1/2}$: in 15/18 GCs, [Ca$^{2+}$]$_i$ did not decline to one-half of the peak during the 0 Na$^+$ wash. In the remaining three GCs, which initially lacked the shoulder in the decay curve, $T_{1/2}$ increased by about twofold. Overall, for phasic GCs, [Ca$^{2+}$]$_i$ was approximately 80% of the peak value at the end of the 0 Na$^+$ wash (Fig. 3). In control experiments, where the second 60 mM [K$^+$] pulse was followed by a NS wash, the decay in [Ca$^{2+}$]$_i$ was similar to the first pulse (Fig. 3). Thus Na/Ca exchange plays a prominent role in Ca$^{2+}$ extrusion from both tonic and phasic GCs.

**Role of mitochondria in Ca$^{2+}$ clearance from tonic and phasic GCs**

We investigated the role of mitochondria in Ca$^{2+}$ clearance at the tonic and phasic GCs by using the mitochondrial inhibitors Antimycin A1 and CCCP. Antimycin A1 causes a rundown of the mitochondrial inner membrane potential by inhibiting electron transport, which blocks the Ca$^{2+}$ uptake by mitochondria. Antimycin A1 (1 µM) was applied 3 min before the second pulse of a pair of high [K$^+$] pulses (Fig. 4). We did not attempt to wash out Antimycin A1 since this is difficult with aqueous solutions (Slater 1973). Antimycin A1 had no significant effect on [Ca$^{2+}$]$_i$ decay in tonic GCs ($T_{1/2}$ for 1st (27.36 ± 0.08 s) and 2nd (25.51 ± 0.07 s; $n$ = 12, 8; $P = 0.76$) pulses) or the peak [Ca$^{2+}$]$_i$ (1st (1.84 ± 0.20 µM) and 2nd (1.59 ± 0.21 µM) pulses). In contrast, Antimycin A1 eliminated the shoulder in the [Ca$^{2+}$]$_i$ decay curve for phasic GCs (Fig. 4); the absence of a shoulder in the presence of the inhibitor indicated that mitochondria were no longer sequestering Ca$^{2+}$ during the test pulse and releasing it later during [Ca$^{2+}$]$_i$ decay. In addition, there was a trend towards a higher peak [Ca$^{2+}$]$_i$ during depolarization in the presence of Antimycin A1.

Studies with carbonyl cyanide m-chlorophenyl hydrzone (CCCP), a rapidly acting mitochondrial inhibitor, provided further evidence for Ca$^{2+}$ uptake/release by mitochondria in phasic GCs. CCCP rapidly dissipates the H$^+$ gradient across the inner mitochondrial membrane, thereby collapsing the membrane potential. Thus application of CCCP should inhibit mitochondrial Ca$^{2+}$ uptake and also trigger the release of any previously sequestered Ca$^{2+}$. Indeed, the addition of CCCP in the wash for the second high [K$^+$] pulse ($n$ = 7, 3) caused a further increase in [Ca$^{2+}$]$_i$ after the initial peak (presumably due to the release of Ca$^{2+}$ from mitochondria) and eliminated the shoulder in [Ca$^{2+}$]$_i$ decay in phasic GCs as seen with Antimycin A1.

To confirm that the Ca$^{2+}$ released from mitochondria was taken up during the high [K$^+$] pulse, we determined whether mitochondria contained significant amounts of Ca$^{2+}$ before the pulse. When CCCP was added before the second depolarizing pulse, there was only slight increase (0.06 ± 0.02 µM; $n$ = 7, 3) in resting [Ca$^{2+}$]$_i$ in phasic GCs, indicating that the mitochondria sequestered Ca$^{2+}$ during the pulse.

**Do other mechanisms effectively clear Ca$^{2+}$ from phasic and tonic GCs**

We inhibited both Na/Ca exchange and mitochondria to determine whether other mechanisms were effective in clearing
Ca\(^{2+}\) from these GCs. In phasic GCs, [Ca\(^{2+}\)]\(i\) did not decline after the high [K\(^+\)] pulse when both Ca\(^{2+}\) extrusion by Na/Ca exchange and uptake by mitochondria were eliminated (Fig. 5, top). In fact, [Ca\(^{2+}\)]\(i\) increased in the 0 Na\(^+\) plus Antimycin A1 wash, probably due to Ca\(^{2+}\) influx through the Na/Ca exchanger (Na/Ca exchanger can operate in the Ca\(^{2+}\)-entry mode in 0 mM [Na\(^+\)]\(i\)). This indicated that other mechanisms are not very effective in clearing large Ca\(^{2+}\) loads from phasic GCs. 

In tonic GCs, when Ca\(^{2+}\) extrusion by Na/Ca exchange and Ca\(^{2+}\) uptake by mitochondria were inhibited, [Ca\(^{2+}\)]\(i\) still declined during the wash (Fig. 5, top), indicating the presence of another Ca\(^{2+}\) clearance mechanism. This appeared to be the plasma membrane Ca\(^{2+}\) ATPase since [Ca\(^{2+}\)]\(i\) did not decay when the Ca\(^{2+}\) ATPase was blocked by adding 0.1 mM La\(^{3+}\) (Quist and Roufogalis 1975) to the 0 Na\(^+\) wash. Again, like phasic GCs, tonic GCs show an increase in [Ca\(^{2+}\)]\(i\) during the wash, presumably due to Ca\(^{2+}\) entry through the Na/Ca exchanger.

Finally, the role of Ca\(^{2+}\) uptake/release by the endoplasmic reticulum (ER) Ca\(^{2+}\) ATPase was examined. To inhibit the ER Ca\(^{2+}\) ATPase, 1 μM thapsigargin was applied either 3 min before a high [K\(^+\)] pulse or in the wash; however, we found no
evidence that thapsigargin influenced resting $[\text{Ca}^{2+}]_{\text{i}}$ or the decay of $[\text{Ca}^{2+}]_{\text{i}}$.

Greater Na/Ca exchange activity for tonic than phasic GCs

Since Na/Ca exchange plays an important role in $\text{Ca}^{2+}$ clearance from both tonic and phasic GCs, and tonic GCs show more rapid $\text{Ca}^{2+}$ clearance than phasic ones, it seemed likely that tonic GCs have greater Na/Ca exchange activity. To compare Na/Ca exchange activity in tonic and phasic GCs, we reversed the Na/Ca exchange by a step-wise reduction in $[\text{Na}^{+}]_{\text{o}}$ (from 206 to 0 mM) and monitored the increase in $[\text{Ca}^{2+}]_{\text{i}}$ produced by $\text{Ca}^{2+}$ entry through the Na/Ca exchanger (Fig. 6). Theoretically, these reductions in $[\text{Na}^{+}]_{\text{o}}$ should run the Na/Ca exchanger in the $\text{Ca}^{2+}$-entry mode (reverse mode). The exchanger can run in the $\text{Ca}^{2+}$-entry mode when the exchanger reversal potential ($E_{\text{Na/Ca}} = 3E_{\text{Na}} - 2E_{\text{Ca}}$ for a 3Na:1Ca exchanger) is more negative than the resting potential ($E_{\text{Na}}$) (Mullins 1981). $[\text{Ca}^{2+}]_{\text{i}}$ increased in both phasic and tonic GCs as $[\text{Na}^{+}]_{\text{o}}$ was continually reduced; however, the increase was greater in the tonic than phasic GCs. Comparing a number of phasic and tonic GCs showed that reducing $[\text{Na}^{+}]_{\text{o}}$ produces a significantly larger increase in $[\text{Ca}^{2+}]_{\text{i}}$ in tonic than in phasic GCs (Fig. 7).

To confirm that the increase in $[\text{Ca}^{2+}]_{\text{i}}$ resulted from entry through the Na/Ca exchanger, we inhibited the Na/Ca exchanger in tonic GCs using $\text{La}^{3+}$. Low concentration of $\text{La}^{3+}$ (0.1 mM) can selectively block the $\text{Ca}^{2+}$ ATPase; however, higher concentrations can inhibit both the forward and reverse mode of the exchanger (Rahamimoff and Spanier 1984; Shimizu et al. 1997) and are effective in a broad range of systems, including squid (Baker et al. 1969), lobster (Eisenrauch et al. 1995), and rat adrenal chromaffin cells (Herrington et al. 1996). Although $\text{La}^{3+}$ has been applied at 1 mM to inhibit the exchanger (Herrington et al. 1996), a dose-response curve
has not been established. A pair of 300-s 0 mM [Na+] pulses was applied to tonic GCs, and 1 mM La3+ was added during the second pulse. The addition of La3+ reduced the increase in [Ca2+], by 46 ± 19% (n = 6, 5), indicating that Ca2+ entered through the exchanger. The failure to completely block the increase in [Ca2+], could result from an incomplete block of the Na/Ca exchanger and possibly inhibition of the plasma membrane Ca2+ ATPase by 1 mM La3+. (Blocking the Ca2+ ATPase could enhance the increase in [Ca2+], produced by Ca2+ influx through the Na/Ca exchanger.) These results indi-
These differences in Ca\(^{2+}\) allow Ca\(^{2+}\) entry through reverse Na/Ca exchange, thus allowing Ca\(^{2+}\) entry through the Na/Ca exchanger. [Ca\(^{2+}\)](i) goes higher in tonic than phasic GCs. Two classes of GCs were included in this study: tonic axon GCs, with high rates of Ca\(^{2+}\) clearance, and phasic axon GCs, which clear Ca\(^{2+}\) more slowly. These differences in Ca\(^{2+}\) clearance were demonstrated in a previous study in which the Ca\(^{2+}\) ionophore A23187 was added to the GCs (Lnenicka et al. 1998a). Here, these differences were further demonstrated by the more rapid decline in [Ca\(^{2+}\)](i) in tonic GCs compared with phasic ones after high [K\(^{+}\)](o) pulses. Differences in GC morphology cannot explain the differences in Ca\(^{2+}\) clearance from tonic and phasic GCs. When viewed with differential interference contrast (DIC) microscopy, the phasic growth cones appear thinner than tonic growth cones and therefore are likely to have a higher surface-to-volume ratio. Hence, one would expect a more rapid decline in [Ca\(^{2+}\)](i) at the end of a high [K\(^{+}\)](o) pulse in phasic GCs, which is not seen.

**Ca\(^{2+}\) extrusion by Na/Ca exchange and the Ca\(^{2+}\) ATPase**

When Ca\(^{2+}\) extrusion by the Na/Ca exchanger was inhibited with a 0 Na\(^{+}\) wash after a depolarizing pulse, [Ca\(^{2+}\)](i) decay was delayed for both tonic and phasic GCs, thus demonstrating the important role played by Na/Ca exchange in Ca\(^{2+}\) clearance. These results are consistent with immunocytochemistry, which shows high expression of the exchanger in GCs of cultured neurons from *Xenopus laevis* (Luther et al. 1992) and rat hippocampus (Juhaszova et al. 1996).

In phasic GCs, Na/Ca exchange seems to be the only mechanism for effectively extruding large loads of Ca\(^{2+}\) since [Ca\(^{2+}\)](i) did not usually decline to near resting levels until 0 Na\(^{+}\) wash was replaced with normal saline wash. Alternatively, [Ca\(^{2+}\)](i) in tonic GCs often declined substantially in the 0 Na\(^{+}\) wash, apparently due to Ca\(^{2+}\) extrusion by the plasma membrane Ca\(^{2+}\) ATPase. When both the Na/Ca exchange and the Ca\(^{2+}\) ATPase were inhibited in tonic GCs, [Ca\(^{2+}\)](i) did not decline after the high [K\(^{+}\)](o) pulse. The Ca\(^{2+}\) ATPase has a high affinity for Ca\(^{2+}\) but a low transport rate (the maximum transport rate of the Ca\(^{2+}\) ATPase is only about 1/50 of the Na/Ca ATPase) and therefore functions to adjust the final resting [Ca\(^{2+}\)](i) rather than remove large Ca\(^{2+}\) loads (Blaustein and Lederer 1999; Carafoli 1994; Hilgemann et al. 1991).

**FIG. 6.** Fura-2 ratio map showing changes in [Ca\(^{2+}\)](i) in tonic (left) and phasic (right) GCs. [Ca\(^{2+}\)](i) was measured in a series of low [Na\(^{+}\)](o) washes to control tonic and phasic GCs. Low [Na\(^{+}\)](o) reverses Na/Ca exchange, thus allowing Ca\(^{2+}\) entry through the Na/Ca exchanger. [Ca\(^{2+}\)](i) goes higher in tonic than phasic GCs. Top to bottom [Na\(^{+}\)](o), (mM): 206, 140, 80, 40, 20, 0, and 206 (scale bar = 50 μm).

**FIG. 7.** Increase in [Ca\(^{2+}\)](i) for tonic and phasic GCs. Increase in [Ca\(^{2+}\)](i), due to Ca\(^{2+}\) entry through reverse Na/Ca exchange was monitored in series of 300-s low [Na\(^{+}\)](o) pulses. [Ca\(^{2+}\)](i), from images acquired in last 150 s of each [Na\(^{+}\)](o) pulse was averaged and plotted. For all [Na\(^{+}\)](o), the increase in [Ca\(^{2+}\)](i) was significantly greater for tonic than phasic GCs. The number of GCs varied from 12 to 30 in 7–17 culture dishes. *P < 0.001.


Ca2+ uptake/release by mitochondria is more prominent in phasic than tonic GCs

Ca2+ sequestration by mitochondria can occur in neurons during Ca2+ influx and can play an important role in clearance of Ca2+ loads (Herrington et al. 1996; Thayer and Miller 1990; White and Reynolds 1995). Release of Ca2+ from mitochondria during [Ca2+]i decay results in a shoulder in the decay curve (Werth and Thayer 1994), as seen in the phasic GCs. We found that blocking mitochondrial Ca2+ uptake with Antimycin A1 and CCCP eliminated the shoulder in the [Ca2+]i decay curve. It appears that mitochondria temporarily sequester Ca2+ when [Ca2+]i goes high in phasic GCs and subsequently release it during decay. This probably influences the peak and initial decay of [Ca2+]i in phasic GCs, although the role of mitochondria in limiting the increase in [Ca2+]i was most apparent when the Na/Ca exchanger was inhibited. In this case, [Ca2+]i went higher in the presence of Antimycin A1 than in its absence. The most prominent effect of Ca2+ uptake/release by mitochondria was the prolongation of the Ca2+ signal in phasic GCs. This contributes to the large difference in time (T1/2) for [Ca2+]i, decay in phasic and tonic GCs; however, it is not completely responsible for the differences, since in the presence of Antimycin A1, the decay of [Ca2+]i, is still slower in phasic than tonic GCs.

It appears that mitochondrial Ca2+ uptake is greater in phasic GCs than tonic ones. The weaker Ca2+ extrusion mechanisms in phasic GCs might lead to greater Ca2+ uptake by mitochondria. During the high [K+] depolarization, [Ca2+]i goes higher in phasic than tonic growth cones, possibly due to their weaker Ca2+ extrusion; higher [Ca2+]i could lead to greater Ca2+ uptake by phasic GC mitochondria. A less likely alternative is that tonic and phasic mitochondria sequester similar amounts of Ca2+, but strong Ca2+ extrusion from tonic GCs limits the effect of mitochondrial Ca2+ release on [Ca2+]i. Regardless, the relative contribution of Ca2+ extrusion and mitochondrial Ca2+ uptake/release is different for the phasic and tonic GCs. (It is unlikely that differences in mitochondrial density contribute to differences in Ca2+ uptake since tonic motor terminals and axons have a higher density of mitochondria than phasic ones; Lnenicka et al. 1986, 1998b; Nguyen et al. 1997).

Relative importance of Ca2+-clearance mechanisms

Previous studies have demonstrated cell-specific differences in Ca2+-clearance mechanisms, e.g., cell bodies of rat nucleus basalis neurons show strong Ca2+ clearance by Na/Ca exchange (Tatsumi and Katayama 1993), whereas cell bodies of rat DRG neurons (Werth and Thayer 1994) have prominent mitochondrial Ca2+ uptake. For motor axon GCs, we show that the relative importance of Ca2+ extrusion and mitochondrial Ca2+ uptake/release is correlated with the level of impulse activity: GCs with higher impulse activity have stronger Ca2+ extrusion and less prominent mitochondrial Ca2+ uptake/release. The prior level of impulse activity may directly contribute to these neuron-specific differences. Neurons developing with high impulse activity could develop stronger Ca2+-extrusion mechanisms. Earlier findings have demonstrated that GCs developed weaker Ca2+ clearance when impulse activity was eliminated: after growing tonic axons in TTX, their GCs showed a greater increase in [Ca2+]i when exposed to a Ca2+ ionophore (Lnenicka et al. 1998a). Also, in vivo stimulation strengthens Ca2+ clearance in mature crayfish phasic motor axons (Fengler and Lnenicka 2002).

Activity-dependent gene expression leading to long-term changes in neuron structure and function is an important feature of development (Brosenitsch and Katz 2001; Fields 1998; Finkbeiner and Greenberg 1998). High Na/Ca exchange activity in tonic GCs could result from a higher density of Na/Ca exchanger produced by activity-dependent changes in gene expression. In fact, increased activity has been shown to up-regulate Na/Ca exchange expression in cardiac myocytes: an increase in Na+ influx produces a secondary increase in [Ca2+]i and increased expression of the Na/Ca exchanger (Kent et al. 1993).

The relative contribution of Na/Ca exchange and mitochondria to Ca2+ clearance could have important functional consequences. For example, Ca2+ clearance from crayfish motor terminals, like crayfish motor axon GCs, can involve Na/Ca exchange and mitochondria (Mulkey and Zucker 1992; Zhong et al. 2001). The relative contribution of Na/Ca exchange and mitochondria for clearing large Ca2+ loads from these motor terminals could influence transmitter release. For instance, greater mitochondrial Ca2+ uptake/release could result in

Reversal of Na/Ca exchange provides evidence for greater Na/Ca exchange activity in tonic than phasic GCs

The Na/Ca exchanger has been shown to operate in the Ca2+ entry mode in a number of neurons including lobster motor axons (Peterson et al. 1984). On reversing the Na/Ca exchange, the increase in [Ca2+]i produced by lowering [Na+]o is much larger in tonic than phasic GCs, indicating that there is greater influx of Ca2+ into tonic GCs, presumably due to greater Na/Ca exchange activity. Alternatively, the other Ca2+-clearance mechanisms could be stronger in phasic GCs, resulting in a smaller increase in [Ca2+]i. The latter possibility seems unlikely since we have already shown that Ca2+ ATPase is more effective in removing Ca2+ from tonic than phasic GCs. In addition, GCs with slower [Ca2+]i decay after a high [K+] pulse generally had less Ca2+ influx during low Na+ pulses; low Na/Ca exchange activity would produce both results.

The increase in [Ca2+]i produced by 0 [Na+]o in this study is larger than that seen in previous studies of neuronal cell bodies (Bleakman et al. 1993; Duchen et al. 1990; Kennedy and Thomas 1995; Werth and Thayer 1994; White and Reynolds 1995) and motor terminals (Mulkey and Zucker 1992). This may reflect a particularly high Na/Ca exchange activity at tonic GCs and/or their high surface area to volume. Alternatively, the large increase in [Ca2+]i, seen in these GCs could result from applying a series of low Na+ pulses. In fact, we found that when the 0 Na+ pulse was preceded by a series of low Na+ pulses, the increase in [Ca2+]i was larger (1.18 ± 0.18 μM; n = 22, 13) than for a single 0 Na+ pulse (0.39 ± 0.10 μM; n = 6, 5) in tonic GCs. It may be that the prolonged increase in [Ca2+]i, "primed" the Na/Ca exchanger. Previous studies show that the Ca2+-entry mode of Na/Ca exchange is activated by [Ca2+]i, i.e., increases in [Ca2+]i, have a positive feedback on Ca2+ entry through the Na/Ca exchanger (Dipolo and Beaugé 1987; Hilgenmann et al. 1992; Rasgado-Flores et al. 1989).
stronger posttetanic potentiation (PTP), since mitochondrial Ca\(^{2+}\) release after long trains of impulses contributes to the production of PTP at crayfish motor terminals (Tang and Zucker 1997).

Environmental cues can influence advancing GCs by altering [Ca\(^{2+}\)]; e.g., impulse activity and neurotransmitters have been shown to influence the rate and direction of growth cone advance by regulating [Ca\(^{2+}\)]. (Cohan and Kater 1986; Ming et al. 2001; Zhang and Poo 2001). During development or regeneration, it seems likely that phasic and tonic GCs would respond differently to environmental cues due to differences in their Ca\(^{2+}\)-clearance mechanisms.

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