Activity-Dependent Plasticity of Calcium Clearance From Crayfish Motor Axons

BRIAN T. FENGLER AND GREGORY A. LNENICKA
Department of Biological Sciences, State University of New York, Albany, New York 12222

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Fengler, Brian T. and Gregory A. Lnenicka. Activity-dependent plasticity of calcium clearance from crayfish motor axons. J Neurophysiol 87: 1625–1628, 2002; 10.1152/jn.00487.2001. Previous studies of a crayfish explant culture demonstrated that regenerating motor axons with high impulse activity develop more rapid clearance of cytoplasmic free Ca2+ than those with low impulse activity. We examined whether Ca2+ clearance in mature axons also showed activity-dependent plasticity. We studied the phasic and tonic axons of the motor bundle innervating the crayfish closer muscle that display large differences in impulse activity. To compare their Ca2+ regulation, we applied the Ca2+ ionophore Br-23187 (1 μM) and measured the increase in intracellular free Ca2+ concentration ([Ca2+]i) with fura-2. After 55 min of ionophore application, the increase in [Ca2+]i in the phasic axons (1.326 ± 192 nM) was significantly greater than in the tonic axons (359 ± 148 nM). This resulted from stronger Ca2+ clearance in the tonic axon rather than less Ca2+ influx because blocking Ca2+ clearance by Na/Ca exchange and mitochondria eliminated these differences in [Ca2+]i. Next we determined whether Ca2+ clearance from the phasic axon could be strengthened by a prolonged increase in impulse activity. The phasic axon was stimulated in vivo at 5 Hz for 1 h/day for 5 days, and 1–3 days after stimulation, Ca2+ clearance was again examined. After 55 min of Br-23187 (1 μM) exposure, the increase in [Ca2+]i in the stimulated phasic axon was only 232 ± 123 nM, which was much less than in the control phasic axons and similar to that in the tonic axons. Thus Ca2+-clearance mechanisms adapt to changes in impulse activity both in growing and mature axons.

INTRODUCTION

Intracellular Ca2+ plays a fundamental role in the development and regulation of neuronal structure and function. For example, the intracellular free Ca2+ concentration ([Ca2+]i) regulates transmitter release (Katz 1969), membrane excitability (Turrigiano et al. 1994), gene transcription (Morgan and Curran 1991), and neuronal growth and survival (Mills and Kater 1990). Increases in [Ca2+]i are produced by Ca2+ influx through voltage- or ligand-gated channels and release from intracellular organelles; free Ca2+ is cleared by uptake into intracellular organelles, chelation by Ca2+-binding proteins, and extrusion across the plasma membrane (Miller 1991). Given the paramount role of intracellular Ca2+ in many cellular processes, it is important to understand the development and regulation of the mechanisms that control [Ca2+]i.

We have previously shown that impulse activity plays a role in the development of Ca2+-clearance mechanisms. In crayfish explant cultures, regenerating tonic axons, which have high impulse activity, develop stronger Ca2+ clearance than inactive phasic axons (Lnenicka et al. 1998a). The development of this difference is activity-dependent because eliminating impulse activity in regenerating tonic axons reduced their Ca2+-clearance capacity. These differences in Ca2+ clearance influence the response of the advancing axon to Ca2+ influx (Arcaro and Lnenicka 1997) and may influence the subsequent transmitter-releasing properties of the motor terminals.

We examined whether these activity-dependent differences in Ca2+ regulation in growing motor axons are maintained in the adult nervous system and whether Ca2+ clearance in mature axons can be strengthened by increased impulse activity. The phasic and tonic axons innervating the closer muscle are an ideal preparation for these studies: they are easily identified and have large differences in impulse activity (Pahapill et al. 1985). The axons can be isolated and positioned in a perfusion chamber for imaging (Lnenicka et al. 1998b), and the phasic axon can be selectively stimulated in vivo over a period of days to weeks (Lnenicka and Atwood 1985). Previous studies of these axons have demonstrated that chronic stimulation of the phasic axon transforms its motor terminal structure and transmitter-releasing properties so that they become more similar to those of the tonic motor axon (Lnenicka and Atwood 1985; Lnenicka et al. 1986). We found that the tonic axon showed stronger Ca2+ clearance than the phasic one and chronic stimulation of the phasic axon strengthened its Ca2+ clearance.

METHODS

Motor axon isolation

Crayfish (Procambarus clarkii) were obtained from Atchafalaya Biological Supply (Raceland, LA) and maintained in shallow, aerated tanks. Crayfish with carapace lengths 4.5–5.4 cm and claw lengths 3.4–4.6 cm were used in these studies. There are three axons in the motor bundle that innervates the crayfish claw closer muscle: the large phasic excitor, the intermediate-sized tonic excitor, and the small inhibitor (Van Harreveld and Wiersma 1936). As in a previous study (Lnenicka et al. 1998b), the claw nerve was exposed in the carpodite and meropodite. A length of motor bundle, approximately 2.5 cm, was removed from the claw, and placed on a poly-l-lysine-coated coverslip. The coverslip was mounted in a gravity-flow perfusion chamber (RC-21B, Warner Instrument, Aamden, CT), which was
filled with crayfish saline (pH 7.4), containing (in mM) 13.5 CaCl₂, 2.5 MgCl₂, 5.3 KCl, 206.0 NaCl, 1 d-glucose, and 10 Na-HEPES plus 0.2 μM TTX. The axons were visualized with a ×40 objective (Nikon Fluor; NA 1.3) on an inverted microscope (Nikon Diaphot 200) using DIC optics.

Measurement of Ca²⁺ clearance

Crayfish saline containing 2 μM fura-2 AM (Molecular Probes, Eugene, OR) was added to the perfusion chamber and the axons were incubated in the dark for 50–60 min. The axons were then perfused with crayfish saline followed by saline containing the Ca²⁺ ionophore Br-A23187 (1 μM) for a total of 60 min. This solution was prepared by adding 1 mM Br-A23187 (Molecular Probes) in DMSO to the saline and sonicating it. [Ca²⁺], was measured every minute by ratio imaging of fura-2 fluorescence as previously described (Lnenicka et al. 1998a). The fura-2 fluorescence ratio (340:380) was used to estimate [Ca²⁺], using the standard equation (Gryniewicz et al. 1985), a 0.7 viscosity correction factor, a fura-2 Kd of 865 nM, and Rmin and Rmax values were determined in vitro (Delaney et al. 1991). Values of [Ca²⁺], were compared using a Student’s t-test.

Although both axons and surrounding glial cells were likely loaded with fura-2, measurements of axonal [Ca²⁺], do not appear to be influenced by the signals from the glia. The intensity of the fura-2 fluorescence from the intervening glia was much less than from the axons, and when there were large increases in the fura-2 fluorescence ratio [Ca²⁺], in the phasic axon, the ratio dropped sharply at the axon-glia border.

To block Na/Ca exchange, external Na⁺ was replaced with N-methyl-D-glucamine (Blaustein and Lederer 1999). Mitochondria were inhibited with the oxidative-phosphorylation uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). CCCP rapidly collapses the H⁺ gradient across the inner mitochondrial membrane and prevents Ca²⁺ uptake (Miller 1991).

In vivo stimulation of phasic motor axons

The implantation of electrodes for in vivo stimulation of the phasic axon was performed as previously described (Lnenicka and Atwood 1985). The phasic axon was stimulated at 5 Hz for 1 h/day for 5 days. Although the tonic axon may also have been stimulated, the increase in impulse activity was much greater for the phasic axon than for the tonic one. The Ca²⁺-clearance capacity of these phasic axons was examined 1–3 days after the final stimulation period.

RESULTS

Tonic axons show stronger Ca²⁺ clearance than phasic axons

We compared Ca²⁺ clearance in the phasic and tonic axons innervating the claw closer muscle. These axons have dramatic differences in impulse activity: the tonic and phasic axons fire ~6,000 and 1 imp/h, respectively (Pahapill et al. 1985). To examine Ca²⁺ clearance, the Ca²⁺ ionophore, Br-A23187 was added to the axons, and the increase in [Ca²⁺] was measured. At the beginning of the experiment, resting [Ca²⁺] was similar in phasic (206 ± 51 nM, n = 6) and tonic axons (177 ± 49 nM; n = 7). After 5 min, the normal saline was replaced by saline containing 1 μM Br-A23187, and we continued to measure [Ca²⁺] every minute for the next 55 min (Fig. 1, top). For every claw, [Ca²⁺] was found to increase more rapidly in the phasic axon than in the tonic one (Fig. 2). The final increase in [Ca²⁺] at the end of 55 min of ionophore application was significantly higher in phasic axons (1,326 ± 192 nM; n = 6) than in tonic axons (359 ± 148 nM; n = 7; P = 0.002). (One phasic axon was removed from the study due to high resting [Ca²⁺], indicating that the axon was damaged.)

The smaller increase in [Ca²⁺] in the tonic axon is either due to stronger Ca²⁺ clearance or less Ca²⁺ influx compared with the phasic axon. To determine whether Br-A23187 produces similar Ca²⁺ influx in the two axons, we blocked Ca²⁺ clearance and compared the [Ca²⁺] increase in phasic and tonic axons. Experiments were performed as before except the major mechanisms for clearing large Ca²⁺ loads, mitochondrial Ca²⁺ uptake, and Na/Ca exchange (Miller 1991) were blocked by adding 1 μM CCCP and replacing extracellular Na⁺ with N-methyl-D-glucamine before adding 1 μM Br-A23187. For each experiment (n = 3), the increase in [Ca²⁺] was greater in the tonic than the phasic axon, and overall it was 19 ± 8% greater in the tonic than in the phasic axon. The final [Ca²⁺] in tonic axons was 1,483 ± 295 and 1,236 ± 107 nM in phasic axons. Thus the tonic axons have stronger Ca²⁺ clearance mechanisms than the phasic axons not less Ca²⁺ influx.

Increased impulse activity strengthens Ca²⁺ clearance in phasic axons

The phasic axon was stimulated in vivo to determine whether Ca²⁺ clearance is strengthened by increased impulse...
The smaller increase in Ca\(^{2+}\) axons than in tonic ones after 55 min of Br-A23187 application. Its Ca\(^{2+}\) measuring the increase in Ca\(^{2+}\) were added at 10-min intervals. In control claws, tonic axons clearly have a stimulated claw. Data from 1-min intervals were averaged, and error bars in tonic axons from stimulated and control claws (\(n = 8\)).

**DISCUSSION**

**Tonic axons have a greater capacity for Ca\(^{2+}\) removal than phasic axons**

The increase in [Ca\(^{2+}\)] was 3.7 times greater in phasic axons than in tonic ones after 55 min of Br-A23187 application. The smaller increase in [Ca\(^{2+}\)] seen in tonic axons cannot be due to differences in axon size. The smaller diameter of the tonic axon should give it a 29% greater surface/volume than the phasic axon (Lnenicka et al. 1998b), thus favoring a higher rate of increase in [Ca\(^{2+}\)], for the tonic axon. In addition, these differences are not due to Br-A23187 producing greater Ca\(^{2+}\) influx in phasic than in tonic axons. When Ca\(^{2+}\) clearance was blocked, the increase in [Ca\(^{2+}\)], was 19% greater in the tonic than in the phasic axons. (The greater increase in [Ca\(^{2+}\)], in tonic axons probably reflects its greater surface/volume.) Thus the differences in Ca\(^{2+}\) clearance seen in regenerating phasic and tonic motor axons (Lnenicka et al. 1998a) are also seen in the adult nervous system. In addition, this result is consistent with studies of crayfish motor terminals where tonic terminals appear to extrude Ca\(^{2+}\) more rapidly than phasic ones (Mugnana et al. 1999).

The plasma membrane Na/Ca exchanger and mitochondria play an important role in the clearance of large Ca\(^{2+}\) loads, such as those produced by a Ca\(^{2+}\) ionophore. The exchanger does not appear to establish resting [Ca\(^{2+}\)], rather it appears to be specifically activated by high levels of [Ca\(^{2+}\)], and has a high capacity for Ca\(^{2+}\) extrusion (Blaustein and Lederer 1999). The Na/Ca exchanger is found in the squid giant axon (Baker et al. 1969), and we have demonstrated Na/Ca exchange activity in regenerating crayfish axons (G. A. Lnenicka and N. Rumpal, unpublished observations). Thus we assume that the crayfish axons used in this study have Na/Ca exchange activity, which could play a major role in regulating [Ca\(^{2+}\)], during application of the Ca\(^{2+}\) ionophore. The plasma membrane Ca\(^{2+}\)-ATPase also extrudes Ca\(^{2+}\); however, it has a lower transport rate than the exchanger (Carafoli 1992).

Mitochondria could also play an important role in Ca\(^{2+}\) clearance from crayfish motor axons. Mitochondrial Ca\(^{2+}\) uptake has been shown to play a role in clearing large Ca\(^{2+}\) loads from neurons (Werth and Thayer 1994). Because the tonic axons have twice the mitochondrial density of the phasic axons (Lnenicka et al. 1998b), mitochondria could be involved in the differences in Ca\(^{2+}\) regulation, if not through direct Ca\(^{2+}\) clearance, then indirectly through the production of ATP. Maintenance of ATP levels would be important for driving the Ca\(^{2+}\)-ATPase and sustaining the Na\(^+\) gradient for operation of the Na/Ca exchanger.

Control experiments where Na/Ca exchange and mitochondria were inhibited indicate that one or both play an important role in Ca\(^{2+}\) clearance from the tonic axon. The inhibitors appeared to have no effect on Ca\(^{2+}\) clearance from the phasic axon: the final [Ca\(^{2+}\)], was similar in the presence and absence of the inhibitors. Although these results are preliminary, this suggests that the phasic axon has very little capacity to clear large Ca\(^{2+}\) loads; this would not be surprising considering that it normally has very low impulse activity.

**Activity-dependent plasticity of Ca\(^{2+}\) clearance**

In vivo stimulation of the phasic motor axon for 5 days significantly increased the Ca\(^{2+}\)-clearance capacity of the phasic axon. In fact, the increase in [Ca\(^{2+}\)], in the stimulated phasic axon during application of Br-A23187 was similar to that in the tonic axon. This dramatic activity-dependent change in Ca\(^{2+}\) clearance in adult axons is consistent with a previous study of growing axons where Ca\(^{2+}\) clearance was weakened by reduced impulse activity (Lnenicka et al. 1998a). Thus the mechanisms for Ca\(^{2+}\) clearance appear to be particularly sen-

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itive to changes in impulse activity both in developing and mature axons.

An activity-dependent increase in mitochondrial density could play a role in producing stronger Ca\(^{2+}\) clearance. One week of stimulation of the phasic axon was shown to increase its mitochondrial density so that it became similar to that of the tonic axon (Lnenicka et al. 1998b). As discussed in the preceding text, greater mitochondrial density could lead to stronger Ca\(^{2+}\) clearance. In addition, the increased impulse activity could result in greater Na/Ca exchange activity. In cardiac muscle, increased activity resulting in an increase in Na influx and elevated [Ca\(^{2+}\)], produced an increase in the expression of the Na/Ca exchanger (Kent et al. 1993).

An activity-dependent change in Ca\(^{2+}\) clearance could influence a broad range of neuronal processes including growth, firing properties, transmitter release, and susceptibility to Ca\(^{2+}\) neurotoxicity (Chitwood and Jaffe 1998; Choi 1988; Mills and Kater 1990). For example, posttetanic potentiation (PTP) at crayfish motor terminals appears to be dependent on the buildup of residual Ca\(^{2+}\) (Delaney et al. 1989). PTP is produced by lower frequencies of stimulation in phasic terminals than in tonic ones (Pahapill et al. 1987), and chronic stimulation of the phasic axon to the closer muscle reduces the magnitude of PTP (Pahapill et al. 1986). This might be due to stronger Ca\(^{2+}\)-clearance mechanisms in tonic and stimulated phasic terminals compared with control phasic terminals. If the “normal” level of motoneuron impulse activity changed, activity-dependent changes in Ca\(^{2+}\) clearance could reset the amount of impulse activity required to produce PTP; this might occur during seasonal changes in motor activity (Lnenicka and Zhao 1991).

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REFERENCES


