Intracellular EDTA Mimics Parvalbumin in the Promotion of Skeletal Muscle Relaxation

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ABSTRACT   Parvalbumin (PA) is an intracellular Ca^{2+}-binding protein found in some muscle and nerves. Its ability to bind Ca^{2+} and facilitate skeletal muscle relaxation is limited by its Mg^{2+} off-rate. EDTA serves as an "artificial" PA in that it exhibited similar rate constants for Mg^{2+} ($3 \ s^{-1}$) and Ca^{2+} ($0.7 \ s^{-1}$) dissociation at 10°C. When introduced into frog skeletal muscle, EDTA increased the relaxation rate by ~2.7-fold, and with increasing tetanus duration, EDTA lost its ability to contribute to relaxation (and Ca^{2+} sequestration) at its Mg^{2+} off-rate. Intracellular EDTA recovered its ability to contribute to muscle relaxation and Ca^{2+} sequestration at its Ca^{2+} off-rate. Like PA, EDTA's contribution to muscle relaxation and Ca^{2+} sequestration was more clearly observed when the SR Ca-ATPase was inhibited. Introduction of EDTA into rat soleus muscle, which has low [PA], increased the relaxation rate in a manner that was analogous to the way in which PA facilitates relaxation of frog skeletal muscle. Thus intracellular EDTA serves as an effective mimic of PA, and its use should aid in our understanding of PA's function in muscle and nerve.

INTRODUCTION

Parvalbumin (PA) is an 11-kDa cytosolic Ca^{2+}-binding protein that binds two Ca^{2+} ($K_d = 5–20 \times 10^{-9}$ M) or two Mg^{2+} ($K_d = 1–2 \times 10^{-4}$ M) per molecule (Potter et al., 1977, 1980; Haiech et al., 1979; Ogawa and Tanokura, 1986). PA is found in skeletal muscle and nerve, where it is a marker for fast muscle relaxation kinetics and fast-firing neurons, respectively (Heizmann, 1984; Kawaguchi et al., 1987).

Although PA has been shown to increase the rate of fall of the Ca^{2+} transient in motor neurons (Chard et al., 1993), its function has been studied most extensively in skeletal muscle. Briggs (1975) originally proposed that PA acts as a relaxing factor in skeletal muscle, and there is a positive correlation between the absolute speed of skeletal muscle contraction-relaxation and [PA] in various muscles across the animal kingdom and within a given animal (Heizmann, 1984). Gerday and Gillis (1976) observed that the Ca^{2+}-free form of PA could remove Ca^{2+} from troponin (Tn) and inhibit actomyosin ATPase activity of myofibrils and that sarcoplasmic reticulum (SR) could remove Ca^{2+} from PA. PA is thought to serve as a reversible Ca^{2+} buffer that can receive Ca^{2+} from TnC's Ca^{2+}-specific regulatory sites, to facilitate relaxation, until Ca^{2+} is resequestered by the SR (see Gillis, 1985, and Rall, 1996, for reviews).

PA binds Ca^{2+} with ~10,000-fold higher affinity than it binds Mg^{2+}. In the resting state of frog skeletal muscle, free $[Ca^{2+}]$ is low (100 nM) and free $[Mg^{2+}]$ is high (~1 mM), resulting in ~50% of the PA being saturated with Mg^{2+}

(Mg-PA) (Hou et al., 1991). While apo-PA can bind Ca^{2+} rapidly, Mg-PA cannot bind Ca^{2+} and contribute to relaxation until Mg^{2+} dissociates, and this occurs with a rate constant of $2–3 \ s^{-1}$ at 10°C and at $4–5 \ s^{-1}$ at 20°C (Hou et al., 1991, 1992; Jiang et al., 1996). Therefore, Mg-PA is a "slow" Ca^{2+} chelator, and it cannot initially compete with the rapid ($1.2 \times 10^8 \ M^{-1} \ s^{-1}$) Ca^{2+} on rate of the Ca^{2+}-specific sites of TnC (Johnson et al., 1994). Thus, during a rapid cellular Ca^{2+} transient, Ca^{2+} would first occupy TnC to initiate contraction instead of binding to PA. As PA loses Mg^{2+}, it would contribute to Ca^{2+} sequestration and relaxation.

The relaxation rate of frog skeletal muscle slows (about twofold) with increasing tetanus duration. Hou et al. (1991, 1992) have shown that this slowing of the relaxation rate occurs at the rate of Mg^{2+} dissociation from PA. After a long (≥ 1.2 s at 10°C) tetanus, PA is in equilibrium with Ca^{2+} and no longer contributes to Ca^{2+} sequestration and relaxation. Frog skeletal muscle can recover its fast relaxation rate with rest. Hou et al. (1991, 1992) have shown that the relaxation rate increases with the duration of the rest interval at the rate at which Ca^{2+} dissociates from PA (a rate constant of $\sim 0.5 \ s^{-1}$ at 10°C and $\sim 1.0 \ s^{-1}$ at 20°C).

PA's contribution to relaxation can be observed more clearly at low temperatures, where SR Ca-ATPase is less active (Gillis et al., 1982; Hou et al. 1992). Jiang et al. (1996) have shown that inhibition of the SR Ca-ATPase "unmasks" the relaxing effect of PA in frog skeletal muscle. When Ca^{2+} uptake by the SR was inhibited, Ca^{2+} sequestration and relaxation due to the putative effects of PA was clearly observed. Specifically, the relaxation rate became progressively slower in a series of consecutive twitches, and it decreased at the rate of Mg^{2+} dissociation from PA (Jiang et al., 1996). Muntener et al. (1995) have demonstrated that overexpression of PA, by direct injection of its cDNA into rat slow twitch skeletal muscle, produced about a twofold increase in relaxation rate. All of the above studies provide

support for PA being a soluble relaxation factor that is limited in its ability to sequester Ca^{2+} by its Mg^{2+} off rate.

The Ca^{2+} chelator EDTA binds Ca^{2+} ($K_d = 5.5 \times 10^{-8}$M) and Mg^{2+} ($K_d = 3.2 \times 10^{-6}$M) (Smith and Martell, 1989), and it can be loaded into living cells in its acetoxymethyl ester (AM) form. In this paper we show that intracellular EDTA can mimic many of the effects of PA in promoting the relaxation of skeletal muscle.

MATERIALS AND METHODS

Preparation of frog and rat skeletal muscle fiber bundles

Frogs (*Rana temporaria*) were purchased from Charles Sullivan (Nashville, TN) and kept at 3°C. They were sacrificed by decapitation and spinal cord destruction. Bundles of two to four fibers were dissected with intact membranes and tendons from the tibialis anterior muscle. Bundles were bathed in a solution containing (mM) 95 NaCl, 20 NaHCO₃, 2.5 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 11.1 glucose (pH 7.0). After dissection, small holes were made in the tendons as close as possible to the fibers. Fibers were mounted horizontally in an experimental chamber with one tendon attached to the hook of a force transducer (Kulite BG 10; Kulite Semiconductor Products, Ridgefield, NJ), and the other tendon was attached to a hook fixed to a micrometer. Temperature was maintained at 10°C or 20°C by circulating fluid through channels in a Perspex block that contained the chamber. The resting sarcomere length was determined by counting the number of striations in a 100-μm fiber length through a Leitz Diavert microscope at 400× and was set at 2.9 μm. After the control experiments, the bundles were loaded with EDTA by incubation with EDTA-AM as described in the figure legends.

Male Sprague-Dawley rats (170–230 g) were killed by decapitation as approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Bundles of ~40 fibers were dissected from the soleus muscle. These bundles were bathed in a physiological saline solution containing (mM) 121 NaCl, 5.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 24 NaHCO₃, and 5.5 glucose, gassed with 95% O₂/5% CO₂ at pH 7.3 and 20°C. These bundles were stretched to an optimal length for maximum twitch tension and equilibrated for 1 h, and then tension measurements were conducted as described for frog muscle. Nonfused tetani were induced by stimulation at 6.5 Hz, 20 V, with a pulse duration of 2 ms for 1.4 s, followed by a 7-min rest before subsequent contraction.

Experimental set-up for tension and intracellular Ca^{2+} measurements

Fiber bundles were stimulated in solution via a pair of platinum plate electrodes, which were mounted parallel to the bundle in the walls of the chamber. The rectangular pulse magnitude was 40 V/cm, and the pulse duration was 2 ms for a single twitch or an isometric tetanus. The frequency of stimulation for the frog fibers was at 2.5 Hz for nine consecutive twitches or 40 Hz for the tetani. Isometric force was recorded on a chart recorder and a PC workstation arranged in parallel.

The intracellular Ca^{2+} transients in frog fibers were monitored by using the Ca^{2+}-sensitive fluorescent dye fluo-3 (Molecular Probes, Eugene, OR). The fluorescent dye was loaded by incubating an intact fiber bundle for 1 h at 20°C in Ringer's solution containing 20 μM of the membrane-permeable ester form of fluo-3, fluo-3 AM. After loading, the fiber bundle was washed with dye free Ringer's solution and left for 20 min at 20°C to allow further hydrolysis of the dye into the Ca^{2+}-sensitive free acid form. The temperature was then maintained at 10°C or 20°C. After loading, the fluorescence was typically 2.9 times higher than the fluorescence of the unloaded fiber. Because no changes in background fluorescence were observed when the unloaded fiber was contracted, no subtractions for background fluorescence were necessary. No change was observed in the

time to peak force or in the time for 50% relaxation after loading of the fibers with fluo-3. Thus the amount of dye loaded in this procedure is enough to sense the change in Ca^{2+}, but not great enough to cause a change in the contractile response of the bundle. For the fluo-3 studies, the fibers were excited by passing light from a 75-W xenon lamp through a 490-nm narrow band-pass filter and then through a 515-nm dichroic mirror (Leitz 515 DRLP). Emitted light was detected after passing through the dichroic mirror and a 530-nm narrow bandpass filter. The current output from the photomultiplier tube was converted into voltage along with the simultaneously acquired tension response and displayed on the computer screen, using a Photon Technology (South Brunswick, NJ) dual-wavelength system. The data collection rate was set at 650 points/s. No attempt was made to calibrate the fluorescence signals in terms of [Ca^{2+}].

We found that the use of a 10× glass objective, with a 0.25-mm depth of field and a ~3 mm × ~1 mm field of view, reduced any effects of fiber motion on the fluorescence signal. This is perhaps because the same width and length of fiber would be seen through this objective throughout the contraction. The frog bundles were stretched slightly to a resting sarcomere length of 2.9 μm to further reduce motion during contraction and relaxation. We have previously shown that when 5 mM 2,3-butanedione monoxime was used to reduce the twitch force by 90%, similar fluo-3 fluorescence transients were observed, which indicates that motion did not interfere with the measurement of the Ca^{2+} transient (Jiang et al., 1996). After the chamber temperature reached the working temperature (10°C or 20°C), the fibers were allowed to equilibrate for 10 min before electrical stimulation. Twitches were elicited until twitch amplitude and kinetics were identical, and only then were data stored for analysis. Relaxation rates of all tetani were calculated from a linear fit of the isometric phase of relaxation, corresponding to a reduction in tension from 95% to 80% of peak.

Stopped-flow experiments

All of the stopped-flow experiments were done at 10°C or 20°C, using a thermostatically controlled Applied Photophysics model SF.17MV stopped-flow instrument with a mixing time of 1.6 ms.

Chemicals

Fluo-3 AM and EDTA-AM (EDTA tetraacetoxymethyl ester) were dissolved to 2 mM stock in dry dimethyl sulfoxide (DMSO) containing 20% pluronic acid. DMSO (1%) had no effect on tension or Ca^{2+} transients. The stock solutions were kept at −15°C until used and then diluted 100 times with Ringer's solution to a final concentration of 20 μM. 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ) (from Aldrich Chemical Co., Milwaukee, WI) was dissolved in dry DMSO to 4.3 mM, kept at −15°C before use, and diluted with Ringer's solution to the indicated concentration.

Statistics

All statistics are presented as means ± SEM for n observations. Tests of statistical significance were performed using Student's paired t-test with $p < 0.05$.

RESULTS

The transient occupancy of the N-terminal regulatory sites of TnC in the presence of Mg-EDTA

When Ca^{2+} rises in muscle, it binds to the regulatory sites of TnC before it binds to Mg-PA, because they have a very rapid Ca^{2+} on rate. As Mg^{2+} dissociates from PA, it can remove Ca^{2+} from TnC and contribute to Ca^{2+} sequestra-

tion and relaxation. We wished to determine whether Ca^{2+} would initially bind to the regulatory sites of TnC in the presence of Mg-EDTA and whether EDTA could subsequently remove Ca^{2+} from TnC after Mg^{2+} dissociation. To test this, in vitro, we have reacted Ca^{2+} with dansylaziridine-labeled TnC (TnC-danz) in the presence of increasing [Mg-EDTA]. TnC-danz undergoes a twofold increase in fluorescence, which is specific for Ca^{2+} binding to its regulatory sites (Johnson et al., 1978). This allowed us to determine whether Ca^{2+} could initially occupy these sites on TnC and then be subsequently chelated by EDTA. Fig. 1 shows the change in TnC-danz fluorescence when 200 μM Ca^{2+} (and 1 mM Mg^{2+}) is rapidly mixed with an equal volume of TnC-danz (and 1 mM Mg^{2+}) in the presence of increasing concentrations of EDTA. When Ca^{2+} is reacted with TnC-danz without EDTA, Ca^{2+} occupies the Ca^{2+}-specific sites, producing a maximum fluorescence (Fig. 1 *A*, *No EDTA trace*). When Ca^{2+} is reacted with an equal volume of TnC-danz in the presence of 400 μM EDTA (200 μM EDTA after mixing is complete), Ca^{2+} inititally binds to TnC, and then ~50% of it is removed at 9/s, resulting in a fluorescence decrease. When Ca^{2+} is reacted with an equal volume of TnC-danz in the presence of 700 μM EDTA (350 μM EDTA after mixing is complete), Ca^{2+} binds to TnC, and then ~82% of it is removed at 23/s (350 μM EDTA trace). When 600 μM EDTA is present in the reaction mixture, Ca^{2+} is completely removed from TnC at a rate of ~120/s (600 μM EDTA trace). As the concentration of Mg-EDTA is increased, there is less delay before the rapid fall in TnC-danz fluorescence, because Ca^{2+} is more
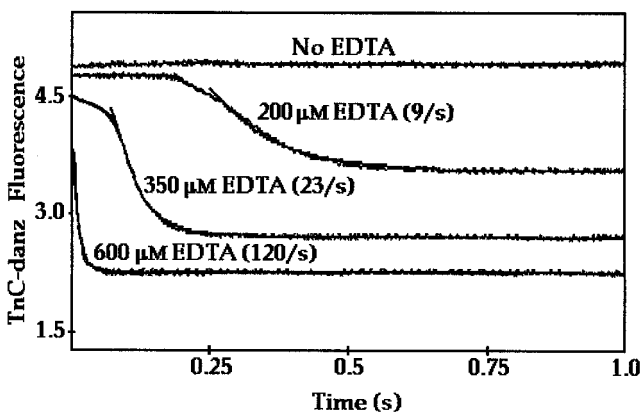
rapidly reduced from the 100 μM range to the low μM range, which is required for Ca^{2+} dissociation from TnC-danz. Furthermore, with increasing [Mg-EDTA], Ca^{2+} is removed from the Ca^{2+}-specific regulatory sites of TnC more completely and more quickly. Both of these phenomena are expected for a second-order reaction. Clearly, at concentrations of 350 μM or more, EDTA can remove Ca^{2+} from the regulatory sites of TnC, even in the presence of physiological concentrations of Mg^{2+}. In the absence of Mg^{2+}, EDTA was even more effective at rapidly removing Ca^{2+} from TnC-danz. EDTA (200 μM) removed all of the Ca^{2+} from TnC-danz at its Ca^{2+} off rate of 120/s at 10°C (data not shown).

These in vitro experiments suggest that in the presence of intracellular Mg-EDTA, a rapid release of the SR Ca^{2+} could result in the transient occupancy of the Ca^{2+}-specific regulatory sites of TnC and produce a transient contraction. Furthermore, like PA, Mg-EDTA should be able to promote relaxation, as it loses Mg^{2+} and chelates Ca^{2+}. In an effort to test this hypothesis in vivo, we have used the acetoxy methyl ester of EDTA (EDTA-AM) to introduce EDTA into intact skeletal muscle fibers and determine its effect on Ca^{2+} transients and tension.

Effect of EDTA on the time course of slowing of relaxation with increasing tetanus duration

It has been known for some time that the rate of relaxation in frog skeletal muscle slows with increasing tetanus duration. Hou et al. (1991, 1992) have shown that the relaxation rate slows as a function of increasing tetanus duration at the rate at which Mg^{2+} dissociates from PA (a rate constant of ~3 s^{-1} at 10°C). In an effort to see whether intracellular EDTA could mimic PA in this regard, we have determined the effect of increasing tetanus duration on the relaxation rate before and after incubation with EDTA-AM. Fig. 2 *A* shows typical force traces from a 0.1-s and a 4-s tetanus in the absence and presence of intracellular EDTA. Fig. 2 *B* shows the normalized relaxation of tension for these traces. For the control, the 0.1-s tetanus relaxed at a rate of 146 ± 13% s^{-1}, and the 4-s tetanus relaxed 1.9-fold more slowly at a rate of 78 ± 10% s^{-1}. The inset in Fig. 2 *B* shows that the rate of relaxation decreased exponentially with increasing tetanus duration with a rate constant of ~3.1 ± 0.25 s^{-1} for the control. After EDTA-AM treatment, the 0.1-s tetanus relaxed 3.0 times more quickly than the control, at a rate of 436 ± 20% s^{-1}, and the 4-s tetanus relaxed 1.4 times more quickly than the control at a rate of 107 ± 18% s^{-1}. After EDTA-AM treatment, the rate of relaxation slowed by about fourfold with increasing tetanus duration, with a rate constant of ~2.9 ± 0.23 s^{-1} (Fig. 2, *inset*). In the presence of intracellular EDTA, the relaxation rate is faster than the control at shorter tetanus duration, but at longer tetanus duration the rates of relaxation approach that of the control. These results suggest that intracellular EDTA mimics PA,
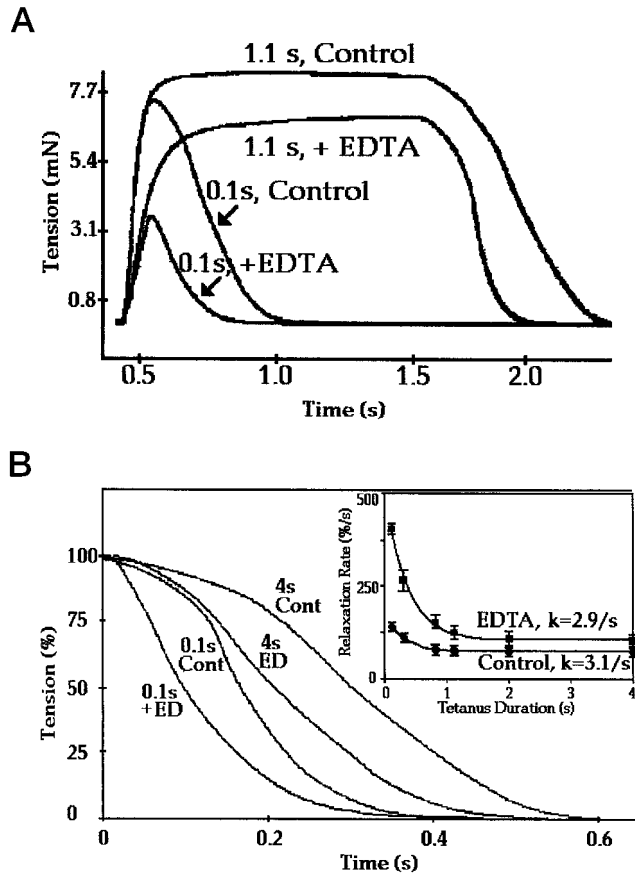

FIGURE 1   The transient occupancy of the Ca^{2+}-specific regulatory sites of TnC-danz in the presence of Mg-EDTA. The time course of the decrease in TnC-danz fluorescence is shown as Mg-EDTA dissociates Ca^{2+} from the Ca^{2+}-specific sites of TnC in the presence of various [Mg-EDTA]. A solution containing TnC-danz (2 μM) and 1 mM Mg^{2+} in the presence of increasing concentrations of EDTA (no EDTA, 400 μM, 700 μM, or 1.2 mM) in 200 mM 3-(*N*-morpholino)propanesulfonic acid, 90 mM KCl, was mixed with an equal volume of Ca^{2+} (200 μM) in the same buffer (with 1 mM Mg^{2+}) at pH 7.0 at 10°C. TnC-danz fluorescence was measured through a 510-nm broad band-pass filter, with excitation at 340 nm. Each trace was the average of seven traces and was fit with a single exponential with a variance of <5 × 10^{-5}. TnC-danz was prepared as described by Johnson et al. (1978).

if a sufficient rest period is allowed between a 1.2-s conditioning tetanus and the test tetanus (Hou et al., 1992). Hou et al. (1992) have previously shown that after a 1.2-s tetanus, the rate of relaxation of a brief test tetanus recovers with rest at a rate that is equal to the rate constant of Ca^{2+} dissociation from PA (0.5 s^{-1} at 10°C). Fig. 3 shows relaxation from a 0.29-s test tetanus delivered just as the 1.2-s conditioning tetanus relaxed to zero force (Cont 0 s) and after a 10-s rest (Cont 10 s) after the conditioning tetanus. With no rest, tension relaxed at 87 ± 5% s^{-1}, and after a 10-s rest, relaxation occurred at 122 ± 15% s^{-1}. The inset in Fig. 3 shows a plot of relaxation rate as a function of increasing rest interval. The relaxation rate increased as a function of increasing rest interval with a rate constant of 0.47 ± 0.14 s^{-1}. After a 2-h incubation with EDTA-AM (*ED traces* in Fig. 3), the 0.29-s tetanus relaxed 1.8 times more quickly than the control at 154 ± 13% s^{-1} with no rest and 1.7 times more quickly than the control at 208 ± 18% s^{-1} after a 10-s rest. EDTA reduced peak tension in the test tetanus by 23% in the fiber with no rest and by 47% in the fiber after a 10-s rest interval. The inset in Fig. 3 shows that in the presence of EDTA, the relaxation rate increased exponentially as a function of increasing rest interval, with a rate constant of 0.50 ± 0.22 s^{-1}. Thus, in the presence of intracellular EDTA, the rate of relaxation is faster at all rest intervals, and the rate of relaxation increases with increasing rest interval with a rate constant of 0.5 s^{-1}. Thus EDTA is similar to PA in that they exhibit similar rate constants for

FIGURE 2   (*A*) Typical force traces for a 0.1-s and a 4-s isometric tetanus before and after incubation with EDTA-AM. (*B*) Normalized force traces during relaxation of a 0.1-s or a 4-s isometric tetanus before and after incubation with EDTA-AM. The tetani were performed on three fiber bundles. The tension traces were recorded before and after (ED) a 2-h incubation with EDTA-AM (20 μM) at 10°C. The inset shows a plot of relaxation rates as a function of tetanus duration (0.1, 0.3, 0.8, 1.1, 2, and 4 s) before (control) and after (EDTA) incubation with EDTA. A tension of 100% corresponded to 7.4 mN for the 0.1-s control, 7.8 mN for the 4-s control, 4.1 mN for the 0.1-s EDTA, and 6 mN for the 4-s EDTA tension traces. Thus incubation with EDTA-AM reduced the peak tension of the 0.1-s and the 4-s tetani by 45% and 23%, respectively. Relaxation rates decayed as a single exponential process with rates of 3.1 ± 0.25 (*n* = 4) for the control and 2.9 ± 0.23 (*n* = 4) in the presence of EDTA. Nonnormalized data exhibited a similar decrease in relaxation rate with increasing tetanus duration (at ~3/s, for control and EDTA-treated fibers), but the relaxation rate of the EDTA-treated fiber remained significantly faster than the relaxation rate of the control, even after a 4-s tetanus.

which loses its ability to speed relaxation with increasing tetanus duration with a rate constant of ~3 s^{-1}. The fact that EDTA loses its ability to buffer [Ca^{2+}] and speed relaxation at the same rate as PA suggests that Mg^{2+} must dissociate from EDTA with a rate constant of ~3 s^{-1} at 10°C.

Effect of EDTA-AM on the recovery of relaxation rate with increasing rest interval

In frog skeletal muscle, the rate of relaxation slows with increasing tetanus duration, and the relaxation rate recovers
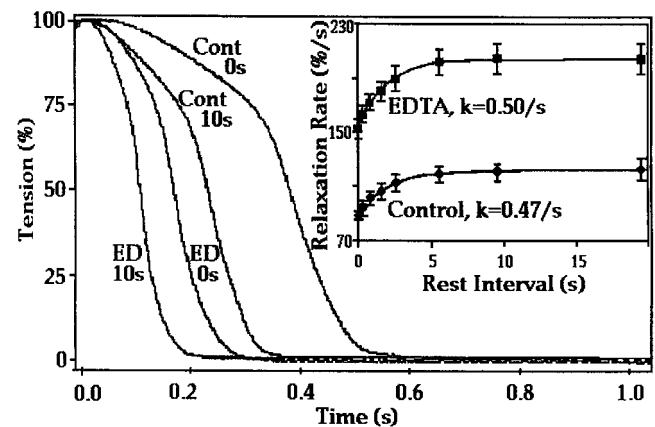
FIGURE 3   Effect of intracellular EDTA on the recovery of the relaxation rate with an increasing rest interval. Normalized force traces of relaxation from a 0.29-s test tetanus with no rest (0 s) and after 10 s of rest (10 s) after a 1.2-s conditioning tetanus are shown before and after (ED) a 2-h incubation with EDTA-AM (20 μM) at 10°C. The rest interval between the conditioning and test tetani was calculated as the period from complete relaxation of the conditioning tetanus to the initiation of the test tetanus. The inset shows a plot of relaxation rate as a function of increasing rest interval (0, 0.5, 1, 2, 3, 6, 10, and 20 s). These plots were fit with a single exponential equation and yielded recovery rate constants of 0.47 ± 0.14 s^{-1} (*n* = 3) and 0.50 ± 0.22 s^{-1} (*n* = 3) before and after incubation with EDTA-AM, respectively. A tension of 100% corresponds to 7.8 mN for the 0 and 10-s rest control and to 6.0 mN for the 0 s + EDTA and 4.1 mN for the 10-s + EDTA tension traces. Thus intracellular EDTA reduced peak tension by 23% and 47% for the no rest and 10-s rest tetani, respectively.

recovery of relaxation rate with a rest interval ($\sim$0.5 s^{-1} at 10°C).

Rates of Ca^{2+} and Mg^{2+} dissociation from EDTA and PA

The above studies suggest that EDTA can mimic PA in the muscle fiber in terms of its facilitation of relaxation. The data in Figs. 2 and 3 suggest that EDTA would have Mg^{2+} (3 s^{-1} at 10°C) and Ca^{2+} (0.5 s^{-1}) dissociation rate constants that are similar to those of PA.

Terbium (Tb) generally has a 1000-fold higher affinity for Ca^{2+} binding sites on Ca^{2+}-binding proteins than Ca^{2+} (Dockter, 1983). The increase in Tb fluorescence that occurs when Tb is reacted with a Ca^{2+}-binding protein or chelator in the presence of Ca^{2+} or Mg^{2+} provides a convenient means of determining Ca^{2+} or Mg^{2+} dissociation rates. Fig. 4 shows the rates of Mg^{2+} (Fig. 4 *A*) and Ca^{2+} (Fig. 4 *B*) dissociation from EDTA and frog PA (IVB isoform), using terbium (Tb) fluorescence. Fig. 4 *A* shows the increase in Tb fluorescence that takes place when EDTA (500 μM) or PA (20 μM) in the presence of Mg^{2+} (10 or 20 mM) is rapidly mixed with Tb (500 μM). Tb fluorescence increased at the rate of Mg^{2+} dissociation from EDTA or PA with a rate constant of 3 s^{-1} at 10°C. In Fig. 4 *B*, EDTA or PA was saturated with Ca^{2+} (500 μM) and then reacted with Tb. Tb fluorescence increased with the rate constant of Ca^{2+} dissociation from EDTA (0.7 $\pm$ 0.1 s^{-1}) or from PA (0.5 $\pm$ 0.1 s^{-1}) at 10°C. These kinetic experiments suggest that EDTA and PA are very similar with respect to their Mg^{2+} and Ca^{2+} off rates.

Relaxation by intracellular EDTA after inhibition of SR-Ca-ATPase

The relaxation rate in frog fibers depends on the combined action of SR Ca^{2+} uptake and Ca^{2+}-Mg^{2+} exchange with PA. We have previously shown that when SR Ca-ATPase is inhibited by TBQ, PA facilitates Ca^{2+} sequestration and relaxation of frog skeletal muscle until it is saturated with Ca^{2+} (Jiang et al., 1996). Fig. 5 shows the Ca^{2+} transients (Fig. 5 *A*) and tension (Fig. 5 *B*) for nine consecutive twitches before (control) and after treatment with TBQ. For the control the twitch Ca^{2+} transients and tension rose and fell rapidly in each consecutive twitch with a similar time course. In the presence of TBQ, the rate of fall in the Ca^{2+} transient for the first twitch was slowed $\sim$2.7-fold, resulting in a $\sim$2.4-fold increase in twitch tension and a $\sim$2.1-fold slower relaxation compared to control. With each subsequent twitch, the Ca^{2+} transient and tension fell more slowly as PA became saturated with Ca^{2+} (Jiang et al., 1996). After six or seven twitches, PA was essentially saturated with Ca^{2+}, and then Ca^{2+} sequestration and relaxation occured very slowly because of the residual Ca-ATPase activity. When this TBQ-treated muscle was incubated with EDTA-AM for 100 min (Fig. 5, TBQ + EDTA
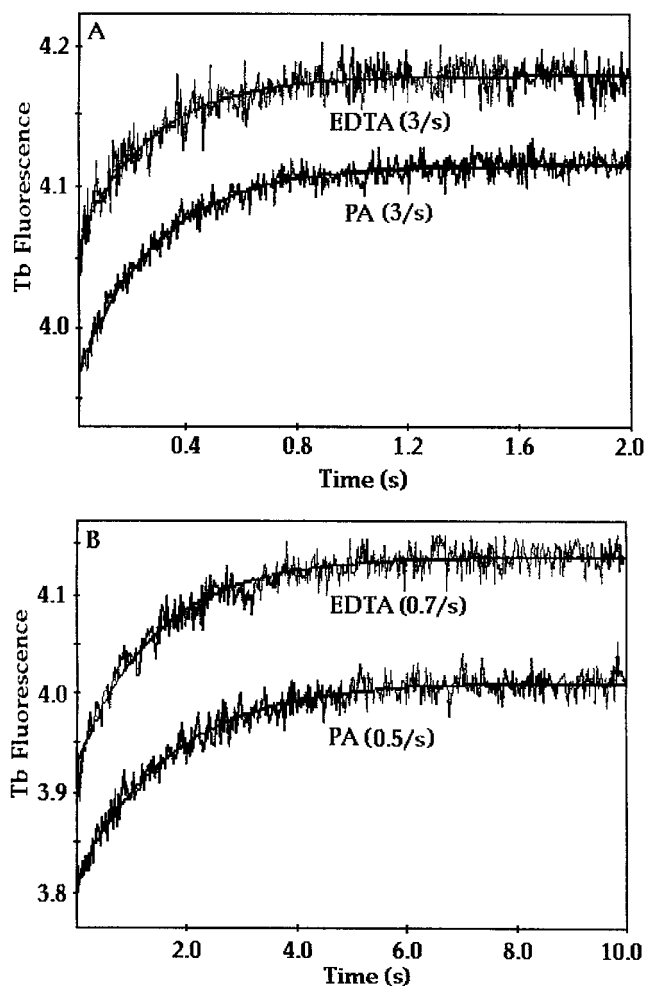

FIGURE 4 The time course of Mg^{2+} and Ca^{2+} dissociation from EDTA and purified PA. The time course of the increase in Tb^{3+} fluorescence is shown as Tb^{3+}-bound EDTA or purified PA saturated with Mg^{2+} (*A*) or Ca^{2+} (*B*). A solution containing EDTA (500 μM) or PA (20 μM) in the presence of Mg^{2+} (10–20 mM, respectively) in 10 mM 3-(*N*-morpholino)propanesulfonic acid and 90 mM KCl was rapidly mixed with an equal volume of Tb (500 μM) in the same buffer at pH 7.0 and 10°C. For the PA experiment, 50 μM EGTA was included to chelate contaminating Ca^{2+}. In *B* the conditions were identical, except that no EGTA was added to the PA solution and 500 μM Ca^{2+} was used (instead of Mg^{2+}) to saturate EDTA and PA. Tb^{3+} fluorescence was monitored through a 500-nm long-pass filter with excitation at 250 nm. Each trace is an average of eight experiments fit with a single exponential (variance < 1.98 × 10^{-4}).

trace), the Ca^{2+} transient and tension fell $\sim$1.5-fold and $\sim$1.3-fold more quickly, respectively, for the first twitch, and both the rate and the extent of their fall approached those observed in the control (non-TBQ-treated fibers). In subsequent twitches, the rate of fall in the Ca^{2+} transient and relaxation rate became progressively slower, as both PA and EDTA came into equilibrium with Ca^{2+}. The ninth twitch relaxed at a similar rate for the TBQ-treated and the TBQ + EDTA-treated fiber. Thus, when SR Ca-ATPase is inhibited and only PA is contributing to Ca^{2+} sequestration, the effect of intracellular EDTA on Ca^{2+} sequestration and relaxation is more evident. EDTA effectively mimics PA in
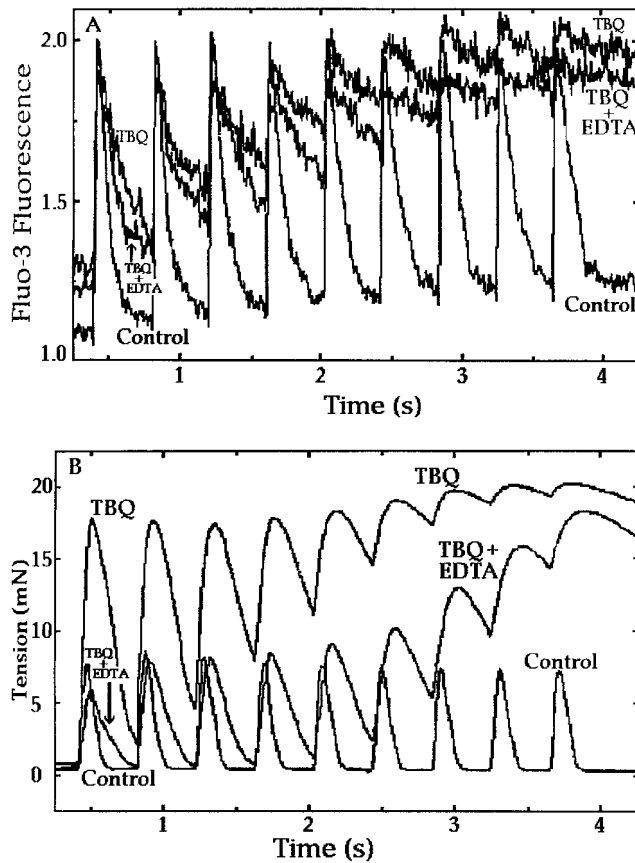
FIGURE 5   Records of fluo-3 fluorescence (*A*) and force (*B*) for nine consecutive twitches for a bundle of control fibers, fibers treated with TBQ, or fibers treated with TBQ and then with EDTA-AM. The TBQ trace was recorded after incubation of the control fibers with TBQ (2.5 μM) for 40 min, and the TBQ + EDTA trace was recorded after a 100-min incubation of the TBQ-treated fibers (still in the presence of TBQ) with EDTA-AM (40 μM). Each trace is an average of three measurements in a bundle of three fibers at 10°C. A 3-min rest period was given between series of consecutive twitches after TBQ treatment to let the SR refill with Ca^{2+} (see Jiang et al., 1996).

its facilitation of relaxation. Furthermore, intracellular EDTA can initially compensate for a partially inhibited SR Ca^{2+} ATPase by facilitating Ca^{2+} sequestration and relaxation.

Effect of EDTA on relaxation of mammalian slow twitch muscle

Mammalian slow twitch skeletal muscle has ~2000-fold lower [PA] than frog skeletal muscle (Heizmann, 1984; Hou et al., 1991), and we wished to determine the effect of intracellular EDTA on the relaxation of this muscle. Fig. 6 *A* shows the effect of EDTA on normalized force records for nine consecutive twitches of rat soleus muscle at 20°C. For the control, peak tension rose steadily with each consecutive twitch. The extent of relaxation decreased considerably with each subsequent twitch, but the rate of relaxation decreased only slightly. The first twitch relaxed ~40% of its peak
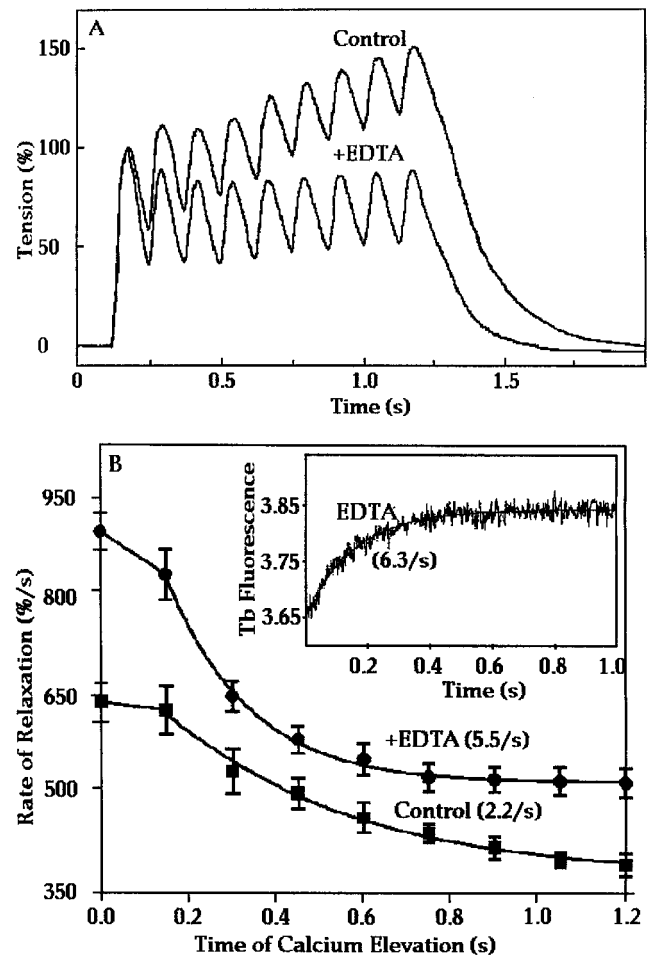
FIGURE 6   Effect of EDTA on relaxation in mammalian slow twitch muscle. (*A*) Normalized force traces for nine consecutive twitches of rat soleus muscle in the absence (Control) and presence of intracellular EDTA. A bundle of ~40 fibers was stimulated at 6.5 Hz for 1.4 s at 20°C. This bundle was then incubated with 20 μM EDTA-AM for 2 h, and the consecutive twitches were repeated. EDTA produced a 50% reduction in peak tension for the first twitch, and the tension traces were normalized for the first twitch tension. A tension of 100% corresponds to 27.4 mN. (*B*) A plot of the relaxation rate of these consecutive twitches is shown as a function of time of contraction for control and EDTA-treated fibers. The relaxation rate was determined by a linear fit of the fall in tension from its peak to the inititation of the subsequent twitch or of the corresponding region of the last twitch. Data are shown as mean ± SE for $n = 3$ experiments. The inset shows the time course of Mg^{2+} dissociation from EDTA at 20°C, using terbium fluorescence as described in Fig. 4.

height at a rate of 640% s^{-1}, whereas the eighth twitch relaxed only 20% of its peak height at a rate of 400% s^{-1}. After a 2-h incubation with EDTA-AM, peak tension increased less with each consecutive twitch, and the initial twitches exhibited greater and faster relaxation than the corresponding control twitches. For example, the first twitch relaxed by 60% of its peak height at a rate of 900% s^{-1}, whereas the eighth twitch relaxed by 40% of its peak height at a rate of 515% s^{-1}. Fig. 6 *B* shows a plot of relaxation rate of these nine consecutive twitches as a function of duration of tension elevation for the control and

EDTA-treated muscle. The rate of relaxation of the control decreases by ~1.6-fold, at a rate of 2.2 s^{-1}, as a function of duration of tension elevation. The rate of relaxation of the EDTA-treated muscle decreases by ~1.8-fold, at a rate of ~5.5 s^{-1}, as a function of duration of tension elevation. Thus the introduction of EDTA into slow twitch mammalian muscle speeds relaxation, and EDTA's ability to speed relaxation is lost with a rate constant of ~5.5 s^{-1} at 20°C. Fig. 6 B (inset) shows that Mg^{2+} dissociates from EDTA with a rate constant of 6.3 ± 0.4 s^{-1} at 20°C.

DISCUSSION

In the present study we have used EDTA as an "artificial" PA. In terms of K_d, EDTA binds Ca^{2+} ~5 times less tightly (50 nM) and Mg^{2+} ~50 times more tightly (3 μM) than PA. Our "in vitro" fluorescence stopped-flow studies with TnC-danz show that in the presence of 1 mM Mg, 300–600 μM EDTA can effectively remove Ca^{2+} from the regulatory sites of TnC (Fig. 1). Our stopped-flow experiments (Fig. 4) also demonstrate that Mg^{2+} dissociates from PA and EDTA with a rate constant of 3 s^{-1} and that Ca^{2+} dissociates from PA and EDTA with a rate constant of ~0.5–0.7 s^{-1} at 10°C. Thus PA and EDTA exhibit essentially identical rates of Ca^{2+} and Mg^{2+} dissociation. These in vitro results suggest that in a muscle cell, EDTA would be more saturated with Mg^{2+} than PA, but that it might still be able to remove Ca^{2+} from TnC and facilitate relaxation at a rate that was limited by its Mg^{2+} off rate. Thus intracellular EDTA could, perhaps, mimic PA's effects on skeletal muscle relaxation.

Because the Ca^{2+} affinity of chelators and TnC can be altered by the intracellular environment (Zhao et al., 1996; Guth and Potter, 1987) and [TnC] is higher in a muscle cell, it was necessary to test the hypothesis that intracellular EDTA can facilitate Ca^{2+} removal from TnC and relaxation with in vivo experiments. We loaded intact muscle fibers with EDTA and examined its effect on relaxation. We found that EDTA produced about a threefold faster relaxation from a 0.1-s tetanus. With increasing tetanus duration, the rate of relaxation decreased with a rate constant of 3 s^{-1}, which is equal to the Mg^{2+} off rate from EDTA (Fig. 2). These results predict that the intracellular free [Mg^{2+}] should increase during contraction at a rate equal to the Mg^{2+} dissociation rate from PA and EDTA. Indeed, [Mg^{2+}] has been shown to increase during a contraction in frog skeletal muscle at a rate equal to the Mg^{2+} dissociation rate from PA (Irving et al., 1989). Thus Mg-EDTA mimics Mg-PA in that they both can sequester Ca^{2+} and speed relaxation until they lose Mg^{2+} and come into equilibrium with Ca^{2+}. For both EDTA and PA this occurs with a rate constant of 3 s^{-1} at 10°C.

During a tetanus of ≥1 s duration, PA and EDTA are in equilibrium with Ca^{2+} and contribute less to relaxation. Their ability to bind Ca^{2+} and augment SR-induced relaxation can be recovered during a rest period. For both PA (in control fibers) and for EDTA (in EDTA-AM-treated fibers)

the relaxation rate recovers with increasing rest interval with a rate constant of ~0.5 s^{-1}, which is equal to the rate of Ca^{2+} dissociation from PA and EDTA (Fig. 3). Thus, in terms of both losing and recovering its ability to facilitate relaxation, EDTA mimics PA. This is as expected from the similarity of their Mg^{2+} and Ca^{2+} off rates.

The Ca^{2+} chelator EGTA has a slow Ca^{2+} on rate (1.2 × 10^6 M^{-1} s^{-1}; Smith et al., 1984) relative to the N-terminal regulatory sites of TnC (1.2 × 10^8; Johnson et al., 1994). We have previously shown that intracellular EGTA can shorten the duration of twitch Ca^{2+} transients, speed relaxation, and reduce twitch tension in frog skeletal muscle (Johnson et al., 1997). EDTA, like PA, must lose Mg^{2+} before it can sequester Ca^{2+}, and this occurs with a rate constant of 3 s^{-1} at 10°C. Because Mg^{2+}-EDTA must lose Mg^{2+} before it can sequester Ca^{2+}, it might be a slower Ca^{2+} chelator than EGTA and produce less reduction in twitch tension. Consistent with this suggestion, a 50% reduction in peak twitch tension is observed after 18 min of incubation with EGTA-AM and after 87 min of incubation with EDTA-AM. These 50% reductions in twitch tension were associated with a 1.7-fold and a 1.3-fold faster relaxation rate (relative to control) with EGTA and EDTA, respectively. Thus EGTA appears to be more effective at speeding twitch relaxation rate than EDTA, for a given reduction in twitch tension. Perhaps because of its slower Ca^{2+} association rate, Mg^{2+}-EDTA produces a more dramatic threefold increase in relaxation rate from a short 0.1-s tetanus than from a twitch. Although Mg-EDTA does speed twitch relaxation and reduce twitch tension, it appears to be kinetically tuned to facilitate relaxation from a short tetanus more than a twitch. Our studies suggest that if the same is true for PA, then PA might also reduce twitch and tetanus tension. This question should eventually be answered by PA knockout in transgenic animals.

Like intracellular EGTA, loading muscle with intracellular EDTA did not affect the height of the fluo-3 Ca^{2+} signal in a series of consecutive twitches (data not shown). This suggests that EDTA is not sequestering Ca^{2+} in the SR, and the cytosolic EDTA is not depleting SR Ca^{2+} stores. Thus intracellular EDTA, like PA, can serve as a "reversable" Ca^{2+} buffer to facilitate relaxation of skeletal muscle.

After TBQ inhibition of SR Ca-ATPase, only PA contributes to the relaxation rate in frog skeletal muscles (Jiang et al., 1996), and the ability of EDTA to mimic the relaxing effects of PA can be clearly visualized. Incubation of TBQ-treated fibers with EDTA produced a faster rate of fall in the Ca^{2+} transient and a more rapid and complete relaxation. Like PA, EDTA lost its ability to facilitate Ca^{2+} sequestration and relaxation with a rate constant of 3 s^{-1} at 10°C. Thus, in all of these regards, the addition of EDTA to a muscle cell is similar to increasing [PA].

One important way to characterize the function of a putative relaxing factor in muscle or nerve is to alter its concentration and determine the change in performance. Our studies demonstrate that EDTA provides a convenient

means of increasing the concentration of a PA-like relaxing factor in muscle.

EDTA is nonfluorescent, and it has no unique absorption properties to allow a determination of its concentration within a muscle cell. Zhao et al. (1997) have shown that the primary factor that determines the rate of AM-dye loading is the molecular weight of the compound, and that there is an inverse relationship between molecular weight and rate of loading. From their figure 6 we estimate an EDTA loading rate of over 600 μM/h. Thus our 2-h incubation with EDTA could introduce 1–2 mM concentrations of this chelator into the cell. Our in vitro and in vivo studies indicate that, at this concentration, EDTA can effectively remove Ca^{2+} from the regulatory sites of isolated TnC and can promote relaxation.

Although intracellular EDTA can mimic the relaxing effects of PA, and has similar Ca^{2+} and Mg^{2+} exchange rates, it does not provide a perfect substitute. In vitro studies suggest that EDTA has lower Ca^{2+} affinity and higher Mg^{2+} affinity than PA. Because intracellular $[Mg^{2+}]$ is high, EDTA might not be as effective as PA in chelating Ca^{2+}. Intracellular EDTA might also remove Ca^{2+} and/or Mg^{2+} from PA. If so, part of EDTA's ability to reduce tension and speed Ca^{2+} sequestration and relaxation could be due to its production of apo-PA. If intracellular EDTA removes Ca^{2+} and/or Mg^{2+} from PA, then apo-PA might prevent some of the Ca^{2+} released from the SR from binding to TnC and produce a reduction in tension. Although this could explain a portion of the tension reduction produced by EDTA, the fact that the height of the fluo-Ca^{2+} signal is not significantly altered by EDTA suggests that apo-PA, if it is produced, is not binding large amounts of Ca^{2+}. Furthermore, our studies of rat soleus muscle show that intracellular EDTA is clearly able to mimic PA's facilitation of relaxation in the absence of PA. Thus it is likely that intracellular EDTA facilitates relaxation by chelating Ca^{2+} itself, at a rate that is limited by Mg^{2+} dissociation, and not by sequestering Ca^{2+} or Mg^{2+} from PA. It is also unlikely that intracellular EDTA is removing Mg^{2+} from ATP, because cross-bridge cycling requires ATP, and nearly full tetanus tension occurs when fibers are loaded with EDTA.

Despite the above potential limitations of EDTA, our studies demonstrate that it can be used effectively as an "artificial parvalbumin." This should allow us to better define the role of PA and Ca^{2+} in muscle relaxation and neuronal cell function. For example, Elliott and Snider (1995) have demonstrated that amyotrophic lateral sclerosis (ALS)-resistant motor neurons express mRNA for PA, whereas ALS-sensitive motor neurons do not. It would be interesting to load these cells with EDTA and see if it could normalize Ca^{2+} homeostasis and function in these impaired neurons.

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