

Measurement of Calcium Transients and Slow Calcium Current in Myotubes

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ABSTRACT The purpose of this study was to characterize excitation-contraction (e-c) coupling in myotubes for comparison with e-c coupling of adult skeletal muscle. The whole cell configuration of the patch clamp technique was used in conjunction with the calcium indicator dye Fluo-3 to study the calcium transients and slow calcium currents elicited by voltage clamp pulses in cultured myotubes obtained from neonatal mice. Cells were held at -80 mV and stimulated with 15–20 ms test depolarizations preceded and followed by voltage steps designed to isolate the slow calcium current. The slow calcium current had a threshold for activation of about 0 mV; the peak amplitude of the current reached a maximum at 30 to 40 mV and then declined for still stronger depolarizations. The calcium transient had a threshold of about -10 mV, and its amplitude increased as a sigmoidal function of test potential and did not decrease again even for test depolarizations sufficiently strong (≥ 50 mV) that the amplitude of the slow calcium current became very small. Thus, the slow calcium current in myotubes appears to have a negligible role in the process of depolarization-induced release of intracellular calcium and this process in myotubes is essentially like that in adult skeletal muscle. After repolarization, however, the decay of the calcium transient in myotubes was very slow (hundreds of ms) compared to adult muscle, particularly after strong depolarizations that triggered larger calcium transients. Moreover, when cells were repolarized after strong depolarizations, the transient typically continued to increase slowly for up to several tens of ms before the onset of decay. This continued increase after repolarization was abolished by the addition of 5 mM BAPTA to the patch pipette although the rapid depolarization-induced release was not, suggesting that the slow increase might be a regenerative response triggered by the depolarization-induced release of calcium. The addition of either 0.5 mM Cd^{2+} + 0.1 mM La^{3+} or the dihydropyridine (+)-PN 200-110 (1 μM) reduced the amplitude of the calcium transient by mechanisms that appeared to be unrelated to the block of current that these agents produce. In the majority of cells, the decay of the transient was accelerated by the addition of the heavy metals or the dihydropyridine, consistent with the idea that the removal system becomes saturated for large calcium releases and becomes more efficient when the size of the release is reduced.

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INTRODUCTION

Excitation-contraction (e-c) coupling in skeletal muscle is initiated by depolarization of the sarcolemma, which causes the release of calcium from an intracellular store, the sarcoplasmic reticulum (SR). The control of calcium release by depolarization has been extensively studied in adult skeletal muscle fibers by means of the combined use of voltage clamp techniques to record transmembrane currents and optical monitoring of calcium-sensitive indicator dyes (Melzer, Schneider, Simon, and Szücs, 1986*b*; Brum, Stefani, and Ríos, 1987; García, Amador, and Stefani, 1989). Another very significant source of information has been the biochemical purification of muscle membrane proteins and their reconstitution into bilayers (Flockerzi, Oeken, Hofmann, Pelzer, Cavalié, and Trautwein, 1986; Smith, McKenna, Ma, Vilven, Vaghy, Schwartz, and Coronado, 1987; Talvenheimo, Worley, and Nelson, 1987; Ma, Mundiña-Weilenmann, Hosey, and Ríos, 1991). An important conclusion from the electrophysiological and biochemical experiments, is that the dihydropyridine (DHP) receptor is a protein of the sarcolemma that functions as a slow calcium channel and as "voltage sensor" which regulates calcium release from the SR (Ríos and Brum, 1987; Tanabe, Takeshima, Mikami, Flockerzi, Kangawa, Kojima, Matsuo, Hirose, and Numa, 1987). The mechanism of the coupling between the voltage sensor and SR has remained uncertain, although a mechanical linkage (Chandler, Rakowski, and Schneider, 1976) is one possibility. Also remaining uncertain is the physiological significance of the function of the DHP receptor as a calcium channel, because the entry of external calcium is not necessary for e-c coupling (Armstrong, Bezanilla, and Horowicz, 1972) and the contribution of the slow calcium current to the calcium transient is very small, amounting to <5% in frog skeletal muscle (García et al., 1989).

Studies of embryonic skeletal muscle cells (myotubes) in primary tissue culture have also contributed to our knowledge of e-c coupling. Myotubes are particularly valuable because they make it possible to use molecular genetic techniques to study the functional consequences of altering the structure of key proteins, an approach that is vastly more difficult with adult skeletal muscle. Unfortunately, information about the control of SR calcium release by voltage in myotubes is rudimentary compared to that in adult muscle. Thus, we have used fluorescent indicators to characterize the voltage dependence and kinetics of calcium transients in voltage-clamped myotubes and to examine the relationship between the transient and the slow calcium current. We have found that the essential features of the control of calcium release by voltage in myotubes are like those of mature muscle cells, indicating that analysis of genetically manipulated myotubes should provide information useful for understanding e-c coupling in adult muscle. Results of this sort of genetic analysis are presented in a companion paper (García, Tanabe, and Beam, 1994).

A preliminary communication of some of the results has been made (Beam and García, 1993).

METHODS

Experiments were performed on cultured cells obtained from skeletal muscle of newborn mice. The procedure for the primary culture is described in detail in Beam and Knudson (1988). Myotubes were examined 7–11 d after initial plating of myoblasts.

Optical Measurements

For the measurement of calcium transients in response to action potentials, intact myotubes were loaded with the fluorescent dye Fluo-3 AM. The cells were washed free of the culture medium with rodent Ringer, exposed to the dye (4–7 μM in rodent Ringer) for 1 h at room temperature, then returned to medium and placed in the incubator for a minimum of 30 min before experimentation. Because the background (dye-unrelated) fluorescence was not measured for these cells, the fluorescence signals are presented in arbitrary units. For the analysis of possible movement artifacts, cells were similarly loaded by exposure to 10 μM 5-Carboxy-fluorescein diacetate AM. For simultaneous measurement of calcium transients and membrane current, cells were loaded with Fluo-3 or Rhod-2 by adding the salt form to the solution contained in the whole-cell patch pipette. Cells in 35 mm culture dishes were mounted on the stage of a Nikon inverted microscope (Diaphot TMD) equipped for epi-illumination with a 75 W xenon bulb. After rupture of the cell membrane and entry into the whole-cell mode (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981), a period of ~ 5 min was required to achieve adequate loading of the cell with indicator dye. A variable, rectangular slit in the epi-illumination pathway was adjusted so that the fluorescent excitation was restricted to a longitudinal portion of the myotube, which excluded the patch pipette. The width of the area of illumination varied from 30 to 70 μm , and the length from 100 to 150 μm . Fluorescent illumination was begun 1 s before the onset of each voltage clamp command sequence by means of a computer-controlled shutter. Fluorescent emission was measured by means of a fluorometer apparatus (Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia, PA), which included analog electronics, a photomultiplier tube which was mounted to the side-port of the microscope and monitored the fluorescent emission signal, and a beam-splitter which was mounted immediately after the computer-controlled shutter and which sent 95% of the incident illumination to the preparation and 5% to a photodiode. As a means of reducing noise in the recorded signal, the apparatus produced an analog ratio of the fluorescent emission signal (changes in fluorescence caused by changes in calcium and by fluctuations in lamp intensity) and the signal measured by the photodiode (fluctuations in lamp intensity). The set of filters used for Fluo-3 and Carboxyfluorescein was as follows: band-pass excitation filter centered at 470 nm (half bandwidth 20 nm); dichroic long-pass mirror centered at 510 nm; long-pass emission filter centered at 520 nm. For Rhod-2, the filters were: band-pass excitation filter centered at 535 nm (half bandwidth 25 nm); dichroic long-pass mirror centered at 580 nm; long-pass emission filter centered at 590 nm. The background fluorescence was measured from each myotube before rupture of the patch and cancelled by analog subtraction. After rupture of the patch and dye entry into the cell, the baseline fluorescence (F_{base}) was monitored. The fluorescence records are expressed as $\Delta F/F$, where ΔF represents an increment in fluorescence from the baseline fluorescence ($\Delta F = F_{\text{transient}} - F_{\text{base}}$), and F is F_{base} .

Electrical Measurements

Transmembrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Analog compensation was used to reduce the effective series resistance, usually to $< 1 \text{ M}\Omega$, so that the linear cell capacitance was charged with a time constant of ≤ 1 ms. Cell capacitance, which was determined by integration of the control current elicited by a 30 mV hyperpolarization from the holding potential, was used to calculate the density of calcium currents (pA/pF). Except during the adjustment of the series resistance compensation or the measurement of linear capacitance, a two time-constant analog circuit was used to cancel the bulk of the linear capacitive current, which allowed for a larger total gain of the current signal; additionally, voltage clamp commands were rounded at 1 kHz with a Bessel

low-pass filter. Test currents were corrected for remaining components of linear capacitive and resistive current by digital scaling and subtraction of the average of 10 control currents.

The prepulse protocol (Adams, Tanabe, Mikami, Numa, and Beam, 1990) was used as voltage clamp command sequence for the measurement of both calcium currents and charge movement. In this protocol, voltage is first stepped from the holding potential (-80 mV) to -30 or -40 mV for 1 s and then to -50 mV for 25–30 ms, varying test potentials for 15 ms, -50 mV for 25–30 ms, and finally back to the holding potential again. Voltage clamp command sequences were applied every 20 s.

Optical signals in response to action potentials were sampled at 0.2 or 1 kHz. In voltage clamp experiments, the electrical and optical signals were sampled simultaneously at either 4 or 10 kHz. Analog filtering was set at 2 kHz for the electrical signals and at 0.1–1 kHz for the optical recordings. All the records shown are single traces for both membrane currents and calcium transients.

Solutions

Normal rodent Ringer for the action potential experiments had the following composition (mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4 with NaOH. Patch pipettes were made from borosilicate glass and had resistances of 1.6–2.1 M Ω when filled with “internal solution,” which contained (mM): 145 Cs-aspartate, 10 HEPES, 5 Mg₂Cl, 0.1 Cs₂EGTA, and 0.2 K₅Fluo-3 or (NH₄)₃Rhod-2. The external solution contained (mM): 145 TEACl, 10 HEPES, 10 CaCl₂, and 0.003 TTX. The pH of the internal and external solutions was adjusted to 7.4 with CsOH.

The acetoxymethyl (AM) ester of Fluo-3, the pentapotassium salt of Fluo-3, the triammonium salt of Rhod-2, Carboxyfluoresceine-AM, and Cs₄BAPTA were obtained from Molecular Probes (Eugene, OR). (+)-PN 200–100 was kindly provided by Drs. A. Lindenmann and E. Rossi of Sandoz Ltd. (Basel, Switzerland); it was prepared as a 10 mM stock solution in ethanol and used at a concentration of 1 μ M. The bath volume of the recording chamber was ~ 0.5 ml. The bath was exchanged with at least 20 ml of any new solution. Temperature (22–24°C) was monitored with a thermistor probe placed within the recording chamber. Data are presented as mean \pm SEM, with the number of experiments in parentheses.

RESULTS

Calcium Transients Due to Action Potentials

Fig. 1 shows calcium transients elicited by action potentials in two different myotubes, which had been previously loaded with the membrane-permeable form of Fluo-3. The calcium transients shown in *A*, at two different time bases, are from a myotube that was spontaneously contracting. Calcium transients from three other spontaneously contracting myotubes were similar to those shown here. The records in Fig. 1 *B* were obtained from another myotube which was not spontaneously contracting and was electrically stimulated (80 V, 4-ms duration) via an extracellular pipette. In this and other myotubes that were not spontaneously contracting, each electrical stimulus caused only a single calcium transient. In Fig. 1 *B*, the uppermost record (control) was acquired when the myotube was bathed in normal, Na-containing rodent Ringer, the middle record after external Na was replaced by choline, and the bottom record after returning to the Na-containing solution. The reversible suppression of the calcium transient by removal of extracellular Na indicates that the transient was produced by an electrically-evoked action potential. Similar results were obtained

when the sodium channels were blocked by the addition of tetrodotoxin to the bath solution. The electrically-stimulated transients decayed somewhat faster than the ones recorded from spontaneously-contracting myotubes. A possible explanation for this difference in decay is that basal calcium levels were higher in the spontaneously contracting cells and thus the calcium removal systems were less effective than in quiescent cells.

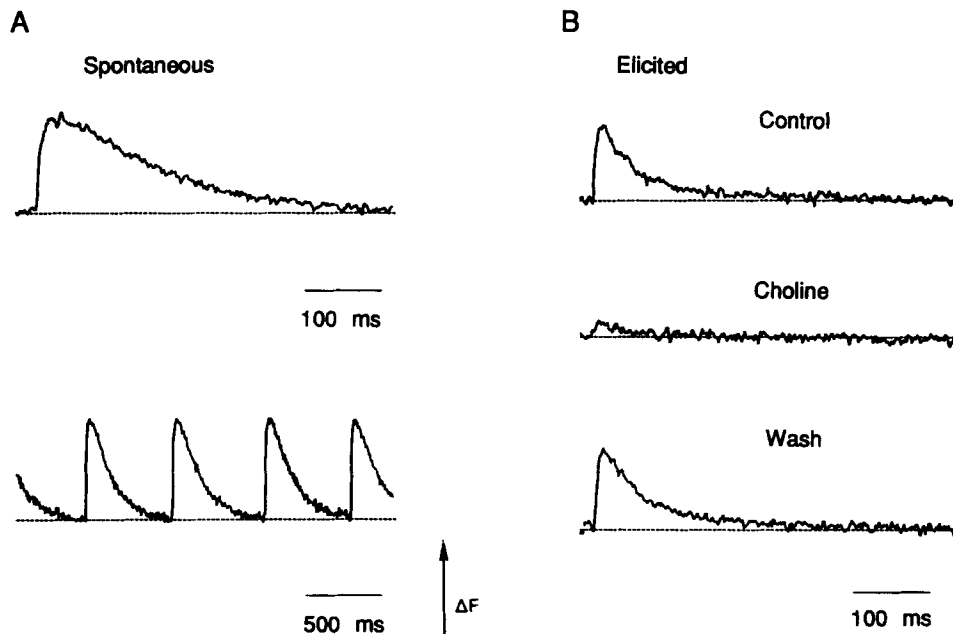


FIGURE 1. Calcium transients of normal myotubes in response to action potentials. Myotubes were loaded by 45 min exposure to Fluo-3 AM. (A) Transients obtained from a myotube that showed spontaneous activity. Note the difference in time base between the upper and lower traces. (B) Transients obtained from a different myotube in response to focal extracellular stimulation. The top trace (*control*) was recorded when the cell was bathed in the normal rodent Ringer, the middle trace (*choline*) was obtained when the sodium in the bath was replaced by choline, and the bottom trace (*wash*) was obtained after return to the sodium-containing solution. The arrow indicates the direction of increasing fluorescence (*arbitrary units*) for both A and B.

Calcium Transients and Slow Calcium Currents Measured Under Voltage Clamp

To allow the measurement of calcium transients and membrane currents simultaneously, myotubes were subjected to whole-cell voltage clamp with a patch pipette containing the pentapotassium salt of Fluo-3. The myotubes were stimulated with the prepulse protocol in which test pulses of varying amplitude were preceded and followed by voltage steps of fixed amplitude and duration (Adams et al., 1990; Materials and Methods). With the prepulse protocol, sodium current and T-type calcium current are inactivated before the onset of the test pulse, but slow calcium current is unaffected (Adams et al., 1990). Fig. 2 A illustrates the calcium transients

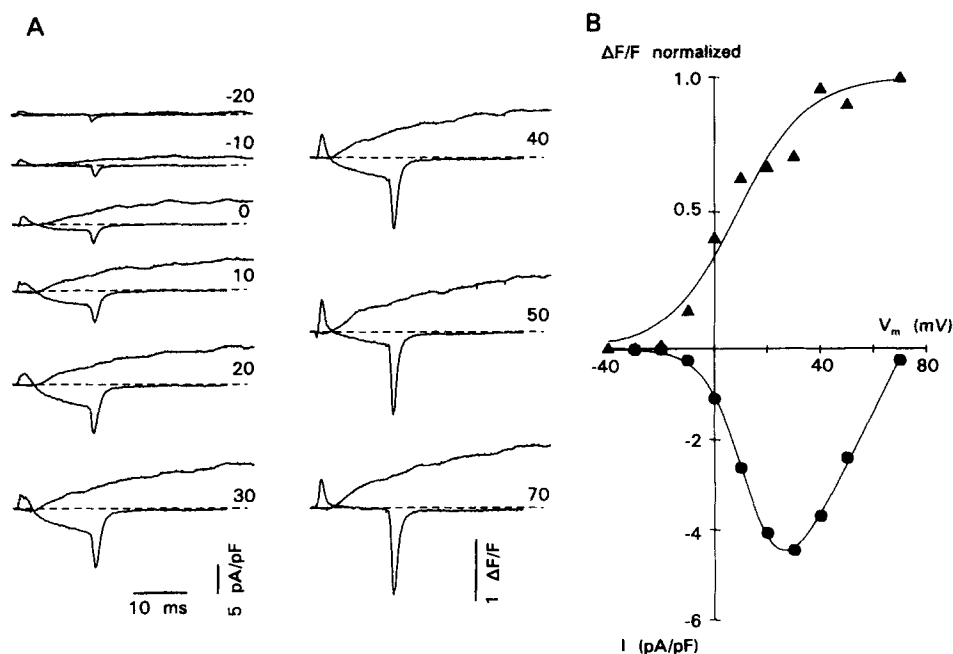


FIGURE 2. Calcium transients and calcium currents recorded under voltage-clamp. (A) Calcium transients (noisier trace which remains at or above the dashed baseline) and membrane currents elicited by test pulses to the potentials indicated at the right; the onset of the test pulses occurred 0.5 ms from the beginning of the illustrated traces. In this and subsequent figures (unless otherwise noted) the test pulses were 15 ms in duration and the prepulse protocol (see Materials and Methods) was used. (B) The peak amplitude of the calcium transient and the calcium current, measured as the average of 10 points (*transient*) or 5 points (*current*) at the end of the 15-ms test pulses, is plotted as a function of test potential. The transients have been normalized to the amplitude of the transient at 70 mV; peak currents have been normalized by linear cell capacitance. Same cell as in A. The smooth curve for $\Delta F/F$ was obtained by least squares fitting (García et al., 1994) of the data points according to the equation:

$$\frac{\Delta F/F}{(\Delta F/F)_{\max}} = 1/[1 + \exp \{(V_{F1/2} - V)/k_F\}],$$

which gave values for $V_{F1/2}$ and k_F of 8.8 mV and 13.2 mV, respectively. The smooth curve for the calcium current data was obtained by fitting of the equation:

$$I = G'_{\max}(V - V_{\text{rev}})/[1 + \exp \{(V'_{G1/2} - V)/k'_G\}],$$

which yielded values of $G'_{\max} = 117.5$ nS/nF, $V_{\text{rev}} = 72$ mV, $V'_{G1/2} = 14.3$ mV, and $k'_G = 7.3$ mV.

and calcium currents elicited in a representative myotube by test pulses to potentials ranging from -20 to $+70$ mV. A test pulse to -10 mV was sufficient to elicit a detectable calcium transient, whereas a stronger depolarization to 0 mV was required to elicit an appreciable slow calcium current. This was typical of the myotubes examined: the calcium transient was first observed for test pulses to -20 or -10 mV

and the calcium current was usually first detected for a 10 mV more depolarized test potential. As is evident in Fig. 2 *A*, the calcium transient activated more rapidly and increased in amplitude with increasing test depolarization. The amplitude of the transient ($\Delta F/F$) and the amplitude of the calcium current, both measured at the end of the 15-ms test pulse, are plotted in Fig. 2 *B* as a function of test potential. Note that the amplitude of the current increased with increasing depolarization up to +30 mV, and then decreased for still stronger depolarizations, whereas the amplitude of the transient increased sigmoidally up to a saturating value. Thus, as previously documented for muscle fibers of amphibians (Miledi, Parker, and Schallow, 1977; Brum et al., 1987; García et al., 1989) and mammals (Eusebi, Miledi, and Takahashi, 1985), voltage-gated calcium entry does not appear to play a role in triggering intracellular calcium release in myotubes. On average, the maximum amplitude of the calcium release transient was $1.8 \pm 0.5 \Delta F/F$ and the maximum amplitude of the current was -2.7 ± 0.3 pA/pF ($n = 31$ myotubes).

Time Course of Transients From Different Longitudinal Regions

Imaging studies with Fura-2 suggest that calcium release within rat myotubes is not uniform: restricted regions of these cells show large changes of intracellular calcium in response to stimulation, whereas other regions show relatively minor changes (Grouselle, Koenig, Lascombe, Chapron, Méléard, and Georgescauld, 1991). Necessarily, the signals from different regions would be spatially averaged by the optical measuring system employed in the studies presented here. As an approach for determining whether spatial inhomogeneities of release might cause distortion of the recorded calcium transients, we compared transients from different longitudinal regions of individual myotubes. Fig. 3 illustrates such an experiment. The myotube, patch pipette and regions of illumination (*rectangles*) are represented schematically. Fluorescence changes were recorded during illumination of three different regions: the right-hand rectangle adjacent to the pipette, the left-hand rectangle more distant from the pipette, and the entire area consisting of both rectangles (which represents the standard region of illumination). Although the amplitude of the transients differs for the three areas of illumination, the time course is very similar, as can be best seen in the bottom of the Fig. 3 where the three records have been normalized to the same amplitude and superimposed. Comparable results were found in three cells. The similarity of delay and time course of the fluorescence signals recorded from different regions is evidence that the measured calcium transients are not distorted by the presence of large-scale, longitudinal inhomogeneities, such as slowly propagating waves of calcium release.

Continued Release of Calcium after Repolarization

Calcium transients recorded for test potentials of -10 to $+30$ mV are illustrated on an intermediate time base in Fig. 4 *A*. These intermediate time base recordings reveal that the fluorescence signals continued to increase for a period of time after repolarization and then decayed very slowly. Both the slow decay and the continued fluorescence increase after repolarization, which is also visible in the records shown in Fig. 2 *A*, were most prominent for strongly depolarizing test pulses and were characteristic of the majority of myotubes examined. We considered a number of

possible explanations for the continued fluorescence increase and slow decay, the first being that both are artifacts of fiber movement. In particular, because Fluo-3 is not a ratiometric dye, an increase in fluorescence would result if contraction caused the volume of dye-containing myoplasm to increase within the illuminated region.

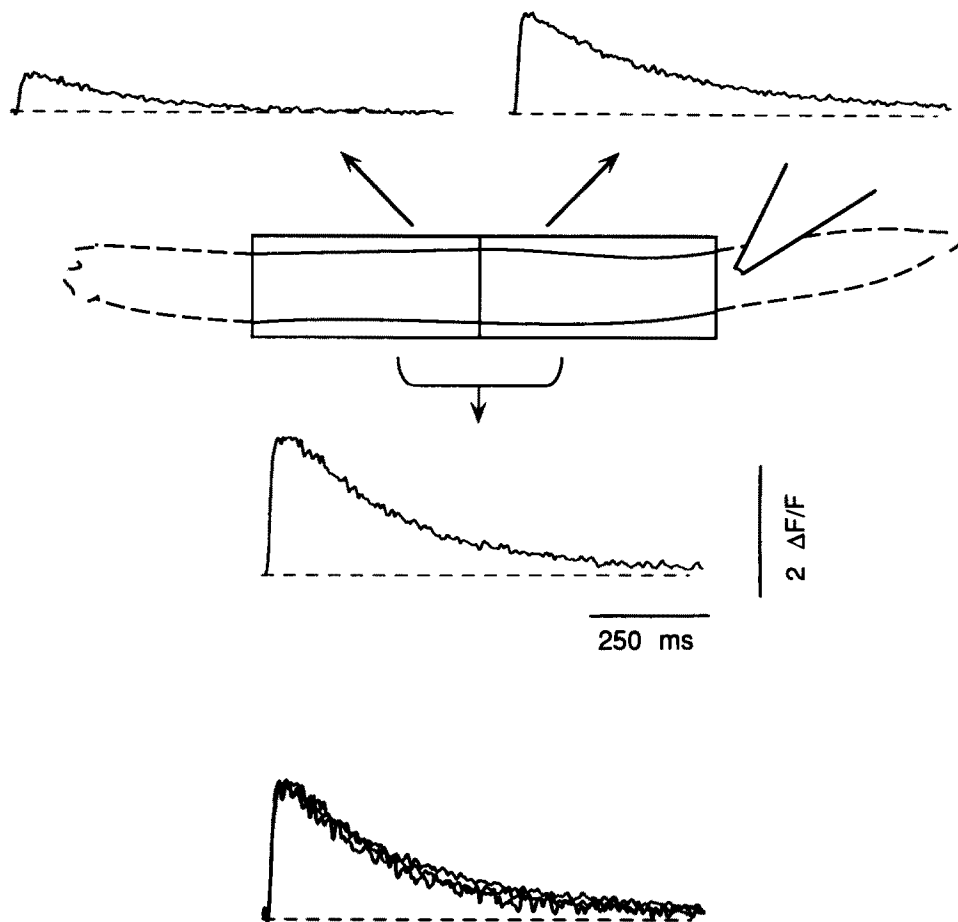


FIGURE 3. Comparison of the calcium transients recorded from different regions along a myotube. The diagram in the middle of the figure represents a myotube with fields of illumination (*rectangles*) and the patch pipette at the right. The single calcium transient shown below the myotube was obtained when the whole field (*both rectangles*) was illuminated. The two transients shown above the myotube were obtained when the indicated rectangular portions were illuminated. For all the transients, the test pulse was to 0 mV. At the bottom of the figure, the three traces were scaled to the same maximum amplitude and superimposed.

Thus, control experiments were carried out in which intact myotubes were loaded with 5-Carboxyfluorescein diacetate AM, a fluorescent dye that is not affected by calcium. These experiments revealed that the change in illuminated volume produced by contraction in response to an action potential caused a change in

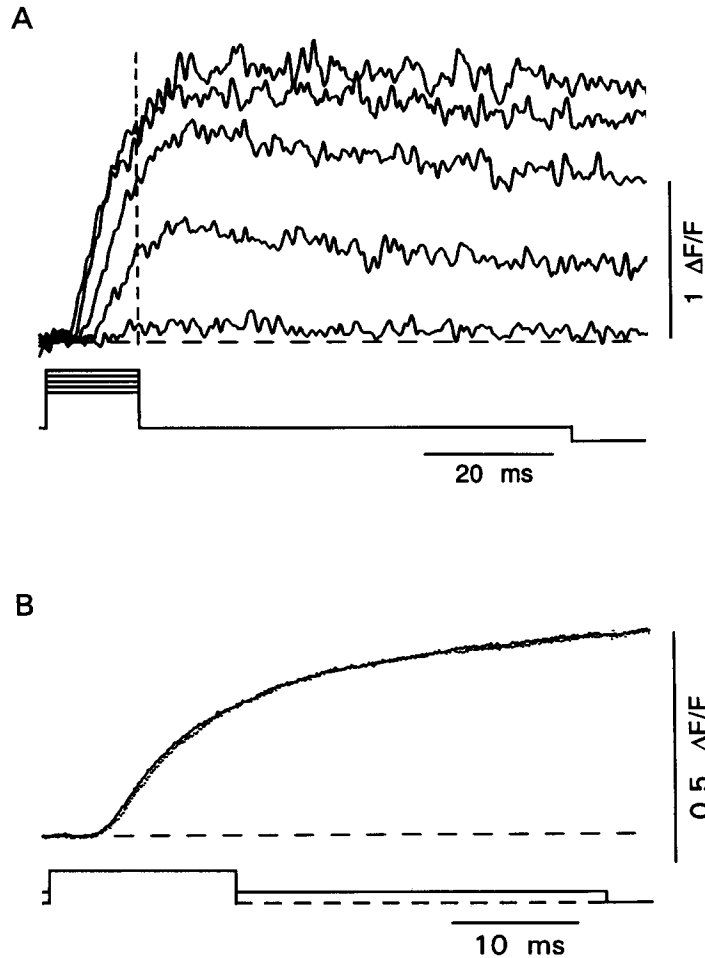


FIGURE 4. Continued rise of calcium transients after repolarization. (A) Calcium transients recorded on an intermediate time base, which makes the continued rise more evident. Test pulses (schematized at the bottom) ranged from -10 to 30 mV in 10 mV steps and were applied from the pedestal potential of -50 mV that constitutes part of the prepulse protocol (see Materials and Methods). The dashed, vertical line indicates the time at which repolarization to -50 mV occurred. (B) Comparison of calcium transients for a test pulse to 30 mV applied either from the pedestal potential of -50 mV (*continuous trace*) or a holding potential of -80 mV (*dotted trace*). The time course of the transient was identical for the two cases. The voltage clamp commands are schematized below the calcium transients.

fluorescence ($\Delta F/F$) that was $< 5\%$ of the fluorescence change of similar myotubes loaded with Fluo-3 AM. Thus, it appears that the fluorescence change recorded with Fluo-3 reflects primarily the change in intracellular calcium, with little contamination by movement artifact.

Another possible explanation for the continued rise in the calcium transient after repolarization is that it is a consequence of the use of the prepulse protocol, in which

the 15-ms depolarizing test pulses are terminated by repolarization back to a "pedestal potential" of -50 mV rather than to a more negative potential (e.g., -80 mV) that might better approximate the resting potential of mammalian muscle cells. Conceivably, repolarization to -50 mV would not have caused the voltage sensors for e-c coupling to reset rapidly to the resting state and thus would allow the continued release of calcium from the SR. To test this idea, we compared calcium transients for repolarization to either -50 or -80 mV. Fig. 4 B demonstrates that the calcium transient is almost identical when a 15-ms step to $+30$ mV was applied from a pedestal potential of -50 mV (*continuous line*) or directly from a holding potential of -80 mV (*dotted trace*). Thus, the continued rise of the calcium transient after repolarization does not appear to be an artifact resulting from the use of the prepulse protocol.

Comparison of Transients Recorded with Fluo-3 and Rhod-2

Because the optical system that we used reports a spatial average of the fluorescence signal, this signal could continue to increase after the cessation of a release that caused localized saturation of an indicator dye. Specifically, diffusion of calcium out of a region of local saturation would not affect the fluorescence of that region (as long as the calcium remained above the saturating level), whereas diffusion of calcium into neighboring regions (in which the indicator was not saturated) would cause the fluorescence of those regions to increase. Because relatively large regions of illumination, like those used in the experiment of Fig. 3, could have obscured the effects of local dye saturation, we compared calcium transients recorded with Fluo-3 and those recorded with Rhod-2, an indicator which has an ~ 2.5 -fold lower affinity for calcium (Minta, Kao, and Tsien, 1989) and is thus less susceptible to saturation. Fig. 5 illustrates calcium transients obtained from two different myotubes, one of which was loaded with Fluo-3 (*top*) and the other with Rhod-2 (*bottom*). The transients illustrated for Rhod-2 are representative of transients recorded from seven other Rhod-2-filled myotubes. The amplitude of the fluorescence changes recorded with Rhod-2 (0.34 ± 0.06 , $n = 8$) were considerably smaller than those recorded with Fluo-3. This smaller amplitude is a consequence of both the lower calcium affinity of Rhod-2 and the smaller change in its fluorescence between the calcium-free and calcium-complexed states is only 3.4-fold, compared to ~ 40 -fold for Fluo-3 (Minta et al., 1989). In Fig. 5, it is apparent that for both Fluo-3 and Rhod-2, the fluorescence signal decayed much more slowly following stronger depolarizations. Additionally, for a brief period (up to 75 ms) after such stronger depolarizations, the fluorescence signal for both dyes continued to increase, although the continued increase was less prominent for Rhod-2. The similarity of the slow decay for Fluo-3 and Rhod-2 argues that this slow decay is not a consequence of dye saturation. The role of dye saturation in the continued increase in fluorescence after repolarization is not as clear.

Effect of Intracellular BAPTA on the Calcium Transient

As another means of examining the factors that govern the kinetics of calcium transients in myotubes, we increased the calcium buffering capacity of the myoplasm by including 5 mM BAPTA in the patch pipettes rather than the usual 0.1 mM

EGTA. The increased buffering had no obvious effect on calcium currents, but markedly altered the calcium transients (Fig. 6). With 5 mM BAPTA in the pipette, a calcium transient was still present which indicates that voltage sensors and SR calcium release are operative in low intracellular calcium. However, the high concentration of BAPTA reduced the amplitude of the transient (maximum $\Delta F/F$ of 0.4 ± 0.1 , $n = 7$) and slowed the decay, both of which can be simply explained if the added BAPTA served as an effective sink for calcium released from the SR. Such a sink would both reduce the increase in free calcium that was detected by the fluorescent indicator and slow the removal of free calcium from the myoplasm. Another important difference in the calcium transient with 5 mM BAPTA was that it did not continue to rise after

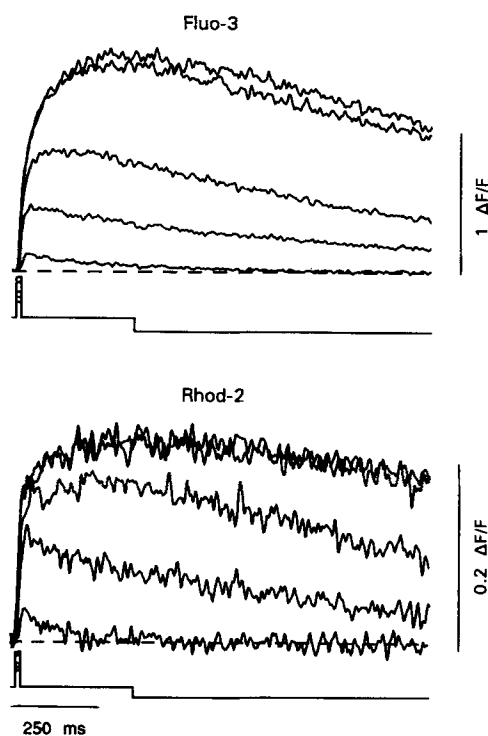


FIGURE 5. Similarity of calcium transients recorded with Fluo-3 (*top*) or Rhod-2 (*bottom*). The concentration of the calcium indicator dyes inside the patch pipettes was 200 μ M. Test pulses to -10 , 0 , 10 , 30 , and 40 mV. For both dyes, the transient continued to rise after repolarization and showed a slow rate of decay. For both Fluo-3 and Rhod-2, the test pulses are schematically illustrated below the transients. The potential preceding and following the 15-ms test pulses was -50 mV and the last downward step indicates repolarization back to the holding potential of -80 mV.

repolarization. If it is assumed that the primary control of calcium release in myotubes is like that in adult muscle, then repolarization would be expected to cause the rapid termination of calcium release (Melzer, Ríos, and Schneider, 1986*a,b*; Simon and Schneider, 1988). If this is the case, then an explanation for the continued increase in fluorescence after repolarization, seen with 0.1 mM EGTA in the pipette, would be that the primary release caused calcium to exceed the threshold for a secondary, calcium-induced calcium release from an intracellular compartment within the myotube. A high concentration of BAPTA might prevent calcium from exceeding the threshold for this secondary release.

Effect of Calcium Channel Blockers on the Calcium Transient

Fig. 7 illustrates the effects of calcium channel blockers on calcium currents and calcium transients. Fig. 7 *A* shows that 0.5 mM Cd²⁺ + 0.1 mM La³⁺ completely blocked the calcium current (cf Adams et al., 1990), but did not eliminate the calcium transient, providing further evidence that calcium entry is not the primary trigger for calcium release in myotubes. The amplitude of the transient in the presence of Cd²⁺ + La³⁺ was smaller (maximum $\Delta F/F$ of 1 ± 0.3 , $n = 6$) than in the absence of the blockers (1.8 ± 0.5 , $n = 31$). The data in Fig. 2 argue against an essential contribution of the calcium current to the calcium transient. Thus, the reduction in

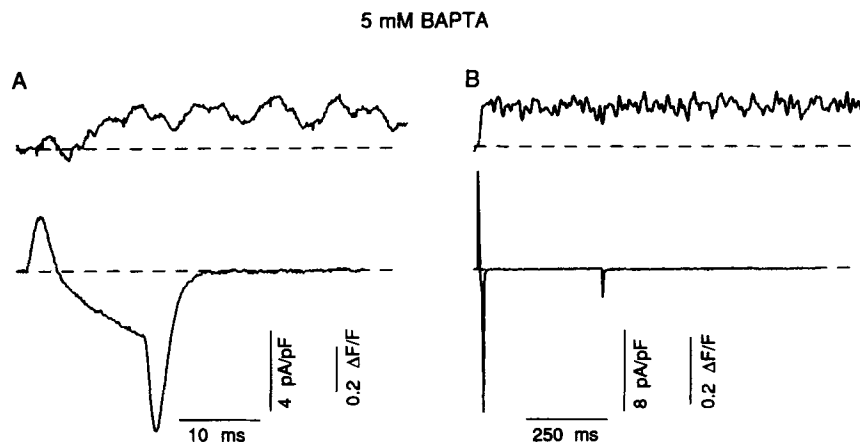


FIGURE 6. Calcium transients recorded in the presence of intracellular BAPTA. Calcium transients and calcium currents, recorded on a fast (*A*) or slow (*B*) time base, were measured for a test pulse to 40 mV. The pipette solution was identical to the standard pipette solution, except that 0.1 mM EGTA was replaced by 5 mM Cs₄BAPTA. With BAPTA in the pipette, the calcium current was similar to currents recorded with 0.1 mM EGTA in the pipette, whereas the calcium transient was much smaller and did not show the continued rise after repolarization (compare with Fig. 2). In *B*, linear components of the membrane current were not subtracted; the smaller, inward tail of current corresponds to the voltage change from -50 mV back to the holding potential of -80 mV.

the size of the transient by the heavy metals requires a different explanation. If the metals entered the cell, they would be expected to cause partial quenching of the dye (cf Kao, Harootunian, and Tsien, 1989). However, this does not appear to be the only effect because the metals routinely caused basal fluorescence to increase ($8.0 \pm 1.2\%$, $n = 6$), as also reported by Grouselle et al. (1991). The increase in basal fluorescence may indicate a higher resting calcium resulting from altered homeostasis.

Fig. 7 *B* illustrates the effect of the DHP antagonist (+)-PN 200-110 (1 μ M). In eight myotubes, the antagonist reduced the calcium current by $92 \pm 3\%$ and the amplitude of the transient by $40 \pm 7\%$ (test pulse to 20 mV). On a longer time scale (Fig. 7 *C*), it is evident that the DHP caused the transient to decay more rapidly, an

effect also observed with the heavy metals (not shown). In three of the eight cells exposed to (+)-PN 200-110, we were able to obtain recordings after extensively washing the cells with drug-free solution. In those three cells, the amplitude of the calcium current showed partial recovery (up to 94% in one cell) but the amplitude of the transient showed no recovery. This result argues against the idea that the

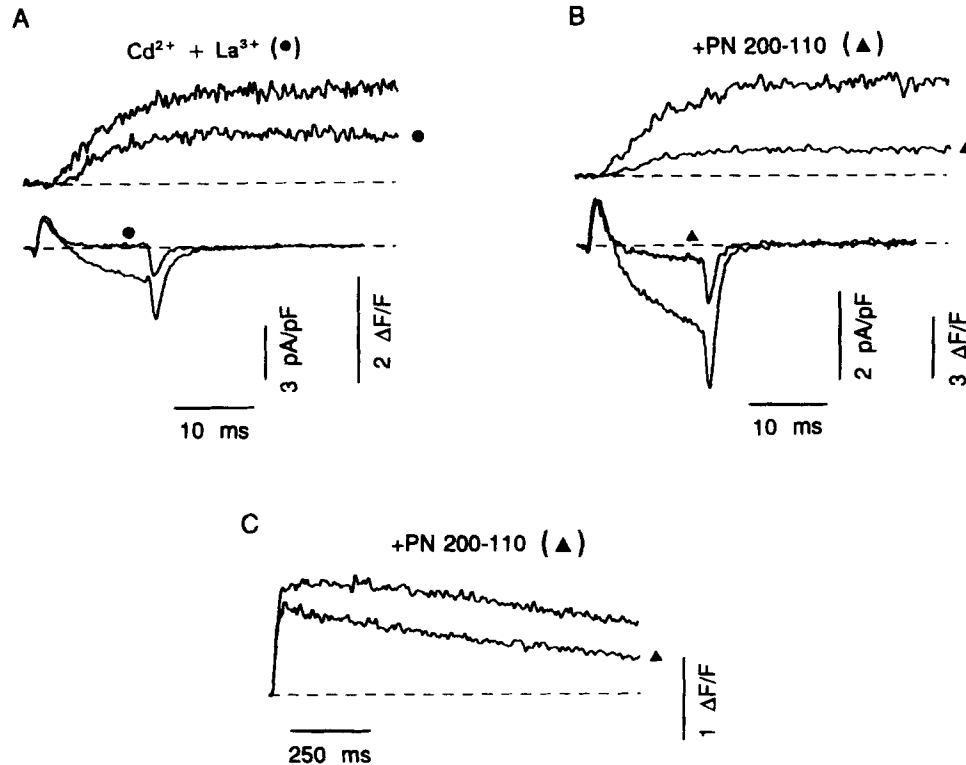


FIGURE 7. Effect of calcium channel blockers on calcium transients and calcium currents. (A) Calcium transients and calcium currents obtained in control conditions and after the addition of 0.5 mM Cd²⁺ + 0.1 mM La³⁺ (●). Note that after this addition, calcium current was eliminated whereas a calcium transient was still present. The reduction in amplitude of the transient likely represents effects of Cd²⁺ and La³⁺ unrelated to block of calcium current (see Text). (B) Recordings from a different myotube in control conditions and after the addition of 1 μ M (+)-PN 200-110 (▲). The DHP caused a reduction in the amplitude of both the calcium current and the calcium transient. (C) Recordings from a myotube at a slower time base before and after addition of 1 μ M (+)-PN 200-110 (▲). The decay of the transient was faster after the addition of the DHP, an effect also seen after the addition of Cd²⁺ + La³⁺ (not shown). All the records were obtained for a test potential of 20 mV.

reduction of the transient results from block of the calcium current and supports instead the idea that the reduction in the transient is caused by an interaction of the DHP with the voltage sensor for excitation-contraction coupling, as first suggested by Ríos and Brum (1987).

DISCUSSION

We have used the whole-cell configuration of the patch clamp technique and the calcium indicator Fluo-3 to measure calcium transients and calcium currents in myotubes. Control experiments indicated that the transients in different longitudinal regions have a similar time course and that the effect of movement artifacts is negligible. With the prepulse protocol and 15-ms test pulses, the threshold for activation of the calcium transient was usually ~ 10 mV hyperpolarized compared to that for activation of calcium current. In contrast to the calcium current, which first increased and then decreased again as a function of test depolarization, the amplitude of the transient increased sigmoidally to a saturating value. Thus, the dependence of the calcium current and calcium transient on voltage in myotubes is qualitatively similar to that of adult muscle (Brum et al., 1987; García et al., 1989).

Compared to calcium transients in adult rat muscle (García and Schneider, 1993), the transients in myotubes decayed very slowly. Because the measurements on myotubes were performed with the whole cell configuration of the patch clamp technique, dialysis of the internal ATP and, therefore, a reduction in the SR calcium ATPase activity could have contributed to this slow decay. In some experiments ($n = 8$), 5 mM ATP was included in the pipette solution, but the presence of ATP did not noticeably alter the time course of the transient. Thus, the myotubes appeared able to maintain levels of ATP adequate for function of the SR calcium ATPase, in agreement with earlier results showing that myotubes retained the ability to contract for long times (30 min) after they were voltage clamped in the whole cell configuration (Tanabe, Mikami, Numa, and Beam, 1990). Moreover, the decay of the transient following weaker depolarizations under voltage clamp was very similar to the decay of transients evoked by action potentials in intact myotubes (compare Figs. 1 and 5) and to the relaxation of twitches recorded from intact myotubes with a photodiode (Tanabe, Beam, Powell, and Numa, 1988; Tanabe et al., 1990; Adams and Beam, 1991). The non-physiological temperature of our experiments (around 23°C) could have contributed to the slow decay, but even at temperatures close to physiological, the decay is slow for calcium transients determined by spatial integration of Fura-2 images (Grouselle et al., 1991). Thus, the slow decay appears to be an inherent property of myotubes, which evidently have a low capacity for calcium removal. Consistent with the idea that the capacity of calcium removal systems have a lower capacity and are easily saturated in myotubes, calcium transients decayed more slowly after stronger depolarizations that released more calcium (Fig. 5).

An interesting property of the calcium transient in myotubes is that it continued to rise for tens of ms after the test depolarization was terminated by repolarization to a negative potential. This continued rise was usually present only after depolarizations of ≥ 10 mV (see Fig. 5), suggesting the presence of a threshold. After repolarization, the voltage sensors are thought to return to the resting state and shut off calcium release rapidly (Melzer et al., 1986b; Simon and Schneider, 1988). Thus, if processes that remove free calcium are operative, the optical signal should begin to decline soon after the cell is repolarized, as is observed in frog skeletal muscle. Therefore, during the continued increase of the calcium transient in myotubes, it seems that the calcium input flux into the myoplasm must exceed the removal flux (i.e., the release

of calcium must be continuing even after the voltage sensors have been reset to the resting state by repolarization). One way to explain this is to suppose that the primary calcium release that is under control of voltage sensors can, if it is sufficiently large, trigger a secondary release that is regenerative. Consistent with this idea, the continued rise after repolarization was not observed in the experiments when 5 mM BAPTA was present in the patch pipette. However, an alternative explanation for this experimental result is that BAPTA prevented the released calcium from causing local saturation of the indicator dye. An argument against this latter idea is that the continued rise after repolarization was seen not only with Fluo-3 but also with Rhod-2 which has a ~ 2.5 -fold lower affinity for calcium (Minta et al., 1989).

Calcium transients were still present after the exposure of myotubes to either 0.5 mM Cd²⁺ + 0.1 mM La³⁺ or 1 μ M (+)-PN 200-110. Because both kinds of agents greatly reduced calcium currents, this is further evidence that calcium entry is not required for e-c coupling in myotubes. However, the blockers did modify the transient, reducing its amplitude and speeding its decay. These modifications of the transient did not appear to be a consequence of blocking the calcium current. The heavy metals increased resting fluorescence, both in our experiments and in the experiments of Grouselle et al. (1991) with Fura-2. This increase in basal fluorescence is suggestive that the heavy metals have a deleterious effect on myotubes. If this is true, then the addition of cadmium or lanthanum to the bath may not be a useful approach for obtaining a quantitative estimate of the role of calcium entry in myotube e-c coupling. An indication that calcium entry contributes negligibly is that the amplitude of the calcium transient for a depolarization that elicited maximal slow calcium current was very similar to that of the transient elicited by a much stronger depolarization that elicited little inward calcium current (Fig. 2).

The DHP (+)-PN 200-110 caused a decrease in both calcium current and calcium transient. The effect of the DHP on the transient can be explained in terms of the effects of the drug on the voltage sensor. In myotubes, immobilization-resistant charge movement is reduced about one third by 1 μ M (+)-PN 200-110, with 10 μ M of the drug having no further effect (Adams and Beam, unpublished observations). A similar quantity of total charge is suppressed by DHPs in adult rat and rabbit muscle (Lamb and Walsh, 1987). Because the DHP receptor is thought to be the voltage sensor for e-c coupling, a reduction in amount of immobilization-resistant charge can account for the reduction of the calcium transient (40%) that we observed in response to application of 1 μ M (+)-PN 200-110 to myotubes. Indeed, a reduction of the calcium transient and charge movement by application of DHPs to frog skeletal muscle fibers was the first evidence that the DHP receptor was the voltage sensor for e-c coupling (Ríos and Brum, 1987).

Both the heavy metals and (+)-PN 200-110 caused the decay of the calcium transient to become more rapid (Fig. 7). This effect can be accounted for by the reduction caused by these agents in the input flux of calcium into the myoplasm. Because calcium removal systems in myotubes appear to have a low capacity and to be easily saturated (see above), a reduction of the input flux would be expected to speed decay.

In summary, myotubes provide a useful system for examining the control of calcium release by voltage. In the following paper, we characterize the quantitative

relationship between charge movements, calcium transients and slow calcium conductance in normal myotubes and in dysgenic myotubes expressing cDNAs for the skeletal muscle or cardiac DHP receptors (García et al., 1994).

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