Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers

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ABSTRACT The role of elevated intracellular calcium concentration ($[Ca^{2+}]$) in activating calcium release from the sarcoplasmic reticulum (SR) was studied in skeletal muscle fibers microinjected with strong calcium buffers. After the injection of 3.8 ± 0.5 mM (mean ± S.E. of mean, n = 16) BAPTA (1,2-*bis*[*o*-aminophenoxy]ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid) or 2.2 – 2.8 mM fura-2 the normal increase in $[Ca^{2+}]$ during a depolarizing pulse was virtually eliminated. Even though calcium was released from the SR the kinetics of this release were markedly altered: the extensive buffering selectively eliminated the early peak component of SR calcium release with no effect on the maintained steady level. Microinjections of similar volumes but with low concentrations of fura-2 had no significant effect on the release waveform. The calcium released by voltage-dependent activation during depolarization may thus be involved in activating further calcium release, that is, in a calcium-induced calcium release mechanism.

INTRODUCTION

The activation of a skeletal muscle fiber is preceded by the depolarization of the surface- and transverse-(*T*-) tubular membranes. This change in *T*-tubular membrane potential causes the movement of charged voltage sensors (Schneider and Chandler, 1973), within the dihydropyridine receptors (Rios and Brum, 1987; Tanabe et al., 1987), located in the *T*-tubule membrane. The voltage sensors then activate calcium release (Miledi et al., 1977; Blinks et al., 1978) through channels in the ryanodine receptors (Fleischer et al., 1985; Pessah et al., 1986; Imagawa et al., 1987; Lai et al., 1988) of the apposed but distinct sarcoplasmic reticulum membrane. Several alternative mechanisms have been proposed (see for recent review Fleischer and Inui, 1989) as to how the movement of the voltage sensor may be linked to the opening of the SR calcium release channels. Although calcium-induced release of calcium from the SR has been documented in skinned skeletal fibers (Endo et al., 1970; Ford and Podolsky, 1970) and in SR vesicles (Miyamoto and Racker, 1982; Meissner et al., 1986) and SR calcium release channels (Smith et al., 1986) from skeletal muscle, its role during physiological activation of intact skeletal muscle fibers has been strongly questioned (Endo, 1985; Baylor and Hollingworth, 1988). We have investigated this issue by injecting sufficient amounts of the high-affinity calcium buffers 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) (Tsien, 1980) or fura-2 into voltage clamped frog skeletal fibers to almost completely suppress the elevation of myoplasmic free calcium concentration during depolarization. Under these conditions the early peak of calcium release (Melzer et al., 1984) was virtually eliminated, indicating that the activation of this component is $[Ca^{2+}]$ dependent. On the other hand the steady level of release was unaffected showing that this part of the release was independent of changes in $[Ca^{2+}]$ and controlled by voltage.

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METHODS

The methods of fiber preparation, the optical setup and the data acquisition and analysis have been described in detail previously. In brief, single skeletal muscle fibers dissected from the m. semitendinosus of cold adapted frogs (*Rana pipiens*) were mounted in a double vaseline-gap chamber and stretched to sarcomere length beyond 3.8 μm to avoid movement (Kovacs et al., 1983). After forming the vaseline seals the solution in the open end pools was exchanged to an Internal solution containing (mM) 102.5 Cs-glutamate, 5.5 $MgCl_2$, 5 ATP, 4.5 Na-Tris-maleate buffer, 13.2 Cs-Tris maleate buffer, 0.1 EGTA, 5 creatine phosphate and 6 glucose; in the middle pool to an External solution containing (mM) 125 $TEACH_3SO_3$, 2 $CaCl_2$, 5 Cs-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and 10^{-7} g/ml tetrodotoxin. Fibers were voltage clamped to −100 mV, all measurements were carried out at 8–10°C.

$[Ca^{2+}]$ was monitored at rest and during depolarizing pulses by the simultaneous application of the relatively low affinity calcium indicator antipyrylazo III (AP III) and the higher affinity calcium indicator fura-2 (added to the Internal solution in 1 mM and 50 μM concentration, respectively). $[Ca^{2+}]$ was measured using either the changes in absorbance at 700 nm due to AP III calcium binding or the ratio of fura-2 fluorescence when excited at 358 and 380 nm. Recording of optical signals, calculation of $[Ca^{2+}]$ from the AP III absorbance, calculation of fura-2 saturation from the fluorescence record and the determination of ON and OFF rate constants for the Ca-fura-2 reaction were carried out as previously described (Kovacs et al., 1983; Klein et al., 1988).

BAPTA was pressure injected into fibers from glass microelectrodes

containing (in mM) 0.5 fura-2, 10-50 BAPTA, 3–15 $CaCl_2$ (nominal free $[Ca^{2+}]$ 40–50 nM using K_D = 100 nM for BAPTA) and 5 Hepes (osmolarity: 65–240 mosm). In some experiments the BAPTA in the microelectrode was replaced by fura-2. Control injections used pipettes containing 0.5 mM fura-2 in diluted Internal solution (170 mosm) to mimic the osmolarity of the BAPTA solutions.

The fiber was observed continuously during each impalement and injection using low intensity long wavelength transmitted light video microscopy via a water immersion objective (40×, 0.75 NA; Ziess, Thornwood, NY) and an image intensifier (KS-1381; Video Scope International, Herndon, VA) coupled to a video camera and monitor. Every 5 s during the injection the fiber was epiilluminated for 1 s (at 358 nm) so that the fluorescence image of the dye was superimposed on the transmitted light image of the fiber. The pressure applied to the injection pipette was kept sufficiently low that there was no sign of fiber swelling during most injections; whenever any swelling was observed the pressure was decreased. The fiber fluorescence was also monitored during each period of epiillumination and the increase was used to calculate the fura-2 and BAPTA concentration injected into the fiber.

The rate of calcium release from the SR before injection was calculated as described earlier (Melzer et al., 1984, 1987) using the parameters for the calcium binding and removal systems in the myoplasm given by Klein and co-workers (1990). After injection the increase in $[Ca^{2+}]$ during depolarizing pulses was < 100–200 nM. The released calcium was almost completely (97–99%, see Results) bound to the injected BAPTA and/or fura-2 and hence the release after injection was estimated as the time derivative of the Ca-BAPTA and Ca-fura-2 complex. Because fura-2 and BAPTA have similar binding properties (Tsien, 1980; Pethig et al., 1989) the time course of Ca^{2+} binding to BAPTA was assumed to be identical to that of the time course of Ca^{2+} binding to fura-2, that is, the percent saturation of BAPTA was assumed to equal the percent saturation of fura-2 obtained from the fluorescence. The ratio of fura-2 to BAPTA injected into the fibers was taken to be the same as in the pipette; possible errors due to different diffusional properties should have been minimal since the first pulse after injection was used throughout the analysis.

Fura-2 and BAPTA were purchased from Molecular Probes, Inc. (Eugene, OR), AP III was from ICN Biochemicals, Inc. (Cleveland, OH), and all other reagents were of analytical grade. Statistical significance was calculated using Student's paired *t*-test.

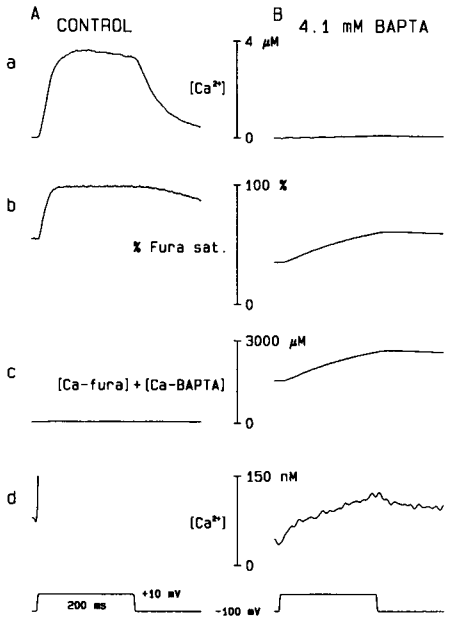

FIGURE 1 Changes in intracellular calcium distribution in a skeletal muscle fiber caused by the microinjection of the Ca buffer BAPTA. (*a*) AP III calcium transients accompanying a 200-ms pulse to +10 mV (*bottom*) measured before (*A*) and for the first pulse after (*B*) microinjection. Even though AP III did not detect any significant change in $[Ca^{2+}]$ during depolarization after injection, there was an increase in the saturation of fura-2 (*Bb*) indicating that calcium was released from the SR. (*c*) The increase in calcium bound to fura-2 and BAPTA during the pulse, calculated as percent saturation × concentration, was negligible before the injection (26 μM) but became dominant in the presence of BAPTA (1,046 μM). The $[Ca^{2+}]$ records determined from the fura-2 fluorescence (*d*) show a lower resting $[Ca^{2+}]$ and also reveal a slight (74 nM) increase in $[Ca^{2+}]$ during the pulse after injection (*Bd*). The pressure-micropipette contained (in mM) 0.5 fura-2, 10 BAPTA, 3 $CaCl_2$ (nominal free calcium 43 nM) and 5 HEPES (osmolarity: 67 mosm). Fiber 806, diameter (*d*): 67 μm, sarcomere length (sl): 4.0 μm, temperature (*T*): 10°C; $[AP\ III]_i$: 668 and 812 μM; $[fura\text{-}2]_i$: 58 and 263 μM before and after injection, respectively; $[BAPTA]_i$ after injection: 4.1 mM.

RESULTS

Before injection (Fig. 1 *A*) a 200-ms depolarization of a frog skeletal fiber from a holding potential of −100 to +10 mV resulted in an ≈4 μM increase in $[Ca^{2+}]$ (Fig. 1 *Aa*) as derived from the absorbance change of AP III. The signal recorded simultaneously by the higher affinity indicator fura-2 was fully saturated during much of the pulse (Fig. 1 *Ab*), but enabled the determination of the resting $[Ca^{2+}]$ (79 nM, Fig. 1 *Ad*) before stimulation.

Microinjection of BAPTA to a concentration of 4.1 mM (Fig. 1 *B*) completely abolished the AP III transient (Fig. 1 *Ba*), presumably due to the high affinity and fast calcium binding properties of the buffer (Tsien, 1980). However, calcium was released from the SR during depolarization since fura-2 saturation increased from 35.4% before the pulse to 59.5% by the end of the pulse (Fig. 1 *Bb*). The increase in the amount of calcium bound to fura-2 and BAPTA during the depolarizing pusle was calculated from the fura-2 saturation record as described in Methods and was found to be 1,046 μM after injection (Fig. 1 *Bc*) and negligible in control (26 μM, Fig. 1 *Ac*). The resting calcium concentration decreased to 44 nM after the injection (Fig. 1 *Bd*), indicating that the above effects were not secondary to an increase in resting $[Ca^{2+}]$. The $[Ca^{2+}]$ calculated from the fura-2 record after injection (Fig. 1 *Bd*) revealed a slight (74 nM) increase by the end of the 200 ms depolarizing pulse.

Using the AP III calcium transient and the calcium bound to fura-2 and BAPTA in Fig. 1 the rate of calcium

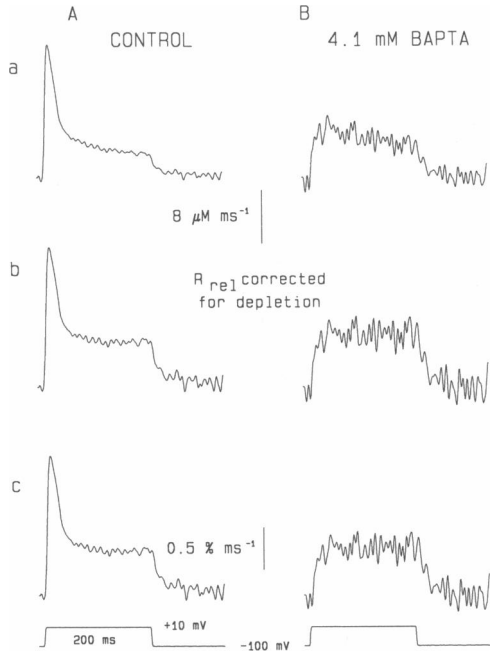

FIGURE 2 Microinjected BAPTA eliminates the peak rate of calcium release from the SR leaving the maintained steady level unchanged. (*a*) R_{rel} before (*A*) and after the injection (*B*). The control R_{rel} exhibits an early peak followed by a sharp decline due to a calcium-dependent inactivation of the release channel and then by a slower decline due to the depletion of calcium from the SR. In the presence of BAPTA both the early peak and the sharp decline are missing, only the slow depletion is present. (*b*) The depletion corrected R_{rel} (C_0 = 2,550 μM and 2,800 μM before and after injection, respectively) in the presence of BAPTA rises steadily, not showing the peak, and reaches the same final level as that in control. (*c*) Because the SR content should be the same before and immediately after injection traces were normalized to SR calcium content to eliminate the uncertainty in absolute scale. Same fiber as in Fig. 1.

release from the SR (R_{rel}) was calculated before and immediately after the microinjection of BAPTA (Fig. 2). Before injection R_{rel} exhibited an early peak followed by a rapid and then a slow phase of decline during the pulse (Fig. 2 *Aa* and Schneider and Simon, 1988). After injection both the early peak and the rapid decline were eliminated leaving only the slowly declining phase (Fig. 2 *Ba*).

The slow phase of decline of R_{rel} before (Schneider et al., 1987) and, presumably, after injection is due to depletion of calcium from the SR. This can be corrected for and used to estimate the SR calcium content before the pulse (Schneider et al., 1987, 1989) by scaling the record at any time, t, by $C_0/(C_0 - \int R_{rel}dt)$, where C_0 is the SR calcium content before the pulse and $\int R_{rel}dt$ is the amount of calcium released from the start of the depolarization until time t. The value of C_0 is determined by the assumption that the rate of release should have reached a steady value by the end of the 200 ms pulse. In control the depletion corrected R_{rel} showed an early peak and then declined to a constant steady level about one-fourth of the peak (Fig. 2 *Ab*). After BAPTA injection the depletion corrected R_{rel} rose monotonically to a final level which was about the same as the final level in control (Fig. 2 *Bb*).

The absolute scale of R_{rel} before injection was determined by the scaling of the AP III calcium transient and by the assumed concentrations and reaction rates for the rapidly equilibrating intrinsic myoplasmic calcium binding sites such as troponin C and the SR calcium pump (Melzer et al., 1987; Klein et al., 1990); the contribution from calcium bound to fura-2 was minimal (4% of total released calcium in Fig. 2). In contrast, after injection the contribution from calcium bound to fura-2 and BAPTA was dominant (99% of total released calcium in Fig. 2). Therefore, to compare R_{rel} based predominantly on calcium binding to intrinsic (CONTROL) or injected (BAPTA) buffers, R_{rel} was normalized to the calcium content of the SR (C_0), in Fig. 2 *c* and in subsequent figures, assuming that the SR contents were not altered by the injection itself.

Fig. 3 shows release records obtained from a fiber in which the SR calcium content determined as described above was quite different before (1,900 μM, if free in myoplasmic water) and after (3,200 μM) the microinjection. This difference in calculated SR content presumably reflects an error in the absolute scale of one or both records. However, when each record was normalized to the SR content the steady levels of release before and after the injection were about the same whereas the peak was eliminated by the injection of BAPTA (Fig. 3, *a* and *b*). Fig. 3 *c* demonstrates the effects of different assumed SR calcium contents (80% and 140% of those used in part *b*). The underestimation (80%) resulted in a secondary rising phase of release whereas the overestimation (140%) did not fully compensate for the slow decline of release. However, neither underestimation nor overestimation of the SR content altered the general conclusion that BAPTA removed the peak R_{rel} with little change in the steady level.

The calculation of R_{rel} in the presence of BAPTA and fura-2 involved the assumption that the two buffers have the same calcium binding properties in the myoplasm. This assumption was necessary because the binding of calcium by BAPTA could not be measured directly. If BAPTA actually bound calcium more rapidly than fura-2, we might have selectively underestimated the peak in the release record calculated after injection using the assumption that fura-2 and BAPTA had the same binding kinetics. For example, when the R_{rel} after injection was recalculated assuming the k_{ON} and k_{OFF} values for Ca–BAPTA to be 10-fold higher (same K_D)

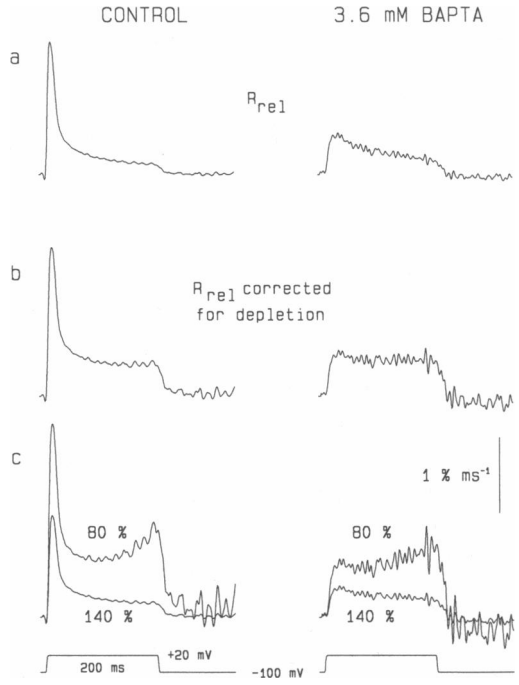

FIGURE 3 The rate of calcium relase in a fiber with different estimated SR calcium contents. R_{rel} before (*a*) and after (*b*) correcting for depletion (C_0 = 1,900 μM before and 3,200 μM after injection). (*c*) Shows that an underestimation (using 80% of that in part *b*) of the SR calcium content results in a secondary rising phase while an overestimation (140%) does not fully compensate for the decline. All traces have been normalized to SR calcium content. The fiber was depolarized from −100 to +20 mV for 200 ms (pulse protocol is shown at the bottom). The pressure micropipette contained the same solution as in Fig. 1. Fiber 804, *d*: 64 μm, *sl*: 3.9 μm, *T*: 10°C; [AP III]$_i$ 782 and 795 μM; [fura-2]$_i$: 60 and 243 before and after injection; [BAPTA]$_i$ after injection: 3.6 mM.

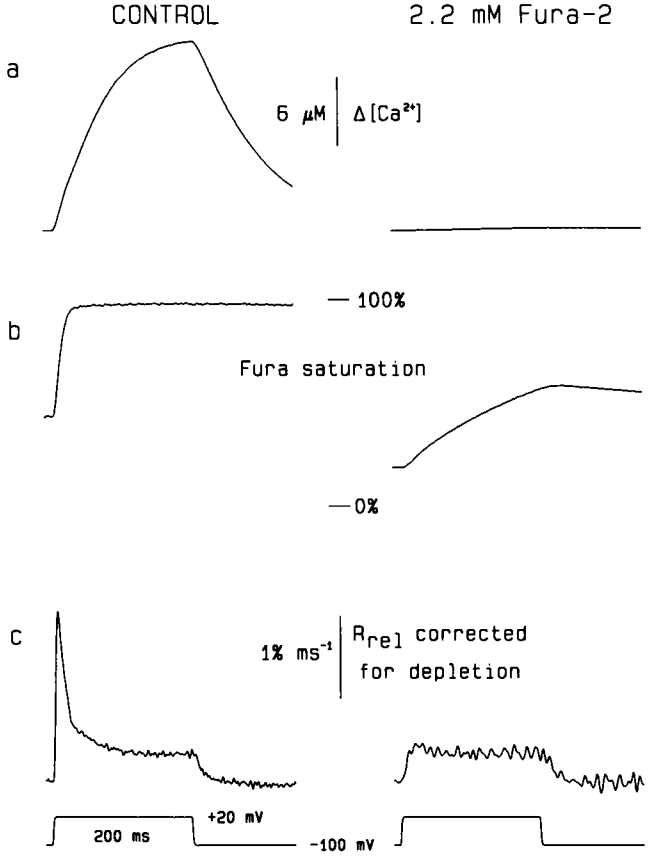

FIGURE 4 Injection of fura-2 suppresses the peak R_{rel}. (*a*) AP III calcium transients before and following the microinjection of 2.2 mM fura-2. Though AP III did not detect any significant increase in free [Ca^{2+}] during the depolarizing pulse following the injection, the increase in fura-2 saturation (*b*) shows that calcium was released from the SR. (*c*) The calculated R_{rel} was first corrected for depletion (C_0 = 1,200 and 1,600 μM before and after injection, respectively) and then normalized to SR content. The pressure-micropipette contained (in mM) 10 fura-2, 3 $CaCl_2$ (nominal free calcium 39 nM) and 5 Hepes. Fiber 846, *d*: 87 μm, *sl*: 4.0 μm, *T*: 9°C; [AP III]: 561 and 626 μM; [fura-2]: 45 μM and 2.2 mM before and after injection, respectively.

than those of Ca–fura-2, the R_{rel} showed an early peak whose amplitude was about twice that of the steady level. It was of interest, therefore, to see whether the injection of fura-2 alone would produce effects similar to those of BAPTA + fura-2 on the R_{rel}.

In two fibers fura-2 alone was injected to sufficiently high concentrations to completely eliminate the AP III calcium transient. Fig. 4 presents the results from one of these fibers in which the fura-2 concentration reached 2.2 mM after the injection. The fiber was depolarized for 200 ms from −100 to +20 mV both before and after the injection. Fig. 4 *a* shows that the calcium concentration increase as measured by AP III during and after the depolarization was eliminated by the injection of fura-2. The fura-2 saturation records (Fig. 4 *b*), nevertheless, demonstrate that calcium was indeed released from the SR and that the resting [Ca^{2+}] decreased after injection. Both these effects and the time course of the record in Fig. 4 *b* in the presence of high concentration of fura-2 qualitatively resembled those measured following the BAPTA + fura-2 injection described in connection with Fig. 1.

The R_{rel} was calculated, corrected for depletion and normalized to SR calcium content the same way as for the BAPTA + fura-2 injection experiments. Fig. 4 *c* shows that the R_{rel} record after injection rose monotonically, not showing the early peak, and reached essentially the same steady level as in control. Another fiber in which fura-2 alone was injected to a concentration of 2.8 mM gave similar results. The general conclusion is that the injection of similar concentrations of BAPTA + fura-2 or fura-2 alone produce qualitatively similar effects on the R_{rel} waveform.

The elimination of the peak R_{rel} after microinjection

appears to be attributable to the presence of millimolar concentrations of BAPTA or fura-2 because injections of solutions with similar volume and osmolarity but with little buffer did not suppress the AP III transient and did not alter R_{rel}. Fig. 5 shows that the calcium transient (Fig. 5 *a*) and the calculated R_{rel} before and after the injection of 322 μM fura-2 were essentially the same. The injected volume was ~1.3 times that injected into the fiber shown in Fig. 1 (estimated from the increase in fura-2 concentration).

The mean value (±S.E.) of the peak and the steady level of R_{rel} are shown in Fig. 6 from 16 fibers in which the concentration of injected BAPTA was sufficient

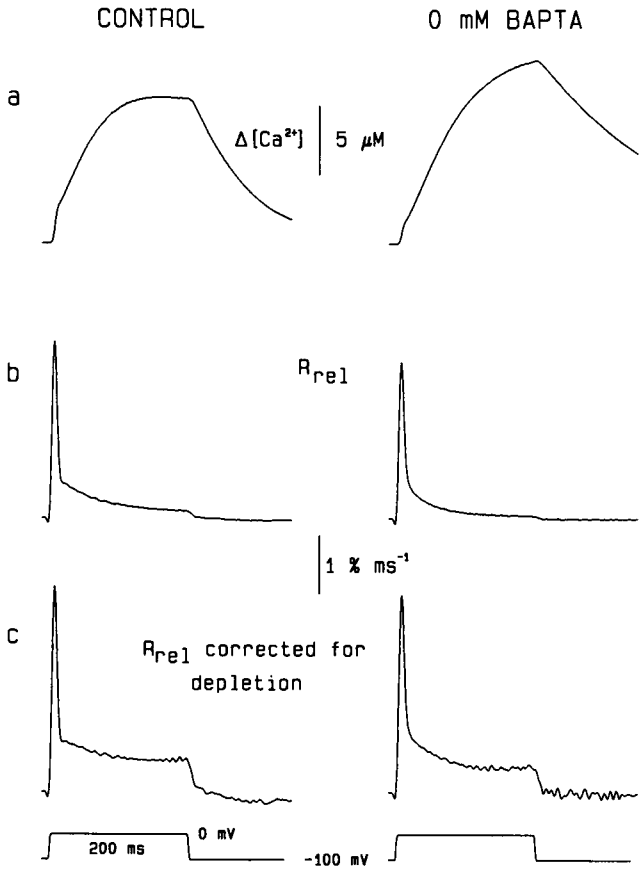

FIGURE 5 Microinjection alone did not alter the rate of release of calcium from the SR. (*a*) The AP III calcium transient measured after an injection (0 mM BAPTA) did not decrease compared to that measured before the injection (CONTROL) when BAPTA was left out of the pipette solution. The calculated R_{rel} waveforms and the absolute magnitudes before (*b*) and after (*c*) correcting for depletion (C_0 = 1,350 and 1,650 μM before and after injection, respectively) are, within experimental error, the same before and after the injection. All records were normalized to SR calcium content. The pulse protocol (200 ms pulse from −100 mV to 0 mV) is shown at the bottom. Fiber 836, *d*: 70 μm, *sl*: 4.0 μm, *T*: 9°C; $[AP\ III]_i$: 560 and 641 μM; $[fura\text{-}2]_i$: 50 and 322 μM before and after injection, respectively.

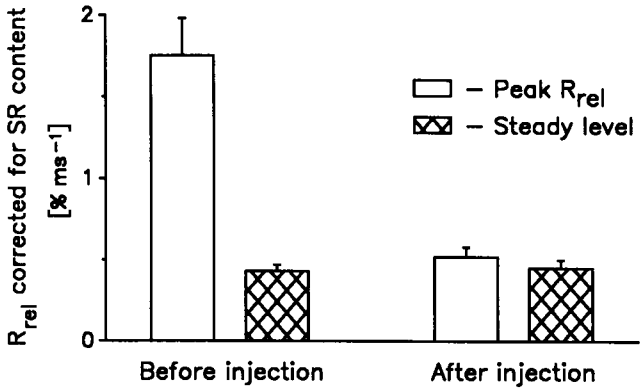

FIGURE 6 The effect of the calcium buffer BAPTA on the rate of calcium release from the SR. The rate of release of calcium from the SR was first depletion corrected then normalized to the SR calcium content (for details see Fig. 2 and related text). The peak was determined as the maximum in the first 100 ms, while the steady level is the average of the values in the last 50 ms of the pulse. Values are expressed as mean ± SEM (n = 16). "After injection" refers to the first depolarizing pulse following the microinjection. $[fura\text{-}2]_i$: 47 ± 3 and 175 ± 26 μM before and after injection, $[BAPTA]_i$: 3.8 ± 0.5 mM after injection.

(>1.5 mM) to almost completely suppress the AP III transient. While the steady levels were not statistically different ($P > 0.5$) before and after injection the peak was significantly ($P < 0.001$) smaller in the presence of BAPTA than in control. The slight difference between the peak and the steady level after injection can be, at least in part, attributed to the procedure used to calculate the peak (maximal value in the first 100 ms) which tends to include the noise into the peak.

DISCUSSION

The present study of the modification of intracellular calcium movements due to added cytosolic calcium buffers was greatly facilitated by the microinjection technique, which allowed measurements to be made within a brief time period shortly before and after the injection. This provided the possibility of a direct comparison of the calcium distribution with and without the added binding sites but presumably with little other change in the fiber. To resolve changes in $[Ca^{2+}]$ both before and after the injection of the strong calcium buffers used in this study the simultaneous use of calcium indicators with different calcium affinities was essential.

The decline of release that gives rise to the peak R_{rel} in control is thought to represent a calcium-dependent inactivation of the SR release channel (Schneider and

Simon, 1988; Simon et al., 1991). Our results demonstrate that strong buffering of myoplasmic [Ca^{2+}] selectively eliminated the peak rate of release during a depolarizing pulse. If the [Ca^{2+}] is buffered low during a pulse, as it was after BAPTA injection, and if the injected calcium buffer simply removed inactivation one would expect the depletion corrected R_{rel} to rise monotonically and not exhibit the early peak as was observed in the present experiments. However, the final level reached due to elimination of inactivation would then be equal to or higher than the peak in control. This was clearly not the case in these experiments. On the contrary, the steady levels of release before and after injection were the same, suggesting that the peak is a different component which is calcium dependent and thus did not activate in the presence of BAPTA.

Other investigators (Baylor and Hollingworth, 1988; Pape et al., 1990) reported increased calcium release in the presence of 0.8-1 mM fura-2 in intact fibers stimulated with action potentials. They interpreted their results as the removal of calcium-dependent inactivation. The fact that in those experiments the calcium transients were not completely suppressed (Fig. 3 of Pape et al., 1990) as were those reported here may account for the apparent discrepancy. Furthermore, when our fibers were injected with lesser amounts (<1 mM) of BAPTA analogues having lower calcium affinity they showed increased peak R_{rel} and slower inactivation. These results can be accounted for if the injected buffers were more effective in cut fibers than in intact fibers, perhaps due to less binding of buffers to myoplasmic constituents (Konishi et al., 1988) in cut fibers, or if the release was lower in cut fibers. In either case less buffer would be required to eliminate the [Ca^{2+}] transient in cut fibers than in intact fibers. The alternative possibility that in muscle fibers BAPTA is a more effective buffer than fura-2 appears to be ruled out since we obtained similar results by injecting BAPTA or fura-2 to similar concentrations.

The effect of BAPTA in the present experiments was likely due to its calcium buffering action since the effectiveness of various injected BAPTA analogues paralleled their calcium affinity (data not shown). Selective suppression of peak release was observed regardless of whether resting [Ca^{2+}] was decreased (Figs 1, 2, and 4) or increased (not shown) after injection, indicating that the suppression of the peak was related to elimination of the [Ca^{2+}] transient and not due to a change in resting [Ca^{2+}].

Though the ability of calcium to activate isolated SR calcium release channels has been clearly demonstrated (see e.g., Smith et al., 1986) the involvement of calcium in the opening of these channels in functionally intact fibers during depolarization has not been demonstrated. A calcium sensitive component of depolarization induced calcium release was described by Stephenson (1985) in skinned fibers, but her measurements were done on a much slower time scale (several seconds).

In cardiac muscle calcium-induced release of calcium (Fabiato, 1985) is well established as a key step in excitation-contraction coupling. The depolarization of a heart cell opens calcium channels in the surface membrane and the entry of extracellular Ca^{2+} triggers further release of calcium from the SR (London and Krueger, 1986; Beuckelmann and Wier, 1988). Though a similar mechanism has been suggested for skeletal muscle (Frank, 1980), it has been repeatedly demonstrated that extracellular calcium plays no direct role in opening SR calcium channels (Armstrong et al., 1972; Miledi et al., 1984; Brum et al., 1987). Thus, calcium leaving the SR through channels which are gated by voltage and which give rise to the steady level of R_{rel} is the most likely activator of the peak component of R_{rel}, as suggested by Rios and Pizarro (1988).

In summary, it has been demonstrated that after the microinjection of ≈ 4 mM BAPTA the inactivatable component of release that is responsible for the peak and the decline of release during a pulse is selectively eliminated while the steady level of release was unaffected. These results lead to the conclusion that released calcium was itself involved in the generation of the inactivatable component, that is, in a calcium-induced calcium release mechanism.

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REFERENCES

Armstrong, C. M., F. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. *Biochem. Biophys. Acta.* 267:605–608.

Baylor, S. M., and S. Hollingworth. 1988. Fura-2 Ca^{2+} transients in frog skeletal muscle fibres. *J. Physiol.* 403:151–192.

Beuckelmann, D. J., and W. G. Wier. 1988. Mechanism of release of calcium from sarcoplsmic reticulum of guinea-pig cardiac cells. *J. Physiol.* 405:233–255.

Blinks, J. R., R. Rudel, and S. R. Taylor. 1978. Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J. Physiol.* 277:291–323.

Brum, G., E. Stefani, and E. Rios. 1987. Simultaneous measurement of Ca current and intracellular Ca concentrations in single skeletal muscle fibres of the frog. *Can. J. Physiol. Pharmacol.* 65:681–685.

Endo, M. 1985. Calcium release from the sarcoplasmic reticulum. *Curr. Top. Membr. Trans.* 25:181–229.

Endo, M., M. Tanaka, and Y. Ogawa. 1970. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibers. *Nature (Lond.).* 228:34–36.

Fabiato, A. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85:247–289.

Fleischer, S., and M. Inui. 1989. Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18:333–364.

Fleischer, S., E. M. Ogunbunmi, M. C. Dixon, and E. A. M. Fleer. 1985. Localization of the Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 87:7256–7259.

Ford, L. E., and R. J. Podolsky. 1970. Regenerative calcium release within muscle cells. *Science (Wash. DC.).* 167:58–59.

Frank, G. B. 1980. The current view of the source of the trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. *Biochem. Pharmacol.* 29:2399–2406.

Imagawa, T., J. S. Smith, R. Coronado, and K. P. Campbell. 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. *J. Biol. Chem.* 262:16636–16643.

Klein, M. G., B. J. Simon, and M. F. Schneider. 1990. Effects of caffeine on calcium release from the sarcoplasmic reticulum in frog skeletal muscle fibres. *J. Physiol.* 425:599–626.

Klein, M. G., B. J. Simon, G. Szucs, and M. F. Schneider. 1988. Simultaneous recording of calcium transients in skeletal muscle using high and low affinity calcium indicators. *Biophys. J.* 55:971–988.

Konishi, M., A. Olson, S. Hollingworth, and S. M. Baylor. 1988. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* 54:1089–1104.

Kovacs, L., E. Rios, and M. F. Schneider. 1983. Measurement and modification of free calcium transients in frog skeletal muscle fibres by a metallochromic indicator dye. *J. Physiol.* 343:161–196.

Lai, F. A., H. P. Erickson, E. Rousseau, Q-Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (Lond.).* 331:315–319.

London, B., and J. W. Krueger. 1986. Contraction in voltage-clamped, internally perfused single heart cells. *J. Gen. Physiol.* 88:475–505.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Melzer, W., E. Rios, and M. F. Schneider. 1984. Time course of calcium release and removal in skeletal muscle fibres. *Biophys. J.* 45:637–641.

Melzer, W., E. Rios and M. F. Schneider. 1987. A general procedure for determining calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. *Biophys. J.* 51:849–863.

Miledi, R., I. Parker, and G. Schalow. 1977. Measurement of calcium transients in frog muscle by the use of arsenazo III. *Proc. Roy. Soc. Lond. Part B.* 198:201–210.

Miledi, R., I. Parker, and P. H. Zhu. 1984. Extracellular ions and excitation-contraction coupling in frog twitch muscle fibers. *J. Physiol.* 351:687–710.

Miyamoto, H., and E. Racker. 1982. Mechanism of calcium release from skeletal sarcoplasmic reticulum. *J. Membr. Biol.* 66:193–201.

Pape, P., M. Konishi, S. Hollingworth, and S. M. Baylor. 1990. Perturbation of sarcoplasmic reticulum calcium release and phenol red absorbance transients by large concentrations of fura-2 injected into frog skeletal muscle fibers. *J. Gen. Physiol.* 96:493–516.

Pessah, I. N., A. O. Francini, D. J. Scales, A. L. Waterhouse, and J. E. Casida. 1986. Calcium-ryanodine receptor complex. Solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 261:8643–8648.

Pethig, R., M. Kuhn, R. Payne, E. Adler, T. H. Chen, and L. F. Jaffe. 1989. On the dissociation constants of BAPTA-type calcium buffers. *Cell Calcium.* 10:491–498.

Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature (Lond.).* 325:717–720.

Rios, E., and G. Pizarro. 1988. Voltage sensors and calcium channels of excitation-contraction coupling. *NIPS.* 3:223–228.

Schneider, M. F., and W. K. Chandler. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature (Lond.).* 242:244–246.

Schneider, M. F., and B. J. Simon. 1988. Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *J. Physiol.* 405:727–745.

Schneider, M. F., B. J. Simon, and M. G. Klein. 1989. Decline of calcium release from the sarcoplasmic reticulum in skeletal muscle cells due to inactivation and calcium depletion. In Physiology and pharmacology of transmembrane signalling. T. Segawa, M. Endo, M. Ui, and K. Kurihara, editors. Elsevier Science Publishers, Amsterdam. 253–260.

Schneider, M. F., B. J. Simon, and G. Szucs. 1987. Depletion of calcium from the sarcoplasmic reticulum during calcium release in frog skeletal muscle. *J. Physiol.* 392:167–192.

Simon, B. J., M. G. Klein, and M. F. Schneider. 1991. Calcium dependence of inactivation of calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. *J. Gen. Physiol.* 97:437–471.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Ca^{2+}. *J. Gen. Physiol.* 88:573–588.

Stephenson, E. W. 1985. Excitation of skinned muscle fibers by imposed ion gradients. I. Stimulation of ^{45}Ca efflux at constant [K][Cl] product. *J. Gen. Physiol.* 86:813–832.

Tanabe, T., H. Takeshima, A. Mikami, H. Matsuo, T. Hirose, and S. Numa. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature (Lond.).* 328:313–318.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry.* 19:2396–2404.