TIME COURSE OF CALCIUM RELEASE AND REMOVAL IN SKELETAL MUSCLE FIBERS

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ABSTRACT The transient increase in free myoplasmic calcium concentration due to depolarization of a skeletal muscle fiber is the net result of the release of calcium from the sarcoplasmic reticulum (SR) and its simultaneous removal by binding to various sites and by reuptake into the SR. We present a procedure for empirically characterizing the calcium removal processes in voltage-clamped fibers and for using such characterization to determine the time course of SR calcium release during a depolarizing pulse. Our results reveal a decline of the SR calcium release rate during depolarization that was not anticipated from simple inspection of the calcium transients.

INTRODUCTION AND THEORY

Depolarization of a skeletal muscle fiber causes a transient increase $\Delta[Ca^{2+}]$ in the myoplasmic-free calcium concentration (1, 2). This calcium transient is the net result of several simultaneous processes. Calcium is released from the sarcoplasmic reticulum (SR) and binds to a variety of different myoplasmic calcium binding sites, the predominant ones being (*a*) the calcium-specific sites on troponin, considered to be rapidly equilibrating (3) compared with the speed of the calcium transient, and (*b*) the more slowly reacting calcium-magnesium sites on troponin and parvalbumin (3). In addition, calcium is returned to the SR by the SR calcium pump.

For determining calcium release, it is convenient to lump calcium removal by the SR pump and calcium binding to all relatively slowly equilibrating sites into an effective overall system for calcium removal. This removal system tends to reduce the total concentration $[Ca]_F$ of fast calcium (free myoplasmic calcium plus calcium bound to all rapidly equilibrating myoplasmic sites). Calcium release from the SR tends to raise $[Ca]_F$. Thus, the rate of change of $[Ca]_F$ will be the difference between the rate of calcium release (RREL) and the rate of calcium removal by the combined removal systems (RREM) so that

$$d[Ca]_F/dt = RREL - RREM. \quad (1)$$

The ratio $[Ca]_F/[Ca^{2+}]$ will be referred to as E. In general, the value of E might vary with time and/or $[Ca^{2+}]$. However, if the rapidly equilibrating sites were in instantaneous equilibrium with $[Ca^{2+}]$ and also far from saturation, E would be approximately constant during each calcium transient and Eq. 1 could be expressed as

$$d[Ca^{2+}]/dt \simeq (RREL - RREM)/E. \quad (2)$$

Eq. 2 serves as the basis for our determination of the time course of SR calcium release.

METHODS

Experiments were carried out on cut segments of single skeletal muscle fibers isolated from the semitendinosus muscle of room temperature-adapted frogs (*Rana pipiens*), mounted in a double Vaseline gap chamber and stretched to sarcomere lengths allowing no overlap of thick and thin contractile filaments (4). The end-pool solution contained the metallochromic indicator dye antipyrylazo III (AP III) (5, 6), which diffused into the fiber segment in the central pool (4). This segment was voltage clamped at a holding potential of -90 mV. Calcium transients $\Delta[Ca^{2+}]$ in response to pulse depolarizations of dye-containing fibers were calculated from measured changes in the fiber's light absorbance at a wavelength of 700 or 720 nm (4, 7) using an apparent dissociation constant of 17,500 μM^2 (4) and assuming instantaneous equilibration of calcium and dye.

RESULTS

Fig. 1 *A* presents the basic pulse protocol used in these experiments. A sequence of pulses of constant amplitude but of varying durations was applied to the fiber. A superimposed set of the resulting calcium transients is presented in the upper part of Fig. 1 *A*. We shall consider first the phase of $\Delta[Ca^{2+}]$ decay after repolarization. Values of the rate constant γ obtained by fitting a single exponential function of time plus a constant, $\Delta[Ca^{2+}]_\infty$, to the decay of $\Delta[Ca^{2+}]$ (4, 7), starting 10 ms after each of the pulses in Fig. 1 *A*, are plotted as a function of pulse duration in Fig. 1 *B*. The decay rate constant clearly

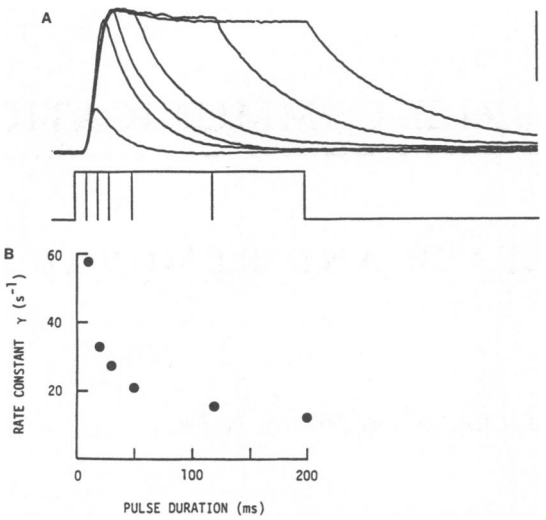

FIGURE 1 Effect of pulse duration on calcium transients and on the rate constant for their decay after repolarization. (*A*) Superimposed transients produced by pulses of 10- to 200-ms duration from −90 to −20 mV, with pulses shown schematically below the records. Each record is an average of two individual pulse applications. In all cases, $\Delta[Ca^{2+}]$ was monitored, stored, and processed as a record of 255 successive points, where each point gave the average value of $\Delta[Ca^{2+}]$ over a 2-ms interval (4). Calibration bar is 1 μM. (*B*) Rate constants obtained by fitting a single exponential plus constant to the decay phase of each record in *A* and plotted as a function of the preceding pulse duration. The fit interval started 10 ms after fiber repolarization. The solution in the central pool contained, in millimoles l^{-1}: tetraethyl-ammonium sulphate, 75; Cs_2SO_4, 5; sodium Tris-maleate buffer, 5; $CaSO_4$, 7.5; tetrodotoxin, 10^{-7} g/ml; pH 7.0. The solution in the end pools contained: caesium glutamate, 108; $MgCl_2$, 5.5; sodium Tris-maleate buffer, 4.5; caesium Tris-maleate buffer, 13.2; EGTA, 0.1; $CaCl_2$, 0.0082; Na_2 ATP, 5; glucose, 5; AP III, 0.5; pH 7.0. The dye concentration measured in the fiber in the central pool ranged from 615 to 685 μM during this pulse sequence. Fiber B140, 4.0-μm per sarcomere, 56-μm fiber thickness along optical axis (10°C).

decreased with increasing pulse duration (4), as previously observed for trains of action potentials of increasing duration (2, 8), and approached a steady relatively low value for the longest pulses used.

Assuming SR calcium release to cease shortly (i.e., within 10 ms) after fiber repolarization, the rate constant, γ, for the decay of $\Delta[Ca^{2+}]$ after each pulse would characterize the state of the combined calcium removal systems at the end of that pulse. The fact that γ decreased with increasing pulse duration would be evidence of a progressive decrease in the overall ability to remove calcium. Assuming negligible release during the decay of $\Delta[Ca^{2+}]$ and considering the case of constant E, Eq. 2 would become

$$d\Delta[Ca^{2+}]/dt_{decay} = -RREM_{decay}/E, \quad (3)$$

where $d\Delta[Ca^{2+}]/dt_{decay}$ and $RREM_{decay}$ are the rate of change of $\Delta[Ca^{2+}]$ and the rate of calcium removal after fiber repolarization. Since the decay of $\Delta[Ca^{2+}]$ closely followed a single-exponential time course, Eq. 3 can be expressed as

$$RREM_{decay}/E = \gamma(\Delta[Ca^{2+}] - \Delta[Ca^{2+}]_\infty). \quad (4)$$

If we assume the calcium removal systems to have the same properties after a pulse as they had just before the pulse was turned off, the calcium decay-rate constants in Fig. 1 *B* would characterize the calcium removal systems both during and after a pulse. The subscript decay could then be dropped from RREM in Eq. 4 and that expression could be used in Eq. 2 together with $d\Delta[Ca^{2+}]/dt$ during a pulse to determine the time course of SR calcium release. The procedure is illustrated in Fig. 2. *A* gives $\Delta[Ca^{2+}]$ for the longest pulse in Fig. 1. *B* gives the continuous time course of the rate constant γ for the calcium removal processes, obtained from the values in Fig. 1 *B* by interpolation as described in the Fig. 2 legend. The relatively small change in γ during the decay of $\Delta[Ca^{2+}]$ was taken into account by expressing γ as a function of $\int\Delta[Ca^{2+}]dt$ (Fig. 2 legend). The product $\gamma(\Delta[Ca^{2+}] - \Delta[Ca^{2+}]_\infty)$, *C*, gives the time course of $RREM/E$ (Eq. 4). *D* in Fig. 2 gives the time course of $d\Delta[Ca^{2+}]/dt$, obtained as the time derivative of *A*.

According to Eqs. 2 and 4, the time course of calcium release is given by

$$RREL/E = d\Delta[Ca^{2+}]/dt + \gamma(\Delta[Ca^{2+}] - \Delta[Ca^{2+}]_\infty), \quad (5)$$

which is presented as *E* in Fig. 2. This record shows that the rate of SR calcium release reached a peak relatively early during the pulse and then declined to a much lower roughly steady level, as briefly described previously (9). The same general time course of release rate has been determined from almost all calcium transients analyzed according to the procedure of Fig. 2, indicating that a marked decline in release during step depolarization appears to be a general characteristic of SR calcium release. Using a somewhat different method of analysis applied to calcium transients measured with Arsenazo III in intact fibers, Baylor et al. (10) have also observed a decline in release during voltage-clamp pulses to potentials near the threshold for calcium release and during a train of action potentials.

The voltage- and time-dependence of the calcium release process is examined in Fig. 3 for two different fibers (left and right panels). Panels *A* present calcium transients for pulses (panels *D*) to two different membrane potentials in each fiber. In addition, a variety of shorter duration pulses to the same voltages were also applied (not shown) and used to characterize the decline in calcium removal ability. The rate of calcium release was then calculated for each pulse using the procedure of Fig. 2. Panels *B* present the resulting calcium release rate records. For both the smaller and larger pulses, the release rate attained a peak value relatively early and then declined to a lower relatively steady level. For these two fibers, the peak rate of release during the larger pulse was 2.5 or 3.0 times higher

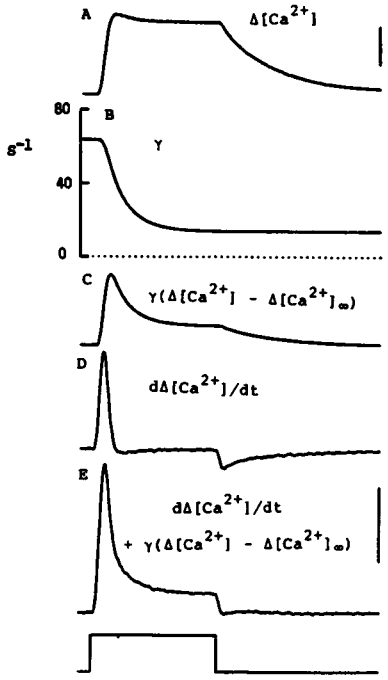

FIGURE 2 Determination of the rates of calcium removal and release during a depolarizing pulse. (*A*) Calcium transient $\Delta[Ca^{2+}]$ elicited by a 200-ms depolarizing pulse from −90 to −20 mV (shown schematically at bottom). Calibration bar is 1 μM. (*B*) Time course of the rate constant γ for decay of $\Delta[Ca^{2+}]$ after a pulse. It was obtained by considering the values in Fig. 1 *B* to be a function of $\int\Delta[Ca^{2+}]dt$, where each integral was evaluated from the start of a $\Delta[Ca^{2+}]$ record to one time constant into the decay phase of $\Delta[Ca^{2+}]$. The mathematical function $\gamma = \gamma_{NS} + \gamma_S \exp(-\int\Delta[Ca^{2+}]dt/A)$ was then fit to the data, where $\gamma_{NS} + \gamma_S$ corresponds to the maximum value of the decay rate constant, γ_{NS} is the value it approached for the longest pulses, and A is a positive constant. This equation provided a good fit to the rate constant data in Fig. 1 *B* and to data for similar pulse sequences in many other fibers. The time course of γ was calculated using the fit together with the running integral of $\Delta[Ca^{2+}]$. (*C*) Rate at which $\Delta[Ca^{2+}]$ would decline due to calcium removal, calculated as $\gamma(\Delta[Ca^{2+}] - \Delta[Ca^{2+}]_\infty)$. (*D*) Rate of change of $\Delta[Ca^{2+}]$, calculated by taking the difference between values of $\Delta[Ca^{2+}]$ two points before and two points after each point in the $\Delta[Ca^{2+}]$ record and dividing each difference by 8 ms. (*E*) Rate at which $\Delta[Ca^{2+}]$ would increase due to calcium release, calculated as $d\Delta[Ca^{2+}]/dt + \gamma(\Delta[Ca^{2+}] - \Delta[Ca^{2+}]_\infty)$ (Eq. 5). Lower calibration bar is 0.1 μM/ms and applies to records *C–E*. Same fiber and pulse sequence as in Fig. 1.

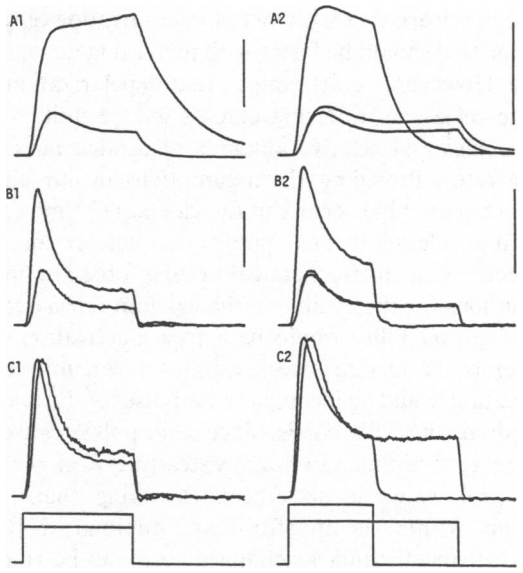

FIGURE 3 Effect of pulse amplitude on calcium transients and rates of calcium release. The records on each side were obtained from a different fiber. (*A*) Calcium transients produced by 200-ms pulses from −90 to −35 or −15 mV (*A1*) and for 300 or 150 ms pulses from −90 to −25 or 0 mV (A_2), as shown schematically at bottom. For A_2, the pulse to −25 mV was applied both before and after the pulse to 0 mV, and gave the smaller $\Delta[Ca^{2+}]$ at its first application. Calibration bar is 1 μM for *A1* and 0.5 μM for *A2*. (*B*) Rate of calcium release calculated from each $\Delta[Ca^{2+}]$ record in *A* and its related sequence of $\Delta[Ca^{2+}]$ records (not shown) for pulses of a variety of shorter durations to the same potential. The release rate is expressed in terms of the resulting rate of change of $\Delta[Ca^{2+}]$. Note that in *B2* the calculated release rates were virtually identical for the initial and final sequences of pulses to −25 mV, whereas $\Delta[Ca^{2+}]$ was appreciably larger during the second sequence (*A2*). This indicates a decline in calcium removal ability during the course of the experiment. Calibration bar is 0.05 μM/ms in *B1* and 0.025 μM/ms in *B2*. (*C*) Records from *B* scaled so as to have the same peak value. The two release records for the smaller pulses in *B2* were averaged for *C2*. Solutions same as for Fig. 1 except that AP III concentration in end pools was 1 mM. *Left* column: fiber B129, measured dye concentration 572 to 625 μM, 4.0-μm per sarcomere, 70-μm fiber thickness along optical axis (10°C). *Right* column: fiber B135, measured dye concentration 979 to 1,126 μM, 3.9-μm per sarcomere, 97-μm fiber thickness along optical axis (7°C).

than during the smaller pulse. Panels *C* compare the time courses of calcium release rate for the smaller and larger pulses in each fiber. Here the release records have been scaled so that the peak release rate is approximately the same for the two different amplitude pulses. These panels indicate that the ratio of peak to steady level of calcium release rate during these depolarizing pulses was about the same for pulses of different amplitudes. Furthermore, the time course of the decline in release rate from its peak toward its final level was quite similar during the two different amplitude pulses. However, the peak release rate was attained earlier for the larger pulses. The same general observations regarding relative time courses of calcium release rate have been made on a variety of fibers in which release was determined for pulses of two different amplitudes.

DISCUSSION

It is interesting to speculate about possible mechanisms for the decline in the calcium release rate during a depolarizing pulse. One possibility might be simple depletion of calcium from the release compartment. A second might be a decrease in the electrochemical driving force for calcium movement due to the SR membrane potential approaching the equilibrium potential for calcium ions. This might occur if calcium conductance became a major component of total SR conductance during activation of release. Both of these mechanisms would seem to predict a greater

decline in release rate with increasing activation of release and depletion should be faster with increasing activation of release. However, we determined that depolarizations that activate release to differing extents induce quite similar time courses and relative amounts of decline in calcium release rates. Providing the assumptions in our analysis introduce only minor errors in the calculated time courses of calcium release, it thus appears that calcium depletion or a decrease in electrochemical driving force are unlikely explanations for the decline in the calcium release rate. A remaining possibility would be a true inactivation of the SR membrane channels responsible for calcium release. Inactivation would be incomplete for pulses of durations up to hundreds of milliseconds, since such pulses caused the release rate to decline to a roughly steady level of 14 to 38% of its peak value in six fibers. Providing that $[Ca^{2+}]$ gradients within the myofibril are minimal, a $[Ca^{2+}]$-dependent inactivation mechanism seems to be ruled out by the similarity of degree and time course of inactivation for pulses that produced $[Ca^{2+}]$ transients of very different amplitudes. If the inactivation mechanism were SR membrane potential dependent, the similar time course and relative amount of inactivation for small and large pulses would require the changes in SR membrane potential for both pulses either to be similar or to be in a range that causes similar inactivation. Note that the inactivation of the calcium release rate observed here occurs over a much shorter time span than that required for suppression of mechanical activity during steady depolarization (11). Presumably if we had determined the release rate on a much slower time scale, eventually it would have decayed to zero.

Three major assumptions underlie our calculation of release rate. First, we assumed that release decreased to negligible levels shortly after repolarization. The prompt initiation of decline of $\Delta[Ca^{2+}]$ on repolarization clearly indicates a decrease in release, but we have no direct evidence that the turn off is complete. Our calculations of the calcium removal rate after a pulse and of the removal and release rates during a pulse would be underestimated to the extent that release would not be turned off after repolarization. Our second assumption was that the calcium removal processes had identical properties during and after a pulse. This assumption clearly should be valid for all components of removal that are due to calcium binding to myoplasmic sites. Only the component of removal due to calcium transport could, in principle, have different properties during and after repolarization. Our release calculation would be in error to the extent that this component of removal differs during and after a pulse.

Our third assumption was that the value of E was constant during each calcium transient. If the rapidly equilibrating sites were not in equilibrium with $[Ca^{2+}]$ or if they become significantly saturated, our assumption becomes invalid. The assumption can be avoided by carrying out calculations of RREL starting directly from Eq. 1. For these calculations the calcium bound to rapidly equilibrating sites was assumed to equal the sum of the concentrations [CaTN] of calcium bound to the calcium-specific sites of troponin and $[CaD_2]$ of calcium-AP III. Then the transient change in the total "fast" calcium would be given by

$$\Delta[Ca]_F = \Delta[Ca^{2+}] + \Delta[CaTN] + \Delta[CaD_2]. \quad (6)$$

The time course of Δ[CaTN] was calculated (3, 10, 12) for each $\Delta[Ca^{2+}]$ record by numerical solution of the differential equation

$$d[CaTN]/dt = k_{ON}[Ca^{2+}] \cdot [TN] - k_{OFF}[CaTN], \quad (7)$$

with $k_{ON} = 57.5\ \mu M^{-1}\ s^{-1}$, $k_{OFF} = 115\ s^{-1}$, and a total concentration of troponin sites in myofilament space water of 240 μM, the values of model II of Baylor et al. (10). The time course of $\Delta[CaD_2]$ was obtained directly from the absorbance change (4). In analogy with Eq. 3, RREM was estimated from the rate of decay of $[Ca]_F$ after pulses of various durations. Again assuming RREM to be the same during and after the pulse, RREL was then calculated as the sum of $d([Ca]_F)/dt$ during the pulse and RREM. These calculations also yielded RREL records that exhibited relatively early peaks and then declined to much lower, roughly steady levels during the pulse. Thus the assumption of constant E does not appear to be crucial for the conclusion that the rate of release declines appreciably during steady depolarization.

Assuming E to be constant during each pulse but to increase with increasing dye concentration, we obtained estimates for the value of E from an analysis of the effect of AP III concentration on the rate constants for decay of $\Delta[Ca^{2+}]$ using a previously described procedure (4), modified to include the decrease in the calcium removal rate with pulse duration (W. Melzer, E. Rios, and M. F. Schneider, in preparation). The value obtained for the component of E due to intrinsic rapidly equilibrating sites (4) for the fiber in Figs. 1 and 2 was 25. Adding this to the components, $([Ca^{2+}] + [CaD_2])/[Ca^{2+}]$, contributed by free calcium and by the calcium-dye complex gave a value of 52 for E. Thus, the removal and release records in Fig. 1 would be 52 times larger than shown if expressed in terms of rates of change of free calcium plus calcium bound to all rapidly equilibrating sites. Fifty-two times the integral of the rate of release record in Fig. 2 gives a total release of 507 μM, expressed as the increase in calcium concentration that would have been produced if all calcium released during the pulse remained free in the myofilament space water. For the records in Fig. 3 *A* the value of E was 51 and the total release calculated from the integrals of the two records would give 207 and 511 μM. The value of E was not determined for the fiber in Fig. 3 *B*.

We have shown previously that for pulses of constant

the contribution of the saturable sites to γ decreases as they are increasingly occupied.

In conclusion, our results demonstrate a decline in the rate of calcium removal during the course of a depolarizing pulse. This decline was empirically characterized and then used together with $\Delta[Ca^{2+}]$ to calculate the calcium release-rate time course. The results indicate that the release system becomes partially inactivated during a depolarizing pulse, and the rate and extent of inactivation of release are independent of its degree of activation.

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