A GENERAL PROCEDURE FOR DETERMINING THE RATE OF CALCIUM RELEASE FROM THE SARCOPLASMIC RETICULUM IN SKELETAL MUSCLE FIBERS

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ABSTRACT A general procedure for using myoplasmic calcium transients measured with a metallochromic indicator dye to calculate the time course of calcium release from the sarcoplasmic reticulum in voltage-clamped skeletal muscle fibers is described and analyzed. Explicit properties are first assigned to all relatively rapidly equilibrating calcium binding sites in the myoplasm so that the calcium content (Ca_F) in this pool of "fast" calcium can be calculated from the calcium transient. The overall properties of the transport systems and relatively slowly equilibrating binding sites that remove calcium from Ca_F are then characterized experimentally from the decay of Ca_F following fiber repolarization. The rate of calcium release can then be calculated as dCa_F/dt plus the rate of removal of calcium from Ca_F. Two alternatives are assumed for the component of Ca_F that is due to fast binding sites intrinsic to the fiber: (*a*) a linear instantaneous buffer or (*b*) a set of binding sites having properties similar to thin filament troponin. Both assumptions yielded similar calcium release wave forms. Three alternative methods for characterizing the removal system are presented. The choice among these or other methods for characterizing removal can be based entirely on convenience since any method that reproduces the decay of Ca_F following fiber repolarization will give the same release wave form. The calculated release wave form will be accurate provided that the properties assumed for Ca_F are correct, that release turns off within a relatively short time after fiber repolarization, that the properties of the slow removal system are the same during and after fiber depolarization, and that possible spatial nonuniformities of free or bound calcium do not introduce major errors.

INTRODUCTION

In skeletal muscle, depolarization of the transverse tubular (T)-system induces an increase in the calcium permeability of the sarcoplasmic reticulum (SR) membrane leading to a flux of calcium ions out of the SR. The resulting increase in myoplasmic free Ca leads to calcium binding to troponin and contractile activation. To quantify the primary voltage-dependent process of excitation–contraction coupling in skeletal muscle, it is therefore necessary to determine the relationship between the T-system membrane voltage and the SR calcium permeability. The situation is complicated by the fact that the voltage across one membrane (T-system) remotely controls the flux of calcium ions across another adjacent but structurally distinct membrane (SR). SR calcium permeability cannot at present be measured directly under conditions where the T-system–SR junction is preserved and the T-system membrane voltage is controlled. Only the result of an increase in SR calcium permeability, the transient increase in myoplasmic free calcium concentration in response to controlled depolarizations, can be detected in relatively intact cells by a number of calcium probes (Blinks et al., 1982).

In a recent brief communication to this journal (Melzer et al., 1984) and in another short report (Schneider et al., 1985) we introduced a general procedure for using myoplasmic calcium transients obtained under voltage-clamp control to derive the rate of calcium release from the SR. Our approach is to conceptually divide the calcium binding sites in the myoplasm into those that equilibrate relatively rapidly with free calcium and those that equilibrate more slowly. The free calcium plus the calcium bound to all relatively rapidly equilibrating sites constitutes a pool (Ca_F) of "fast" calcium. The slowly equilibrating sites together with all systems that transport calcium out of the myoplasm are considered as an overall "removal" system that removes calcium from the pool of fast calcium. We begin by assigning set properties to the rapidly equilibrating sites. The time course of Ca_F can then be calculated

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directly from the calcium transient. We next characterize the overall removal system by analyzing the decay of Ca_F when release is turned off after fiber repolarization. This characterization of removal together with the time course of Ca_F during a depolarizing pulse are then used to calculate the rate of calcium release from the SR during the pulse.

In this paper we describe our procedure for calculating calcium release in more detail and consider various alternative implementations of the general procedure. Two alternative sets of properties are assumed for the rapidly equilibrating binding sites intrinsic to the fiber: (*a*) a set of instantaneously equilibrating sites that are far from saturation and therefore bind calcium in direct proportion to the free myoplasmic calcium or (*b*) a set of binding sites that have the calcium binding properties of the calcium specific sites of thin filament troponin C. Our conclusion from using these two alternative sets of properties for the fast intrinsic sites is that in both cases the calculated rate of calcium release wave form is quite similar, exhibiting an early rise to a peak and a later decline to a lower maintained level during a 100–200-ms pulse.

We also describe three alternative methods for characterizing removal and consider the theoretical basis and practical aspects of each of the methods. An important characteristic of our general procedure is that, provided the correct properties are assigned to the fast calcium binding sites, any method for characterizing removal that correctly predicts the decline in Ca_F following repolarization will give the correct calculation of calcium release. Thus, although the mechanistic details underlying the decline of myoplasmic calcium may be interesting in other contexts and in fact have been examined and analyzed elsewhere in detail by us and others (Melzer et al., 1986*a*; Cannell, 1986), a major advantage of the present calculation of calcium release is its total independence from the mechanism assumed to underline calcium removal.

The major conclusion from the present paper is that the calculated release wave form is rather independent of the assumptions made concerning the fast intrinsic buffer. In that case, it seems appropriate to carry out routine calculations of release in the manner that is most expedient experimentally namely (*a*) to assume that the intrinsic fast calcium sites bind calcium as an instantaneous linear function of free myoplasmic calcium and (*b*) to use a simplified model of the calcium removal system (Melzer et al., 1986*a*) to characterize calcium removal in the fiber.

METHODS

The apparatus and methods for voltage clamping and measuring calcium transients were the same as described in a previous publication (Melzer et al., 1986*a*; see also Kovacs et al., 1983). In brief, single twitch muscle fibers from the M. semitendinosus of American frogs (*Rana pipiens*) were dissected in a relaxing solution, stretched to sarcomere lengths allowing little or no overlap of contractile filaments, and voltage clamped in a double gap perspex chamber. Vaseline seals separated and electrically isolated the cut ends of the fiber segment from a 500–800 μm middle region, which was voltage clamped and used for absorbance measurements. The metallochromic indicator dye antipyrylazo III (0.5–1 mM) was included in the solution applied to the cut ends of the fiber and entered the voltage-clamped segment by diffusion (Kovacs et al., 1983). A rectangular region of the fiber ~50 × 250 μm parallel to the fiber axis was illuminated by light derived from a stable halogen light source passed through interference filters. Intensity changes were recorded with a modified compound microscope (Microstar; American Optical Corp., Buffalo, NY) equipped with a long working distance water immersion objective (40×, 0.75 numerical aperature; model 561702; Carl Zeiss, Inc., Thornwood, NY) and a photodiode (model PDS-050GB; Electro-Nuclear) as light sensor in the trinocular head. The light-induced current of the photodiode was passed through a current-to-voltage converter and corrected for the DC light level just before the pulse by using a track-and-hold circuit and differential amplifier. After suitable preamplification signals were digitized by a voltage-to-frequency converter and counter interfaced to a laboratory minicomputer (model PDP 8E; Digital Equipment Corp., Marlboro, MA).

Changes in light intensity recorded at a wavelength of 700 nm, ΔI_{700}, were converted to absorbance changes using the formula

$$\Delta A_{700} = \log_{10} [I_{700}/(\Delta I_{700} + I_{700})], \quad (1)$$

where I_{700} is the intensity of the light transmitted by the resting fiber at 700 nm. In many cases, ΔA_{700} had to be corrected for dye-independent intrinsic absorbance changes in the fiber (see Melzer et al., 1986*a*) by using reference measurements at 850 nm. Using previous cuvette calibrations of the dye antipyrylazo III, ΔA_{700} was converted to concentration changes of the calcium–dye complex (CaD_2) and of free calcium (Ca) by the equations

$$CaD_2 = \Delta A_{700}/\Delta\epsilon_{700}\, 0.7\, P \quad (2)$$

and

$$Ca = K_D\, CaD_2/(D_T - 2\, CaD_2)^2. \quad (3)$$

$\Delta\epsilon_{700}$ is the differential extinction coefficient at 700 nm, D_T the total dye concentration in the fiber, and P (pathlength) the measured vertical thickness of the fiber. The factor 0.7 corrects the pathlength for cell compartments that are not accessible to the dye (Baylor et al., 1983). The physical constants used in these calculations, which had been determined for our internal solution (Kovacs et al., 1983), were as follows: $\Delta\epsilon_{700} = 1.34 \times 10^{-6}\ \mu M^{-1}\ \mu m^{-1}$ and $K_D = 17,500\ \mu M^2$. Note that $\Delta\epsilon_{720} = 1.64 \times 10^{-6}\ \mu M^{-1}\ \mu m^{-1}$, which had been found in cuvette calibrations, was used when working with a 720-nm narrow-band interference filter (10-nm half-peak band width; Ditric Optics, Inc., Hudson, MA). For the 700 nm broad band filter (70-nm half-peak bandwidth; model 5763; Oriel Corp., Stratford, CT), which we used in most of the present experiments to get higher signal intensities, we had to scale this value by an experimentally determined factor of $\Delta A_{700}/\Delta A_{720} = 0.82$. D_T was derived from the resting absorbance A_{550} of the fiber at 550 nm and the extinction coefficient $\epsilon_{550} = 2.55 \times 10^{-6}\ \mu M^{-1}\ \mu m^{-1}$ using the 790-nm resting absorbance scaled by 790:550 to correct for fiber intrinsic extinction (see Kovacs et al., 1983):

$$D_T = [A_{550} - A_{790}\,(790/550)]/\epsilon_{550}\, 0.7\, P. \quad (4)$$

To demonstrate various alternative procedures for calculating calcium release, we use in this paper only one set of data from one experiment that was selected as a representative example of many experiments in which we calculated calcium release in the ways shown here.

THEORY

General Flux Balance Equation for Calculating the Rate of Calcium Release

Since the free calcium transient (Ca) is the result of simultaneous release and uptake by the SR as well as

complexation of calcium by a number of different binding sites in the myoplasmic space (Robertson et al., 1981), one can in principle use the calcium transient to calculate the rate of calcium release provided binding and uptake can be characterized as a function of free calcium. If the calcium indicator had a sufficiently high affinity for the calcium introduced into the myoplasm from the release compartment so that binding to other components in the cell was negligible, one could simply take the derivative of Ca–dye to get the rate of calcium release from the SR. In our case, the affinity of the dye is relatively low (Kovacs et al., 1983) so that free calcium increases significantly (micromolar range) and serves as a driving force for other fluxes of calcium to binding sites and sequestering systems. These fluxes must be taken into account.

Fig. 1 *A* presents a scheme of the calcium binding and transport fluxes believed to be quantitatively most important in determining the calcium transient resulting from a given time course of calcium release from the SR (*left*). The free myoplasmic calcium (Ca) equilibrates relatively rapidly with both the dye (D) antipyrylazo III (Rios and Schneider, 1981) and with the calcium-specific sites on troponin (TN), whereas it binds more slowly to parvalbumin (PARV). Given these kinetic differences, Ca, CaTN, and CaD_2 can be considered as forming a pool (Ca_F) of fast calcium (Fig. 1, *center*) and PARV and the SR calcium pump can be considered as a "removal" system (Fig. 1, *right*) that takes calcium away from Ca_F. Other fluxes, presumably less important quantitatively, have not been represented in Fig. 1 *A* (cf. Robertson et al., 1981).

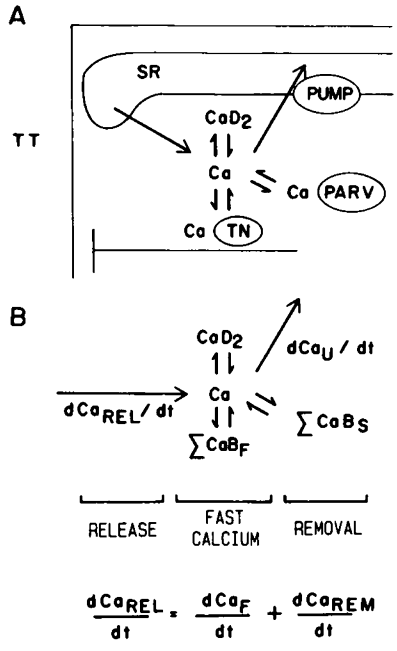

FIGURE 1 Scheme for the calcium release, binding, and transport fluxes in skeletal muscle fibers. (*A*) Representation using the binding sites thought to be quantitatively most important in our experiments. Calcium complexes with dye (D), with calcium-specific sites on thin filament troponin (TN) and with parvalbumin (PARV) are illustrated. The release flux (dCa_{REL}/dt) into the myofilament space from the SR is shown as is the transport system (PUMP) that gives rise to the uptake flux (dCa_U/dt) of calcium back into the SR. (*B*) Generalization of the scheme in *A*. ΣCaB_F and ΣCaB_S represent, respectively, calcium complexes with rapidly and slowly equilibrating binding sites. The labels "release," "fast calcium" (Ca_F), and "removal" (Ca_{REM}) apply to the elements above each label in both *A* and *B*, as does the general equation for the rate of release at the bottom of the figure.

Without specifying the chemical nature of each component, one can generalize the concept of fast and slow binding sites and transport systems as shown in Fig. 1 *B*. Here, dCa_{REL}/dt is the rate of calcium release across membranes and into the myofilament space, ΣCaB_F represents the calcium bound to all rapidly equilibrating myoplasmic calcium binding sites intrinsic to the fiber, ΣCaB_S represents the calcium bound to all slowly equilibrating myoplasmic sites, and dCa_U/dt is the rate of calcium "uptake" by transport across membranes and out of the myofilament space. Using the formalism in Fig. 1 *B*, one can write the flux balance equation

$$\frac{dCa_{REL}}{dt} = \frac{dCa}{dt} + \frac{dCaD_2}{dt} + \frac{d\Sigma CaB_F}{dt} + \frac{d\Sigma CaB_S}{dt} + \frac{dCa_U}{dt}. \quad (5)$$

Taking Ca, CaD_2, and ΣCaB_F to be the pool (Ca_F) of fast calcium and ΣCaB_S and $\int dCa_U/dt$ to be the pool (Ca_{REM}) of calcium removed from Ca_F,

$$\frac{dCa_{REL}}{dt} = \frac{dCa_F}{dt} + \frac{dCa_{REM}}{dt}. \quad (5a)$$

The first term in Eq. 5a gives the rate of change of rapidly equilibrating calcium (corresponding to Fig. 1 *B, center*), whereas the second term gives the rate of removal of calcium from the fast pool by slow binding and transport (Fig. 1 *B, right*). Eq. 5a is identical to Eq. 1 of Melzer et al. (1984) and Eq. 1 of Schneider et al. (1985) but simply uses different notation. In the earlier publications, dCa_{REL}/dt was denoted by RREL and dCa_{REM}/dt was denoted by RREM. The present notation explicitly specifies the relationship between the total amounts of calcium released (Ca_{REL}) or removed (Ca_{REM}) and the instantaneous rates of release (dCa_{REL}/dt) or removal (dCa_{REM}/dt) at any time during a calcium transient. dCa_{REL}/dt as calculated by Eq. 5 or 5a is equal to the net outward flux of calcium from the SR through the voltage-gated release channel plus any entry of calcium into the myoplasm via calcium channels in the surface membrane or in the membrane of the transverse tubular (T) system.

The term $dCaD_2/dt$ is used in Eq. 5 to represent the rate of change of dye-bound calcium since the predominant calcium/dye complex is 1:2 for antipyrylazo III under the conditions of our experiments (Rios and Schneider, 1981; Hollingsworth et al., 1986). However, Eq. 5 could be used for any other calcium indicator by simply substituting the

appropriate expression for dye-bound calcium in place of $dCaD_2/dt$.

It should be noted that we use interchangably the terms flux and rate (of release, of binding, of uptake, etc.) to mean specifically the rate at which the total concentration of calcium would change in the available myoplasmic volume resulting from the particular calcium movement or movements under consideration. This applies to each term in Eq. 5 and to any combination of those terms. With this definition, each flux or rate is expressed as concentration/time. The units used here will be micromolar per millisecond.

Each of the terms in Eq. 5 represents a rate of change averaged over the fiber volume illuminated by the incident light beam so that the rate of release and each of the individual terms in Eq. 5 is the average value in the illuminated volume. This introduces no problem per se in Eq. 5. However, the use of the volume average dye signal to calculate the average concentration of free or bound calcium in subsequent equations could introduce errors if spatial nonuniformities caused local saturation of either dye or binding sites. The existence of spatial gradients of free and bound calcium within a single half sarcomere during calcium release has been predicted by the recent modeling of Cannell and Allen (1984). In principle, our analysis could be applied to each of a large number of small volume elements within the half sarcomere in order to avoid any errors arising from spatial nonuniformities. However, the recording of optical signals from each of these small volume elements has yet to be achieved so that we are limited to the use of average calcium transients for the present calculations.

Eq. 5a is completely general and involves no simplifying assumptions. However, to use Eq. 5a to find dCa_{REL}/dt, one has to quantify both dCa_F/dt and dCa_{REM}/dt. This requires various assumptions.

Alternative Assumptions Regarding the Intrinsic Fast Calcium Binding Sites

The first step in using Eq. 5a to calculate calcium release is to specify the relationship between free calcium and the calcium content of the fast pool. Since the CaD_2 component of Ca_F is determined experimentally, it is only necessary to specify the properties of the intrinsic rapidly equilibrating calcium binding sites. We have used two alternative sets of assumptions regarding the properties of the intrinsic fast sites. In one set of calculations, we assume them to be instantaneously equilibrating and far from saturation. In other calculations, we assume the intrinsic fast sites to have properties similar or identical to those reported for the calcium specific sites on thin filament troponin C.

Instantaneously Equilibrating Fast Binding Sites. In previous analyses, we have found it convenient to specify Ca_F in terms of an "expansion" factor that relates the fast calcium to the concentration of free myoplasmic calcium (Kovacs et al., 1983; Melzer et al., 1984, 1986*a*) and we will continue to use this approach in the present analysis of instantaneously equilibrating sites.

For the case in which the fast calcium binding sites are in instantaneous equilibrium with Ca, Eq. 5 and 5a can be expressed as

$$\frac{dCa_{REL}}{dt} = \frac{dCa}{dt}\left(1 + \frac{dCaD_2}{dCa} + \frac{d\Sigma CaB_F}{dCa}\right) + \frac{dCa_{REM}}{dt}. \quad (6)$$

Defining the expression in parenthesis in Eq. 6 as the "slope expansion" $E(Ca)$,

$$\frac{dCa_{REL}}{dt} = \frac{dCa}{dt}E(Ca) + \frac{dCa_{REM}}{dt}. \quad (7)$$

$E(Ca)$ specifies the binding characteristics, including possible saturation, of each instantaneously equilibrating component including the indicator. However, it should be noted that the indicator component ($dCaD_2/dCa$) of $E(Ca)$ is always measured experimentally and only the intrinsic fast buffer component ($d\Sigma CaB_F/dCa$), which is defined as E_I, is not directly measurable. E_I would in general be a function of Ca and time. Only if all the fast intrinsic components were not only in instantaneous equilibrium with Ca but were also far from saturation for a given calcium transient would E_I be a constant.

Intrinsic Fast Binding Sites with Kinetic Delay in Equilibration. For the case in which the fast sites are not in instantaneous equilibrium with the free calcium it is not convenient to use an expansion factor to express the relationship between free and fast calcium since the relationship depends not only on the instantaneous value of Ca (as above) but also on the preceding Ca time course. In this case, which is exemplified here by our use of troponin C as fast intrinsic site, we must carry out kinetic modeling using on and off rate constants for the calcium-site interaction. The details will be presented in the results section.

Characterizing the Removal System from the Decay of Fast Calcium after Release

Having specified a relationship between free and fast calcium, we can now directly calculate the first term (dCa_F/dt) in Eq. 5a for any measured calcium transient. The next step is to calculate the removal term dCa_{REM}/dt in Eq. 5a. To do this, we have developed an experimental procedure for determining dCa_{REM}/dt at any arbitrarily chosen point in time during a calcium transient. The procedure involves analysis of the decline of Ca_F following pulses of various amplitudes and/or durations and is based on the following two assumptions: (*a*) that calcium release can be turned off rapidly by step repolarization of the membrane and (*b*) that the properties of the fast and slow binding sites and of the calcium transport systems are all independent of voltage.

According to assumption *a*, shortly after repolarization dCa_{REL}/dt is zero so that

$$\frac{dCa_{REM}}{dt_{off}} = -\frac{dCa_F}{dt_{off}}, \tag{8}$$

where the subscript off denotes the condition of zero release after fiber repolarization. If assumption *a* is valid, the decline of fast calcium at the end of a depolarizing pulse will be governed purely by the flux of calcium from the fast components to the slow removal components, uncontaminated by release. The flux to the slow components at the instant of repolarization can be determined as

$$\frac{dCa_{REM}}{dt} = \lim_{t_{off} \longrightarrow t^+} \left(\frac{dCa_{REM}}{dt_{off}}\right) \tag{9}$$

or

$$\frac{dCa_{REM}}{dt} = \lim_{t_{off} \longrightarrow t^+} \left(-\frac{dCa_F}{dt_{off}}\right), \tag{9a}$$

where the limits refer to the extrapolation from later times back to the time t of repolarization that would have applied if release has been negligible both at long and short times after fiber repolarization. Since Ca declines following fiber repolarization, the sign of dCa_F/dt_{off} will be negative so that dCa_{REM}/dt is positive.

Using Eq. 9a, Eq. 5a can be rewritten:

$$\frac{dCa_{REL}}{dt} = \frac{dCa_F}{dt} + \lim_{t_{off} \longrightarrow t^+} \left(-\frac{dCa_F}{dt_{off}}\right) \tag{10}$$

Eq. 10 simply states that the rate of release is equal to the sum of the rate of change of Ca_F (first term) plus the initial rate at which Ca_F would be removed from the fast pool at any point in time if release were to be instantly halted at that time (second term).

In our experiments, we monitor calcium transients for pulses of several amplitudes and/or durations in each sequence of pulses. We thus have data regarding the off time course of several specific calcium transients. To calculate the removal term in Eq. 10, two further steps are necessary. We must first devise an approach for using the later phase of each measured off time course, when release can safely be assumed to be zero, to extrapolate back in time to calculate the limit as $t_{off} \longrightarrow t^+$ that would have applied had release been instantly turned off. We must then devise a procedure for using such measurements from a discrete set of pulses to obtain a continuous time course of the limit term throughout each calcium transient.

Numerical Methods for Calculating Slow Removal

The approach just outlined to determine dCa_{REL}/dt experimentally from Ca records could be implemented using any method that correctly predicts dCa_{REM}/dt as a function of time. In practice, we have employed three different numerical methods. It should be noted that the choice of a method is based simply on convenience, since in principle any of the methods would give the same calculated release wave form if they correctly predict the value of the limit term in Eq. 10.

Method 1 for characterizing removal is the method that we now use routinely (Melzer et al., 1986*b*). It is based on a specific model for the calcium removal system (Melzer et al., 1986*a*). The parameter values for this model can be determined from the decay of Ca following a small number of selected pulses. This method is thus more convenient to use than methods 2 and 3, both of which require analysis of the decay of Ca for a range of different duration pulses for each pulse amplitude at which release is to be determined.

Method 1 works well if the fast intrinsic calcium sites are assumed to be linear and instantaneous functions of Ca. However, it has been found to be inconsistent with published values for the properties of thin filament troponin C (Melzer et al., 1986*a*). We therefore introduce here method 3, which is completely general and can be used to calculate release assuming the fast intrinsic sites to have the properties of thin filament troponin C. Finally, method 2 is described here for completeness since it was the method used in our original report (Melzer et al., 1984). We show here that method 2 is in fact a special case of method 1.

Method 1 for Calculating Slow Removal. The method routinely applied in our current analyses of calcium release uses a model for the calcium distribution in the myoplasm, which was described at length in a previous paper (Melzer et al., 1986*a*). In this model, the removal system was approximated by just two components, a nonsaturable sequestering component, NS, and a saturable binding component, S, which accounted for the slowing of calcium relaxation when large amounts of calcium had been released. For this model the two components of removal were

$$\frac{dCaNS}{dt} = k_{NS}\,Ca \tag{11}$$

and

$$\frac{dCaS}{dt} = k_{on}\,(S_T - CaS)\,Ca - k_{off}\,CaS. \tag{12}$$

CaS was assumed to equal zero in the resting fiber before each depolarization pulse.

For the case of instantaneously equilibrating fast sites, the decay of each calcium transient following turn off of release was described by the differential equation

$$\frac{dCa}{dt_{off}} = \frac{k_{NS}}{E(Ca)}\,Ca + \frac{S_T}{E(Ca)}\,F(Ca,t), \tag{13}$$

where

$$F(\mathrm{Ca},t) = k_{on}\,\mathrm{Ca}\,(1 - \mathrm{CaS}/\mathrm{S_T}) - k_{off}\,\mathrm{CaS}/\mathrm{S_T}. \quad (14)$$

In our routine calculations of release using method 1, the rapid intrinsic binding term $\Sigma\mathrm{CaB_F}$ was approximated by just one nonsaturable instantaneously equilibrating binding site, i.e., $E_I\mathrm{Ca}$ with E_I constant. Values of the parameters k_{NS}, k_{on}, k_{off}, $\mathrm{S_T}$, and E_I were found by simultaneously fitting Eq. 13 to the relaxation phases of a number of calcium transients induced by pulses of different duration and amplitude (Melzer et al., 1986*a*). The value of E_I could be determined only if data were obtained at various times during the experiment when the dye concentration was rather different (Melzer et al., 1986*a*). When the dye concentration did not vary much during the sequence of calcium transients, there was not enough information in the experimental data to derive E_I from the fit. In this case, E_I was arbitrarily set and only k_{NS}, k_{on}, k_{off}, and $\mathrm{S_T}$ remained as free parameters. To account for the fact that release cannot switch off instantaneously, the fit started 14 ms after repolarization. After this time, we assumed the release to be zero. Using the best fit parameters from this model analysis, the occupancy of S could be determined directly from Ca. The flux to the removal system could be calculated using Eqs. 11 and 12 as

$$\frac{d\mathrm{Ca_{REM}}}{dt} = \frac{d\mathrm{CaS}}{dt} + \frac{d\mathrm{CaNS}}{dt}. \quad (15)$$

Method 2 for Calculating Slow Removal. The second method for characterizing slow removal was described and employed by Melzer et al. (1984). Here a depolarizing pulse to a given potential was interrupted at different times and the decline of free calcium was observed. In many fibers, each relaxation phase could be well fit by a single exponential plus a constant (Ca_∞), thus giving values of Ca_∞ and rate constants of relaxation γ for different duration pulses. In this procedure, the instantaneous relaxation rate of free calcium was approximated by $-\gamma(\mathrm{Ca_0} - \mathrm{Ca}_\infty)$, where $\mathrm{Ca_0}$ is the free calcium concentration at the last instant of the pulse. γ was found to get smaller with increasing pulse duration. The values for γ determined from pulses of different duration were interpolated by fitting a theoretical function to the points. This produced a smooth time course for $\gamma(t)$ during a long pulse.

The steady level to which each of the relaxation exponentials seemed to be decaying (Ca_∞) increased with pulse duration. Since Ca_∞ was always only a small fraction of the free Ca level reached by a transient, we assumed that during each pulse Ca_∞ increased linearly with time to its final level at the end of the pulse, i.e., $\mathrm{Ca}_\infty(t) = \mathrm{Ca}_\infty(t_0)\,t/t_0$, where t_0 is the time of pulse repolarization. With these assumptions, the time course of $d\mathrm{Ca_{REM}}/dt/E(\mathrm{Ca})$ during a pulse could be approximated by $-\gamma(t)\,(\mathrm{Ca}(t) - \mathrm{Ca}_\infty(t))$. The theoretical function used for describing the temporal decline of the removal rate constant was

$$\gamma = \gamma_{NS} + \gamma_S \exp\left(-\int \mathrm{Ca}\,dt/A\right), \quad (16)$$

where $\gamma_{NS} + \gamma_S$ corresponds to the maximum value of the decay rate constant (at zero time), γ_{NS} is the value it approached at long pulse durations, and A is a constant. Thus γ_S and γ_{NS} are the saturable and nonsaturable components of the maximal decay rate constant.

Eq. 16 was given in our original description of the calcium release calculation without much explanation (Melzer et al., 1984). It should be mentioned here that Eq. 16 originated from a simpler version of the removal model used in method 1. If the saturating binding site, S, is assumed to bind calcium without causing any backflow, i.e., $k_{off} = 0$, then

$$\frac{d\mathrm{CaS}}{dt} = k_{on}(\mathrm{S_T} - \mathrm{CaS})\mathrm{Ca}, \quad (17)$$

$$\int (\mathrm{S_T} - \mathrm{CaS})^{-1}\,d\mathrm{CaS} = \int k_{on}\,\mathrm{Ca}\,dt \quad (17a)$$

and

$$\mathrm{S} = \mathrm{S_T} - \mathrm{CaS} = \mathrm{S_T}\exp\left(-k_{on}\int \mathrm{Ca}\,dt\right). \quad (17b)$$

This means that the saturating part of the rate constant for the decline of Ca following fiber repolarization (Eq. 16) is an exponential function of the area under the calcium transient, which in turn is a function of pulse duration, and the constant, A, of Eq. 16 is equal to $1/k_{on}$.

We have found in several fibers that Eq. 16 gives a good fit to the variation of γ for pulses of different durations but the same amplitude (cf. Melzer et al., 1984). However, when the pulse amplitude was altered to a different set value, γ still could be described by Eq. 16 but with different parameter values in at least some of the fibers studied. This indicates that the assumption used to obtain equation 16, that $k_{off} = 0$, is not valid. Despite this limitation, Eq. 16 is still valid as an empirical means of interpolating between γ values for pulses of various durations to the same potential for purposes of calculating release using method 2 as we have done previously (Melzer et al., 1984). This simplification did not introduce significant errors because k_{off} was always quite small (Melzer et al., 1986*a*). No records of calcium release using this method of calculation will be presented in this paper. The method is described here for completeness and to establish its theoretical basis. The results were essentially identical to those obtained with method one.

Method 3 for Calculating Slow Removal. A third method employed the same pulse protocol as in method 2, but the instantaneous rate of decline of the rapidly equilibrating calcium $\mathrm{Ca_F}$ (Eqs. 8 and 9) was directly determined by taking the numerical derivative of the relaxation phases of the calculated $\mathrm{Ca_F}$ records without

making any assumptions concerning the properties of the removal system. This method will be described in more detail in the Results in relation to Fig. 4.

Determination of E_I

In all three methods, the determination of E_I is crucial for establishing the absolute size of the calcium release. In our routine analyses, E_I was approximated by a constant. Saturation of the intrinsic fast buffers will make E_I a function of calcium and therefore of time. The influence this may have on the time course of the rate of release will be considered in the Results.

The quantification of intrinsic fast buffering uses the dye-concentration dependence of the decay of Ca following fiber repolarization to assign a constant value to the parameter E_I; the method was carefully described elsewhere (Melzer et al., 1986*a*). The best way at hand to determine E_I was to use the calcium distribution model mentioned in conjunction with method 1, which gave E_I as a best fit parameter. A very similar value can be obtained by linear extrapolation of the dye dependence of the relaxation time constant τ to zero (Fig. 12 in Kovacs et al., 1983 or Fig. 14 in Melzer et al., 1986*a*). Only if a significant change in dye concentration occurred during an experiment was it possible to find a numerical value for E_I. In all other cases, E_I was arbitrarily set and therefore the scale factor E(Ca) of Eq. 7 was uncertain.

According to Eq. 7, each of the three methods of approximating dCa_{REM}/dt could be used in combination with any procedure for determining E_I to calculate the rate of calcium release dCa_{REL}/dt. All calculations of the rate of release use the same quantitative information in the indicator signal in slightly different ways and they therefore lead to very similar results.

Assumptions for the Determination of the Rate of Calcium Release

A number of reasonable but so far untested assumptions have to be kept in mind when applying any of the procedures for calculating the rate of calcium release. (*a*) The indicator dye is assumed to behave in the same way in the fiber as was found in calibrating solutions and all the detected dye is assumed to have the same ability to bind calcium. (*b*) Stability of the removal and binding characteristics (but not of the release properties) was assumed over the time in which the records used for our analysis were taken. (*c*) Spatial gradients of calcium resulting from nonhomogeneous distribution of release or binding sites have not been considered. (*d*) Release is assumed to stop shortly after repolarization to -90 or -100 mV so that most of the decaying phase of free calcium reflects only removal activity uncontaminated by residual release. (*e*) It is assumed that neither fast nor slow binding nor transport is directly voltage dependent, which means that the extrapolation of the removal determined during the "off" following a pulse to the last instant during the "on" of the pulse is valid. In other words, the function describing the rate of removal is a continuous function of time, even at the end of a pulse. In formal terms

$$\lim_{t_{on} \to t^-} \frac{dCa_{REM}}{dt_{on}} = \lim_{t_{off} \to t^+} \frac{dCa_{REM}}{dt_{off}}, \qquad (18)$$

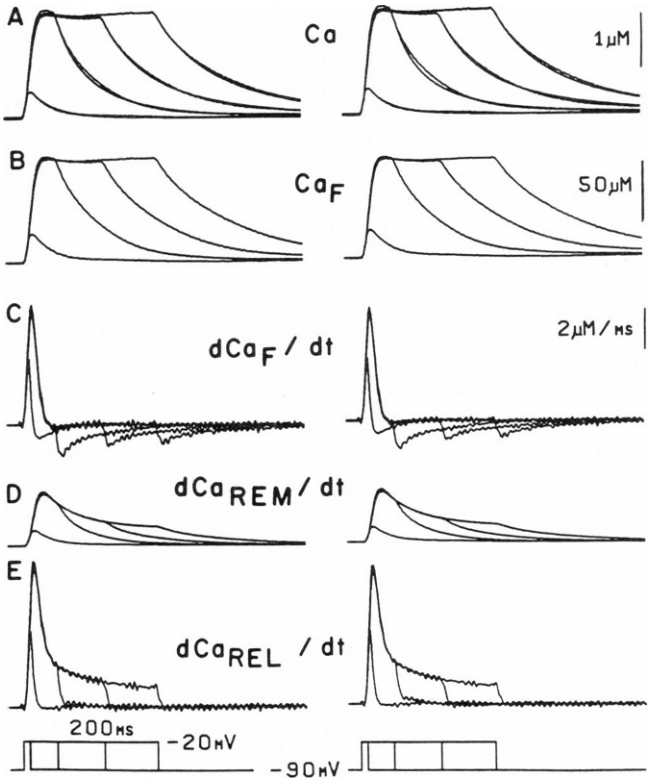

FIGURE 2 Numerical procedure for calculating the rates of voltage-dependent calcium release starting with transients of free myoplasmic calcium. A calcium distribution model (see text and Melzer et al., 1986*a*) was used to simultaneously fit the relaxation phases of a set of calcium transients and thus characterize slow removal (method 1). The total number of records included in this fit was 41. Of these, only four records are presented here. The left and right columns differ in the way the rapidly equilibrating intrinsic calcium binding (ΣCaB_F) was taken into account. (*Left*) This term was approximated by E_ICa, where the value of E_I was determined by the fitting routine to be 18. (*Right*) ΣCaB_F was represented as a saturable binding component with on rate constant and total concentration of sites set to values that had been reported in the literature for troponin C (k_{onTN} = 57.5 $\mu M^{-1} s^{-1}$, TN_T = 240 μM, Baylor et al., 1983) while the value of the off rate constant was determined by the fitting routine to be 747s^{-1}. (*A*) Experimentally measured calcium transients with the calculated time courses of the relaxation phase superimposed on the experimental records. (*B*) Sum of all the rapidly equilibrating calcium species $Ca_F = Ca + CaD_2 + \Sigma CaB_F$ calculated for the four records shown in *A*. (*C*) Numerical derivative of the "fast calcium transients" shown in *B*. (*D*) Rates of slow removal derived from the model analysis (method 1 of calculating removal). (*E*) Rates of calcium release dCa_{REL}/dt calculated as the sum of *C* and *D*. Fiber B140, sarcomere length = 4.0 μm, 56-μm fiber thickness along optical axis, (12°C). Measured dye concentration 767–790 μM for the four records shown in this figure.

where the limit on the left refers to extrapolation from earlier times ahead to the time, t, at which the pulse was turned off.

RESULTS

Linear Intrinsic Fast Buffer with Method 1 for Calculating Removal

Fig. 2, *left*, summarizes the procedure that we now use routinely to calculate the rate of calcium release underlying a measured calcium transient. In this example, releases were produced by 70-mV depolarizing pulses lasting up to 200 ms in duration. The calculation procedure employed the kinetic model for calcium distribution introduced by Melzer et al. (1986*a*), which enabled us to closely reproduce the relaxation of free calcium after membrane repolarization (for details see Theory). The model analysis gave estimates of the total concentration of calcium that is in rapid equilibrium with free calcium ("fast calcium," Ca_F, Fig. 2 *B*, *left*) and of the amount of calcium (Ca_{REM}) that is removed from the pool of rapidly equilibrating calcium via slow binding and transport processes. Fig. 2, *C* and *D* (*left*) show the numerical derivatives of these two components whose sum (Fig. 2 *E*, *left*) is the depolarization induced rate of calcium release from the sarcoplasmic reticulum. The rate of release calculated in this way rose to a maximum shortly after the onset of depolarization but then declined with a slower time course to a steady value. The delayed decline was more or less pronounced in different fibers but was found in every fiber that we analyzed so far.

Since the result depends on a rather tedious and indirect numerical analysis procedure, one has to ask the question whether the calculated time course of the rate of release could not be produced artificially by the structure chosen for the model used in this calculation. The assumption made in the model, that calcium exchanging rapidly with intracellular binding sites be a linear multiple of free calcium (E_ICa), imposes a questionable restriction on the model. In particular, it does not account for the possibility of saturation of the fast intrinsic binding sites during a rather large elevation of free calcium. We therefore attempted to introduce a possibly more realistic approximation of the intrinsic fast buffer.

Troponin as Intrinsic Fast Buffer with Method 1 for Calculating Removal

The only known binding species inside a twitch muscle cell with possibly fast kinetics and considerable capacity for calcium is troponin C. The kinetics of troponin in frog muscle have not been investigated yet. Even for mammalian troponin, which has been studied intensively, the in vivo kinetic properties are uncertain. However, it seems likely that under normal conditions troponin may not behave like a linear buffer for calcium transients in the micromolar range.

Several groups have modeled binding of calcium to troponin using hypothetical or measured calcium transients and kinetic constants obtained from binding studies on mammalian muscle tissue (Robertson et al., 1981; Gillis et al., 1982; Baylor et al., 1983). In a modification of our model, we substituted calcium bound to troponin C (CaTN) for E_ICa and calculated CaTN using the differential equation for a 1:1 binding site according to Baylor et al. (1983):

$$\frac{dCaTN}{dt} = k_{onTN}(TN_T - CaTN)\,Ca - k_{offTN}\,CaTN \quad (19)$$

This offers the possibility of describing the intrinsic fast buffering in a more realistic way. To replace E_ICa in this model analysis by CaTN requires increasing the total number of free parameters by two (replacing E_I by TN_T, k_{onTN}, and k_{offTN}) in the set of equations that have to be fit to the data. This increases the total number of free parameters to seven, which certainly cannot be determined uniquely by the fitting routines. Thus, we kept two of the new constants (TN_T and k_{onTN}) fixed while varying the third one (k_{offTN}). Fig. 2 (*right*) shows the different steps of this calculation. The same set of records as used in the unmodified fit was analyzed with the modified model routine. TN_T was set to 240 μM and k_{onTN} to 57.5 $\mu M^{-1} s^{-1}$ according to Baylor et al. (1983, model II) while k_{offTN} was

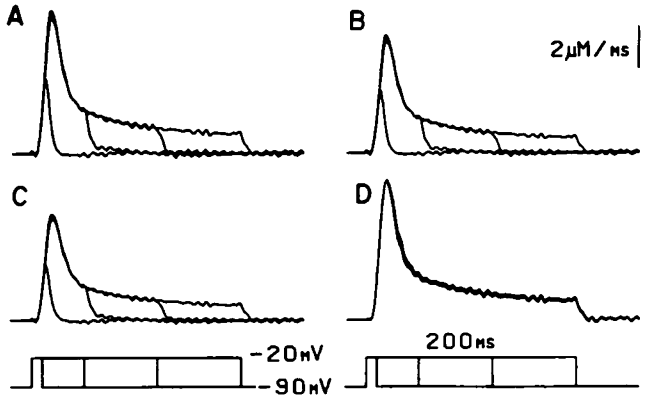

FIGURE 3 Effect of varying the parameters of troponin on the calcium release calculated according to method 1. Rates of release for the same set of calcium transients as in Fig. 2 were calculated by the procedure shown in the right column of that figure. Three different combinations of on rate constant (in $\mu M^{-1} s^{-1}$) and total concentration (in micromolar) of sites of the component representing fast intrinsic binding in the Ca distribution model were used: 57.5 and 240 (*A*, identical to Fig. 2, *right*), 57.5 and 80 (*B*), 5.75 and 240 (*C*). The values of k_{offTN} (in s^{-1}) obtained from the fits were 747 (*A*), 430 (*B*), and 171 (*C*). The results in *A*, *B*, and *C* are shown in their original scale. In *D*, all rate-of-release estimates for the longest record of the sequence (200 ms) were normalized to the peak value and superimposed. Note that all three estimates of dCa_{REL}/dt are almost identical regarding their time course. The scaling factors were 1.00 (*A*), 1.22 (*B*), and 1.33 (*B*).

determined by the fitting procedure, which now allowed the program to take into account saturation of the fast instrinsic calcium buffer troponin and even to make its kinetics rather slow by choosing the appropriate off rate constant if this improved the fit of the relaxation time courses. For simplicity, the free myoplasmic calcium concentration in the resting fiber before stimulation was assumed to be sufficiently low that CaTN was negligible before stimulation for all calculations with troponin as fast buffer.

To summarize the result, the modification did not improve the fit to any extent. On the contrary, it proved more difficult to get a good simultaneous fit of the modified model to all the off phases. Furthermore, k_{offTN} came out to be considerably larger than the literature values thus giving a dissociation constant of 13 μM and making troponin essentially linear in terms of calcium binding for even the largest calcium transients in this experiment. A similar result has already been reported in our detailed analysis of calcium removal (Melzer et al., 1986*a*). The rate of release was calculated as

$$\frac{dCa_{REL}}{dt} = \frac{dCa}{dt} + \frac{dCaD_2}{dt} + \frac{dCaTN}{dt} + \frac{dCa_{REM}}{dt} \quad (20)$$

with CaD_2 and Ca derived from Eqs. 2 and 3, dCa_{REM}/dt given by Eq. 15 with CaS and CaNS determined by Eqs. 11 and 12, and CaTN calculated using Eq. 19. dCa_{REL}/dt was almost indistinguishable from the one obtained with unmodified method 1.

We repeated the analysis with two other sets of fixed troponin parameters: (*a*) decreasing the total concentration of troponin to one-third while leaving k_{onTN} unchanged and (*b*) decreasing k_{onTN} to one-tenth while leaving TN_T unchanged, in this last case imposing rather slow kinetics on troponin. The resulting rate of release estimates are summarized in Fig. 3. Even though there are differences in scaling by at most one-third, the time course of the rate of release in each case came out to be almost the same. In each case, the apparent dissociation constant of the model troponin turned out to be considerably higher than 2 μM (see the figure legend).

As we mentioned in an earlier paper (Melzer et al., 1986*a*) we also set k_{offTN} in the modified fit program to the values used in model II of Baylor et al. (1983) leaving only k_{NS}, k_{on}, k_{off}, and S_T as free parameters. Under these conditions, the program could not reproduce the relaxation phases nearly as well as in the procedures described above. Only a qualitative modification of the removal system structure in the model may be able to compensate for this deficiency. This was not attempted in the present investigation. Nonetheless, it is interesting to check to what degree deviations in the time course of intrinsic fast calcium binding from the one derived in our model analysis would change the rate of release waveform. For this purpose, we developed method 3 of calculating removal.

Method 3 for Removal with either Linear Binding or Troponin as Intrinsic Fast Buffer

Method 3 allows one to estimate the rate of removal independently of any assumption regarding its mechanism. It is only necessary to define the rapidly equilibrating intrinsic component, ΣCaB_F. Thus, method 3 provides the possibility of choosing the properties of the fast component arbitrarily and of investigating its effect on the calculation of the rate of release.

Fig. 4 presents the first step of the release calculation by method 3 for the same Ca record as in Figs. 2 and 3. In this step, the rate of slow Ca removal during the 200 ms pulse is obtained by estimating the initial rate of relaxation of "fast calcium" $Ca_F = Ca + Ca\text{-dye} + \Sigma CaB_F$ (Eq. 8) following several shorter pulses to the same potential. These pulses

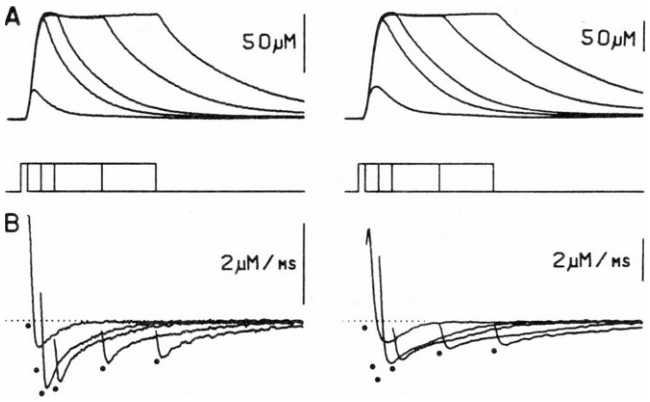

FIGURE 4 Method 3 of calculating slow calcium removal. (*A*) "Fast calcium transient" $Ca_F = Ca + CaD_2 + \Sigma CaB_F$ calculated for five calcium transients elicited by voltage-clamp pulses to −20 mV of 10, 30, 50, 120, and 200-ms duration (pulse schematics, *middle row*). (*Left*) ΣCaB_F was represented as E_1Ca with E_1 derived from the removal model fitting procedure described in Methods applied to the same set of data as in Figs. 2 and 3. Four of the records in *A, left,* are identical to those in Fig. 2 *B, left,* and the record for one additional pulse duration not included in Fig. 2 is shown here. The analysis shown on the right started from the identical Ca records as used on the left but assumed intrinsic rapid Ca binding to originate exclusively from the Ca specific sites of troponin C, which were assigned the kinetic parameters used in model II of Baylor et al. (1983): $TN_T = 240\ \mu M$, $k_{onTN} = 57.5\ \mu M^{-1}\ s^{-1}$, $k_{offTN} = 115\ s^{-1}$. Since these values resulted in a larger amplitude of fast intrinsic binding, the records on the right were scaled for the figure by a factor of 0.52 so that the peaks of the longest records (200 ms) matched. (*B*) Numerical derivatives of each of the records in *A* starting at the first point after pulse off. The ratio of absolute scales between left and right side is the same as in *A*. The dots represent the initial rates of decay of "fast calcium" extrapolated from the maximum negative value of each derivative assuming linearity in calcium for the early decay of Ca_F (Eq. 21). The initial rate of decay is (with inversed sign) the rate of slow removal at the end of the pulse (Eq. 9). Interpolation of the dots by straight lines and changing sign therefore provides an estimate of the rate by which calcium is slowly removed (dCa_{REM}/dt) from the rapidly equilibrating calcium pool during the longest calcium transients shown in the figure. Note: for clarity of the figure we omitted one record of the sequence obtained with a 20-ms voltage-clamp pulse, but included the dot showing the calculated initial rate of decay of Ca_F following the pulse of the omitted record.

were applied very close in time during the course of the experiment, which ensured that the dye concentration did not change appreciably during the sequence. Fig. 4 *A* presents the Ca_F records for each of the pulses. On the left, ΣCaB_F was chosen to be E_1Ca, with E_1 set equal to the value derived from the removal model analysis of Fig. 2 (*left*). Thus, the records in Fig. 4 *A* (*left*) are identical to those in Fig. 2 *B* (*left*). One record was omitted from Fig. 2 for clarity of presentation, but was included in the analysis. In the analysis shown in Fig. 4 (*right*), ΣCaB_F was chosen to be the CaTN calculated using Eq. 19 with the kinetic parameter values given in the Fig. 4 legend (Baylor et al., 1983; model II). Note the difference in scale between the left and right panels of Fig. 4 *A* due to the larger extent of intrinsic fast binding in the latter case.

Fig. 4 *B* shows the numerical derivatives of each of the records in Fig. 4 *A* starting at the first sample point after pulse repolarization. As can be seen in Fig. 4 *B*, the derivative was not at its minimum at the first point after repolarization. For short pulses it was even positive, indicating that release did not stop instantaneously and even exceeded removal at the end of the short pulses. To derive the initial rate of removal, which is equal to minus the initial rate of decline of "fast calcium" only under the assumption of zero release at the end of the pulse (Eq. 8), we took the derivative at some later point after repolarization (when release can be assumed to be zero) and extrapolated back to the last point of the ON using

$$\lim_{t_{off} \longrightarrow t} \frac{dCa_F}{dt_{off}} = \frac{Ca(t)}{Ca\,(t_*)} \left(\frac{dCa_F}{dt}\right)_{t_*} . \qquad (21)$$

t_* in this case was chosen to be the time of the maximum negative value of the derivative during the relaxation period. This extrapolation assumes somewhat arbitrarily that the rate of removal of fast calcium was proportional to the free calcium concentration over the back extrapolation interval. The initial rates of removal derived in this way are represented as dots in Fig. 4 *B*. We also included one value (at 20 ms) whose corresponding record has been omitted for clarity of the figure.

Fig. 5 shows the final steps in the rate of release calculation using method 3. Fig. 5 *A* (*left* and *right*) repeats the 200 ms records of Fig. 4 *A*. In *B*, the numerical derivatives of both records are shown, i.e., the term dCa_F/dt of Eq. 5a. Fig. 5 *C* shows the initial rates of Ca removal estimated in Fig. 4 *B* at discrete points in time and interpolated by straight lines. Fig. 5 *D* shows the sum of the records in *B* and *C* up to the end of the pulse, which gives the rate of calcium release dCa_{REL}/dt (Eq. 5a). There is the same difference in scale between right and left columns here as in Fig. 4, the peak of the rate of release being larger for CaTN than E_1Ca, which again results from the considerably larger extent of fast intrinsic binding when using published troponin parameters. Even though the scale is quite different the time course of dCa_{REL}/dt is not. The

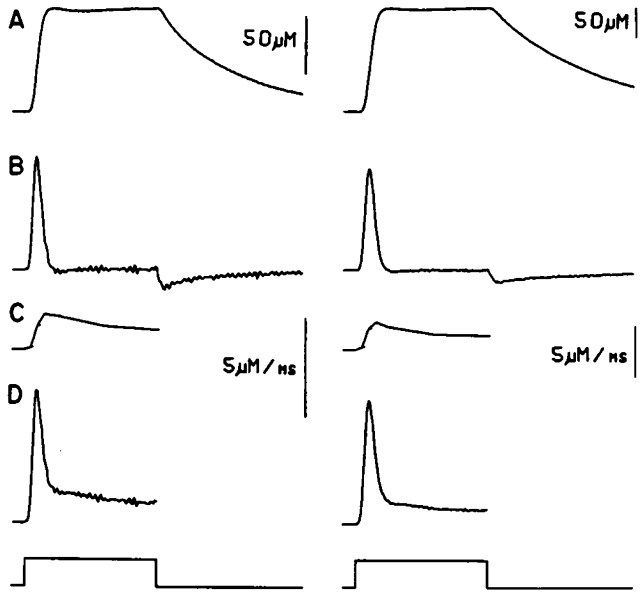

FIGURE 5 Procedure for calculating the rate of release for the longest (200 ms) calcium transient of the previous figure. The estimates of fast intrinsic calcium binding and slow calcium removal for the two sides are from the corresponding sides of Fig 4. (*A*) Fast calcium transients (same as the ones in Fig. 4 for the longest pulse (200 ms). (*B*) Numerical derivatives of *A*, i.e., dCa_F/dt. (*C*) Slow rates of removal obtained using the procedure of Fig. 4 *B* (dCa_{REM}/dt of Eq. 9). (*D*) Rates of release calculated as the sum of *B* and *C*.

characteristic early peak of the release flux and the drastic decline to an approximately steady level are still the same.

In Fig. 6, the two rate-of-release estimates of Fig. 5 and the calculation using method 1 for the same calcium transient are superimposed, in original scale (*left*) and scaled so that the peaks match (*right*). It is clear that substituting CaTN for E_1Ca does not considerably distort the estimate of the time course of rate of release. In particular, the conclusion that a strong inactivation of the release flux takes place is not affected.

Dye Effect on Rate of Release

In some cases, it was possible to carry out the release analysis at different times during an experiment with quite different loading of the muscle cell with the indicator dye. Some of these experiments showed a gradual decline in the peak rate of release occasionally accompanied by other signs of fiber run down like drastic slowing of relaxation kinetics or increase in holding current. In other cases, however, the release estimate did not change much over the course of the experiment. An example of such a case is presented in Fig. 7. There is a considerable difference in the extent of calcium binding to the indicator dye between the first record (459 μM dye) and the last record in this figure (790 μM dye) thus changing the relative size of $dCaD_2/dCa$ and $d\,\Sigma CaB_F/dCa$. If $d\,\Sigma CaB_F/dCa$ was very different from E_1Ca due to saturation, one should expect large changes in the time course of the rate of release estimate the smaller the relative importance of binding to

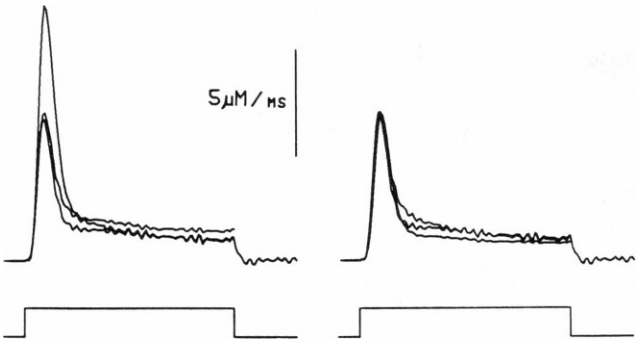

FIGURE 6 Comparison of release calculations using three different procedures. (*A*) Rate-of-release waveforms as calculated in Fig. 5 but here in absolute scale for the same 200-ms pulse. These two calculations both used method 3 for characterizing removal. Also superimposed is the rate of release estimate from Fig. 2 (*right*) for the same calcium transient, obtained using troponin for fast intrinsic binding site and method 1 for characterizing slow removal. The estimate exhibiting the largest peak is the one using troponin C as the fast intrinsic binding component with the parameters given by Baylor et al. (1983). Note that the larger extent of fast intrinsic binding originating from the choice of literature values that are not in accordance with the empirical analysis of this experiment results in a larger $E(\mathrm{Ca},t)$ and therefore larger absolute values for the rate of release (Eq. 10a). The relative time course of the rates of release using the three different calculations is nevertheless very similar. (*B*) Same records as in *A* but normalized to their peak values.

intrinsic buffers over binding to dye molecules gets. This is, however, not the case. From this finding, one can also draw the conclusion that $E_1\mathrm{Ca}$ fits the time course of intrinsic fast buffering quite well. Any of our three procedures should therefore, within the limits of the experimental

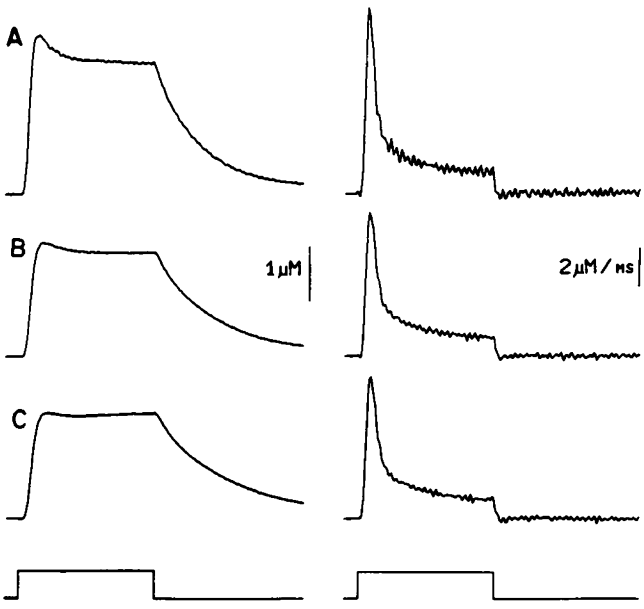

FIGURE 7 Calcium transients (*left*) and calculated rates of release (*right*) at three different dye concentrations during an experiment: 459 μM (*A*), 685 μM (*B*), and 790 μM (*C*). The calcium transient for 790 μM dye (*C*) was the longest transient used for Figs. 2–6. The records at lower dye were obtained earlier during the experiment. For calculating the rate of release the procedure of Fig. 2 (*left*) was used (removal model fit to the relaxation phases of the calcium transients, method 1).

method, give a rather reliable estimate of the voltage-dependent calcium release from the SR.

If we accept that under our experimental conditions $\Sigma\mathrm{CaB_F}$ of Eq. 5 can satisfactorily be approximated by some constant multiple of free calcium, we do not have to know E_1 exactly to derive the time course of rate of release. The deviation of $\mathrm{CaD_2}$ from linearity is usually not very large. This means that over- or underestimating E_1 will basically just cause a difference in scaling of the rate of release and to a good first approximation the time course of the release flux is given by

$$\frac{1}{E}\frac{\mathrm{dCa_{REL}}}{\mathrm{d}t} = \frac{\mathrm{dCa}}{\mathrm{d}t} - \lim_{t_{off} \longrightarrow t^+} \frac{\mathrm{dCa}}{\mathrm{d}t_{off}}. \qquad (22)$$

DISCUSSION

In this paper, we have described and compared several alternative implementations of a general procedure that we have developed for using measured myoplasmic calcium transients to calculate the rate of calcium release from the sarcoplasmic reticulum into the myoplasm via SR calcium channels that are activated during voltage-clamp depolarization of a skeletal muscle fiber. In principle, the calcium release flux that we calculate with any of our analyses should include both calcium release from the SR and any net calcium entry into the fiber via surface or transverse tubular membranes. However, our calculated rate of calcium release peaks at times when the slow calcium channels of surface or T membranes should still be largely closed (Sanchez and Stefani, 1978; Cota et al., 1983). The transmembrane current measured electrically in fibers under the conditions of our present experiments indicates negligible time dependent ionic current across the surface or T membranes during pulses of up to a few hundred milliseconds (Melzer et al., 1986*b*). Thus, the total rate of calcium entry into the myoplasm that we calculate with our procedures should give the rate of release of calcium from the SR, with negligible contribution from calcium influx via surface or T membranes. This conclusion is further supported by recent measurements of Brum et al. (1986) demonstrating that the peak rate of calcium release increases with increasing pulse depolarization up to +40 mV and then becomes constant for further depolarizations up to +90 mV, the largest depolarization explored, whereas current through the slow calcium channel reverses its direction over this voltage range.

Common Aspects of All Our Release Calculations

Each of the particular analysis routines described in this paper follows the same general approach. First, all the calcium binding and transport systems present in the myoplasm are divided conceptually into two categories: (*a*)

those that equilibrate relatively rapidly with the free myoplasmic calcium, Ca, and are represented by the pool of "fast" calcium, Ca_F, and (*b*) those that equilibrate more slowly with Ca or transport calcium out of the myoplasm are referred to as the "removal" system and are represented by the pool of removed calcium, Ca_{REM}. Second, a particular relationship between Ca and Ca_F is assumed. Third, the characteristics of the removal system are determined from analysis of the decline of Ca_F following various pulses. This characterization of the removal system is based on the rate at which calcium is removed from Ca_F following repolarization and depends on the assumption that release is completely turned off throughout the period after repolarization during which removal is characterized. Fourth, the characteristics of the removal system determined after the pulse are used to calculate the rate of calcium removal during the pulse. This depends on the assumptions that the properties of the removal system are independent of both fiber membrane potential and of the degree of activation of calcium release. Finally, the rate of change of Ca_F during the pulse is calculated and added to the rate of removal to give the rate of release of calcium from the SR during the pulse.

The assumptions underlying the analysis of the decay of Ca_F following various pulses to characterize the slow removal system and the use of this characterization to calculate the rate of calcium removal during a pulse are basic to our approach and are common to all routines for calculating calcium release that are described in this paper. In all cases, we assume that the processes of binding and transport by the slow removal system are independent of fiber membrane potential and of activation of SR calcium release. Indeed, the same assumption must be made about the rapidly equilibrating calcium binding components. This assumption does not appear to be questionable for calcium binding to troponin, to paravalbumin, or to dye dissolved in the myofilament solution, all of which should be independent of any direct membrane influence. However, if there were changes in SR membrane potential during release, transport by the SR calcium pump might be altered. If this were the case, the quantity dCa_{REL}/dt calculated here would not be identical to the calcium flux from the SR via the SR release channels during a pulse but would also contain a contribution arising from voltage-dependent changes in the operation of the calcium pump. However, our previous analysis of calcium removal indicates that calcium binding to slowly equilibrating saturable sites, presumably on parvalbumin, may make a larger contribution than the pump to the overall calcium removal system under the conditions of the present experiments (Melzer et al., 1986*a*). Furthermore, at the time of the peak rate of calcium release, the total contribution of flux to the removal system is relatively small compared to dCa_F/dt so that possible voltage-dependent changes in calcium transport should not lead to serious errors in the calculated peak rate of release.

The various individual analysis routines described here differ from each other in one or both of the following respects: the assumptions regarding the properties of the fast calcium system (i.e., instantaneous or noninstantaneous equilibration, etc.) and/or the details of the formalism used to characterize the slow removal system. It should be stressed that if (*a*) the properties assumed for the fast system correspond exactly to the actual properties of the fast binding sites present in the fiber and (*b*) the basic assumptions regarding the characterization of the slow removal system after a pulse and the use of this characterization during a pulse are valid, then any formalism for the slow removal system that accurately describes the decline of Ca_F will lead to a correct calculation of the rate of calcium release. Furthermore, no error would be introduced into the calculated release if any additional binding sites that equilibrate slowly with calcium and are actually present in the fiber were to be included explicitly in the properties assigned to the "fast" system, provided that correct concentrations and rate constants were used for the slow sites assigned to the fast system. In terms of Fig. 1 *B* and Eq. 5, in this case some elements of ΣCaB_S would be included in ΣCaB_F, "fast" calcium (Ca_F) would therefore decay more slowly following a pulse and the resulting characterization of the slow removal system would simply be reduced according to the properties of the slow sites that were assigned to the fast system. The calculation of calcium release (Eq. 5a) would not be altered since the increase in dCa_F/dt would exactly equal the decrease in dCa_{REM}/dt. In contrast, release would be underestimated by our analysis if a group of instantaneously equilibrating sites actually present in the fiber were to be omitted from the properties assigned to Ca_F. In this case, the calculated release would be correspondingly underestimated because there is no way that our procedure for characterizing the slow removal system can compensate for such an omission.

Our general procedure for calculating calcium release from the SR was recently seriously criticized by Luttgau and Stephenson (1986) on two grounds: (*a*) that we did not separate the efflux from the SR via the calcium release channels from the binding and uptake fluxes contributed by the SR calcium pump and (*b*) that we lumped slow calcium removal by binding to slowly equilibrating non-SR calcium binding proteins with binding and transport by the SR calcium pump. Neither of these is a valid criticism of our procedure. Fig. 1 *B* and Eq. 5 show that our approach is explicitly designed precisely to extract the calcium flux from the SR via the calcium release channels without any contribution from the calcium pump. Fig. 1 *B* also shows that for purposes of calculating calcium release from the SR it is not necessary to separate the components due to slow binding from those due to the calcium pump when characterizing the removal of calcium from the pool of fast calcium. Indeed, one of the advantages of our approach is that it does not require separation and characterization of

the individual components contributing to the slow removal system.

Comparison of Methods for Characterizing Removal

In this paper, we have presented three alternative methods for characterizing the removal system. In principle, each method of characterizing removal could be used with various assumed models for the relationship between free and fast calcium. In practice, a particular removal model may be inconsistent with a particular assumed model for fast calcium and thus may not be capable of providing a good description of the decline of Ca_F following fiber repolarization.

Method 1 uses a model of saturable binding and first order transport to represent the slow removal system. A major advantage of this method is that the parameter values for the removal model can be determined from the decay of calcium following a few pulses having an appropriate range of amplitudes and durations. This removal model can accurately reproduce the decay of Ca following pulses of up to a few hundred milliseconds (Melzer et al., 1986*a*), but it requires modification if pulses of up to several seconds are considered (Melzer et al., 1986*a*; Rios, E., and G. Brum, manuscript in preparation). A limitation of method 1 that we have considered in the context of the present results is that it can only be used with a model for Ca_F that is fairly close to an instantaneous linear function of Ca. Since the release wave forms calculated assuming linear fast intrinsic calcium binding sites are similar to those calculated using troponin C as a fast intrinsic site, it seems reasonable to continue to use method 1 for routine studies of calcium release (Schneider and Simon, 1986; Simon and Schneider, 1987).

Method 2 is based on the finding that in many fibers the decay of the calcium transient following fiber repolarization follows a single exponential time course and that the rate constant for this exponential decay decreases with increasing pulse duration. It involves the use of a semi-empirical equation to provide a continuous description of the time course of the decay rate constant during a pulse. This method is somewhat less convenient than method 1 because it requires a range of pulse durations for each voltage to characterize the variation of decay rate constant. For this reason, we no longer use method 2 for routine determinations of SR calcium release, although it was the method used in our original description (Melzer et al., 1984).

Method 3 is the most general of the three methods since it is independent of any specific model for the removal system or for the decay of Ca following fiber repolarization. In this method, a set of properties are assumed for the fast calcium system and the time course of Ca_F is calculated from the calcium transient. The slow removal system is then characterized empirically from the rate of decay of Ca_F following fiber repolarization, which is calculated numerically as the derivative of the Ca_F record. This allows complete freedom in the choice of the assumed fast buffer system since the fast buffer does not have to be consistent with any specified removal system in reproducing the decline of calcium after various pulses. For this reason, method 3 is the approach of choice for evaluating various possible alternative characteristics of the intrinsic fast calcium buffering properties of muscle fibers as we have done in this paper. A major disadvantage of method 3 is that precisely because of its empirical nature and independence from any specific model for calcium removal it does not provide a continuous estimate of the time course of the slow calcium-removal system. The time course of removal must be obtained by numerical interpolation between discrete values of the rate of decay of Ca_F following pulses of various durations. Method 3 is thus rather inconvenient for routine determination of calcium release time courses and has not been used in our other previous or current studies of calcium release.

Other Procedures for Calculating SR Calcium Release

Our general procedure for determining the time course of SR calcium release from a Ca record is not the only one currently available. One alternative approach is to set values for the concentrations of the major calcium binding sites in the myoplasm and for the rate constants for calcium binding and unbinding to each site. With these parameters in hand, the time course of calcium bound to each type of site can be calculated numerically from the calcium transient. The net rate of calcium release is then simply given by dCa/dt plus the sum of the rates of change of concentration of each calcium complex. Theoretical calculations based on this approach using assumed release or Ca waveforms were first carried out by Robertson et al. (1981) and by Gillis et al. (1982). The approach was applied to Ca records actually measured in skeletal muscle fibers by Baylor et al. (1983). Their results indicated a pronounced decline in calcium release following the first action potential in a train of action potentials and during a voltage-clamp pulse to slightly beyond the voltage for a measurable Ca. It should be noted that the calcium release parameter calculated by Baylor et al. (1983) and denoted by them as dCa_T/dt differs slightly in definition from the rate of release (dCa_{REL}/dt) that we calculate. Their dCa_T/dt is the net overall flux from the SR, which is given in our notation by the difference $dCa_{REL}/dt - dCa_U/dt$ between the efflux from the SR via the voltage-gated release channel and the influx into the SR via the calcium pump. In contrast, we calculate only the efflux via the voltage gated channel.

A limitation of the preceding approach is that its accuracy depends on the accuracy of the parameter values used in the calculation and on the accuracy of the absolute

calibration of Ca. Some of the calcium reaction rate constants have been determined only at room temperature for proteins of rabbit muscle, so there is some uncertainty as to the appropriate values for frog fibers at lower temperatures. Also, the concentrations or rate constants for various sites may vary with the state of the fiber or animal. For example, we have observed considerable fiber to fiber variation in intrinsic rapid calcium buffering activity. Finally, the resting levels of free calcium and magnesium have not been determined in fibers under the conditions of our experiments. Since our procedure for determining the time course of SR calcium release relies primarily on a series of measurements carried out on the particular muscle fiber under investigation, it should be able to at least partially take account of some of these uncertainties.

Another approach that has been used to approximate the time course of calcium release from the SR in frog muscle fibers is to use the time derivative of the absorbance change recorded from fibers containing the dye arsenazo III. This approach was suggested and used by Miledi et al. (1982, 1983) without detailed consideration of its theoretical basis and was subsequently analyzed and used by Rakowski et al. (1985). As mentioned at the start of the Theory section, this approach would be perfectly accurate if the rate of change of calcium-dye were the only significant term in Eq. 5. However, even if the pool of calcium bound to intrinsic rapidly equilibrating calcium binding sites were not negligible but were proportional to calcium–dye, and if the slow removal component were small relative to dCa_F/dt, then the use of the derivative of the dye signal would give a good approximation of the calcium release time course. These conditions may apply fairly well at the time of peak calcium release, but they clearly break down at later times during a depolarizing pulse.

The first use of measured calcium transients and an equation similar to Eq. 5 to evaluate calcium movements during muscle activation was that of Ashley and Moisescu (1973) and Ashley et al. (1974), who analyzed simultaneous aequorin light emission and mechanical force measurements from barnacle muscle fibers. In these analyses, the values of the on and off rate constants for the calcium troponin reaction were adjusted so that the measured force transient was well reproduced based on the requirement that force be proportional to the fraction of troponin molecules having both binding sites occupied by calcium. The parameters selected in this way showed troponin to be relatively slowly equilibrating with the free myoplasmic calcium (Ashley and Moisescu, 1972). It is uncertain as to what extent possible internal shortening and the recently documented increase of troponin affinity for calcium with cross bridge attachment (Ridgway et al., 1983; Ridgway and Gordon, 1984), which were not considered by Ashley and Moisescu (1972), may have influenced their choice of troponin rate constants.

For calculating calcium release, Ashley et al. (1973, 1974) assumed that rapidly equilibrating intrinsic calcium binding sites made little contribution and did not consider slow sites other than troponin. Calcium bound to troponin was evaluated using rate constants that reproduced the force records (above) and the remaining calcium movements were assigned to the SR release channel or to the SR calcium pump. Using various assumptions regarding the functional forms of the kinetic equations for the various SR fluxes, Ashley et al. (1973, 1974) showed that a form of calcium-dependent calcium release could be consistent with the barnacle data. However, since release was calculated by assuming a particular functional dependence on Ca, it is not clear to what extent the assumed functional forms of the SR fluxes influenced the calculated release wave form. Our approach contrasts sharply with that of Ashley et al. (1973, 1974) since we make no assumptions regarding the functional form of the SR calcium release system but assume only that release was turned off by ~15 ms after repolarization of our voltage-clamped frog fibers. Aside from this assumption, our analysis was designed to extract the release wave form with minimal assumptions regarding the kinetic forms of the various components in the fiber.

Present Uncertainties and Future Directions

Any procedure for calculating calcium release from measured myoplasmic calcium transients depends on an accurate measurement of both the time course of the calcium–dye complex and of the calcium transient itself. Our calculations are based on calcium transients calculated assuming that all the dye present in the fiber behaves as we have reported for dye in calibrating solutions (Kovacs et al., 1983; Rios and Schneider, 1981). This requires that the dye be in solution in the fiber and available for reaction with calcium. However, recent experiments indicate that an appreciable fraction of the dye in the fiber may be bound to myoplasmic constituents (Irving et al., 1985; Baylor et al., 1986). If the bound dye were in very rapid equilibrium with free dye and if bound dye were unable to react with calcium, the binding of dye to myoplasmic constituents would only increase the apparent dissociation constant of the calcium dye reaction and would simply alter the absolute scale of the calcium transients without altering their time course. At the opposite extreme, if bound dye were to dissociate very slowly compared to the time course of the calcium transient and if bound dye were unable to react with calcium, the dissociation constant of the calcium–dye reaction would not be altered but the effective concentration of dye would be reduced. This might alter the time course as well as the amplitude of the calculated calcium transient if the degree of saturation of the dye by calcium were significantly more pronounced for the lower effective dye concentration. Intermediate dissociation rates of bound dye and/or the ability of bound dye

to react with calcium but in a different way than the free dye would introduce further complications and distortions of the calcium transient. Another possible complicating factor, which has been totally ignored in our analysis, is the possible existence of spatial gradients of calcium that give rise to spatial gradients of saturation of dye or of saturation of any other calcium binding or transport system (Cannell and Allen, 1984). It remains to be determined to what extent these various possibilities actually influence the calculated calcium transients and release wave forms.

As the properties of the dye in the fiber become better understood it will be possible to repeat our procedures using refined estimates of calcium transients so as to calculate correspondingly refined estimates of rates of calcium release from the SR. Since the overall approach used in our procedures is rather general, it might also be applied with appropriate modification in the future to calculate time courses of rates of calcium release from internal stores or rates of calcium entry across surface membranes of various other cell types in which such calcium movements can be halted relatively rapidly by changes in cell membrane potential.

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