LETTER TO THE EDITOR

Determining the Rate of Calcium Release from the Sarcoplasmic Reticulum in Muscle Fibers

Dear Sir:

In the preceding letter (Stephenson, 1987) Dr. Stephenson questions our general procedure for using calcium transients recorded from voltage clamped skeletal muscle fibers to determine the rate of calcium release from the sarcoplasmic reticulum (SR). Dr. Stephenson maintains (*a*) that our approach "... does not lead to determination of the actual rate of Ca^{2+} release from the SR into the sarcoplasm" and (*b*) that we "... cannot extract from [our] data the true rate of Ca^{2+} release from the SR into the sarcoplasm, without [our] knowing the kinetics of calcium movements associated with fast and slow Ca^{2+} binding sites in the sarcoplasm and the rate of Ca^{2+} removal from the sarcoplasm by the SR."

We believe that our paper in the present issue of this journal (Melzer et al., 1987) shows that neither of Dr. Stephenson's contentions is correct and that his present criticisms are based largely on his incorrect representation of our general procedure. In this letter we attempt to point out the errors in Dr. Stephenson's representation of our approach. We also demonstrate, using precisely the two classes of binding sites in Dr. Stephenson's example, that our approach can indeed extract the time course of the rate of calcium release from the SR into the sarcoplasm without any prior information regarding the concentrations or kinetic properties of the fast and slow calcium binding sites in the sarcoplasm.

Dr. Stephenson's Eq. 1 for sarcoplasmic calcium movements,

$$dCa^{++}/dt = (dCa/dt)_{\substack{\text{SR release into the}\\ \text{ionized Ca compartment}}} - (dCa/dt)_{\substack{\text{removal from the}\\ \text{ionized Ca compartment}}}, \quad (1)$$

is a valid equation that can be rearranged to give the rate of calcium release from the SR. Using such an equation for calculating release, the removal term would include the rate of calcium binding to both fast and slow sites as well as the rate of calcium transport back into the SR as Stephenson suggests. However, we do not use Stephenson's Eq. 1 in our analysis.

The first step in our procedure is to assume properties for the fast binding sites so that we can determine the pool of rapidly equilibrating ("fast") calcium Ca_F from the measured Ca^{2+} transient. We then use the equation:

$$dCa_F/dt = dCa_{REL}/dt - dCa_{REM}/dt, \quad (2)$$

where the removal term (dCa_{REM}/dt) now corresponds only to binding to slowly equilibrating sites plus transport back into the SR. Since we use a rearranged form of Eq. 2 to calculate the rate of calcium release (dCa_{REL}/dt) from the SR (Melzer et al., 1987, Eq. 5a), we must only determine the contributions of slow sites and transport in order to evaluate the removal term in our expression for dCa_{REL}/dt. We do not attempt to determine experimentally the calcium binding properties of the fast sites. Thus Dr. Stephenson's valid demonstration that the properties of relatively rapidly equilibrating sites cannot be directly determined from back extrapolation of the decay of a calcium transient is entirely irrelevant as a criticism of our procedure. We make no attempt to characterize rapidly equilibrating sites as part of the removal term in our calculations.

Yet, our release calculations must to some extent depend on the properties assumed for the fast binding sites since those properties determine the conversion from free to fast calcium. For this reason we have carried out calculations using alternative sets of assumed properties for the intrinsic fast binding sites in muscle fibers (Melzer et al., 1987). The results show that aside from variations in absolute size, the calcium release records calculated assuming alternative properties for the fast sites were quite similar, all showing an early peak followed by a decline toward a much lower maintained level (Melzer et al., 1987). Using an entirely different analysis procedure Baylor et al. (1983) also concluded that the properties assumed for the fast sites had relatively little influence on the calculated calcium release wave form.

The second step in our procedure is the characterization of the overall properties of the slow binding sites and transport systems that remove calcium from the fast pool. We do this by analyzing the decay of the calcium transient beginning at a time when the SR is assumed to have stopped releasing calcium. In our Method 3 for calculating removal we carry out an empirical back extrapolation to the instant of fiber repolarization. In this case the relatively small error that Dr. Stephenson found for his slower sites would indeed be present in our calculation. The error arises from the fact that a change in calcium occupancy of the slow sites occurred in Dr. Stephenson's simulation during the interval over which the back extrapolation was carried out. In the actual frog muscle fibers used in our experiments the slow sites are predominately on parvalbumin, which binds calcium more slowly than the slow sites in Dr. Stephenson's simulation. Thus the already small extrapolation error in Stephenson's simulation would be correspondingly reduced in an actual experiment. Furthermore, the extrapolation that Dr. Stephenson criticizes is only used in our Method 3 for calculating removal. In our standard Method 1 we characterize the calcium binding properties of the slow sites and explicitly calculate their occupancy at any time, with no need of an empirical back extrapolation and no introduction of the extrapolation error referred to by Dr. Stephenson.

We do recognize and have clearly stated that our characterization of the removal system does depend on the assumption that release is turned off during the interval over which we use the decay of Ca^{2+} to characterize removal. There are some indications that this assumption is valid (Rios, 1984), but further experimental tests of the assumption would be welcome.

To further evaluate the validity of our procedure, we have carried out simulations using binding sites that have precisely the

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properties used in Dr. Stephenson's simulation. We began by assuming that the rate of SR calcium release followed the time courses in Fig. 1 *A* for pulses of constant amplitude lasting 15, 30, 60, and 120 ms. We also assumed releases of similar time courses but approximately half the amplitude (not shown) for pulses of 20, 40, 80, and 160 ms corresponding to a smaller simulated depolarization. The release was assumed to occur into a system consisting of 100 μM of Dr. Stephenson's faster sites, 500 μM of his slower sites, and 500 μM of antipyrylazo III, with no other binding sites or transport systems present. The resulting free calcium transients are shown in Fig. 1 *B* for the releases in Fig. 1 *A*. The simulated Ca^{2+} transients differ from actual experimental calcium transients in two respects: (*a*) the decay after cessation of release is faster in the simulations and (*b*) the final level of Ca^{2+} after the pulse is much higher in the simulations. Difference *a* occurs because the slow sites in the simulation are faster than in muscle (see above) and *b* occurs because no calcium transport system is present in the simulations.

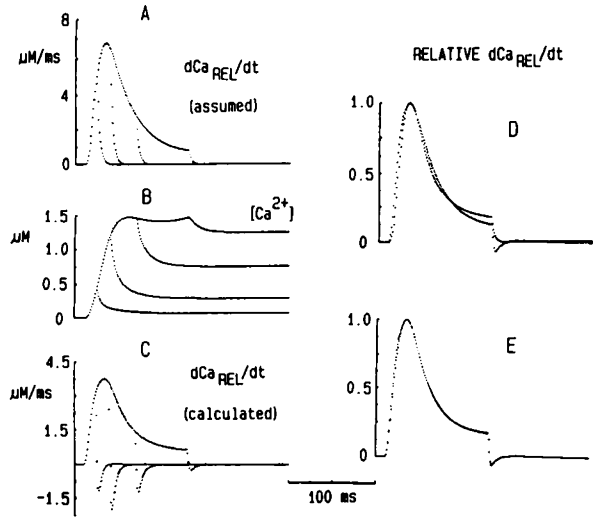

FIGURE 1 Test of the procedure used to calculate the rate of calcium release from the sarcoplasmic reticulum. (*A*) Superimposed records of the rate of calcium release assumed to occur for depolarizing pulses of 15, 30, 60, and 120 ms. During each pulse release was assumed to be the product of the fourth power of an exponentially rising activation variable (time constant = 8 ms) and the first power of an exponentially decaying inactivation parameter (time constant = 25 ms), with steady state activation assumed to be 5% of the completely activated release. After each pulse, release was assumed to turn off exponentially with a 3-ms time constant. (*B*) Simulated free ionized calcium transients generated by assuming the release records in *A* to enter a system consisting only of 500 μM antipyrylazo III (instantaneously equilibrating, apparent K_D = 17,500 μM^2) plus the two types of sites suggested by Stephenson (1987): 100 μM of his faster sites (k_{OFF} = 100 s^{-1}, k_{ON} = 100 μM^{-1} s^{-1}) and 500 μM of his slower sites (k_{OFF} = 10 s^{-1}, k_{ON} = 10 μM^{-1} s^{-1}). Both binding sites have K_D = 1 μM. (*C*) Records of the rate of calcium release calculated for each of the simulated Ca^{2+} records in *B*. For the release calculation the intrinsic fast expansion factor E_I was assumed to equal 30, and removal was characterized by fitting the model of Melzer et al. (1986) simultaneously to the decay of all calcium transients in *B* starting 15 ms after repolarization and to the Ca^{2+} transients (not shown) generated for four other releases similar to those in *A* but about half the amplitude. The least squares removal model parameter values were total site concentration $[S]_T$ = 431 μM with rate constants k_{OFF} = 10.4 s^{-1} and k_{ON} = 5.0 μM^{-1} s^{-1} and a rate constant for linear first order uptake of k_{NS} = −51.1 s^{-1}. To verify that the negative releases calculated after each pulse in *C* were not due to the physically meaningless negative value obtained for k_{NS}, release was recalculated using another fit of the removal model in which k_{NS} was constrained to equal 0.01 s^{-1}. The resulting release records (not shown) were found to be essentially the same as those in *C*. (*D*) Release records for the longest pulses in *A* and *C* scaled to the same peak value and superimposed. (*E*) The release record for the longest pulse in *C* and an analogous record obtained by analyzing the identical Ca^{2+} transients but assuming E_I = 5. The two records are scaled to the same peak and superimposed. The peak rate of release calculated assuming E_I = 5 was 41% of the peak release for E_I = 30.

The calcium transients in Fig. 1 *B* and the set (not shown) corresponding to the smaller releases were analyzed according to our general procedure for calculating release. We assumed a linear instantaneous intrinsic fast binding system giving an expansion factor (Melzer et al. 1986) of E_I = 30 and used our standard Method 1 for characterizing removal. The calculations were kindly carried out for us by Dr. Bruce Simon, who was unaware of both the release records and the parameter values used to generate the calcium transients that he analyzed. The resulting release records obtained from the calcium transients in Fig. 1 *B* are presented in Fig. 1 *C*. The wave form of the calculated release (*C*) for the longest pulse is similar to the actual release record (*A*) used in the simulation. Both rise to an early maximum and then decline toward a small fraction of the peak value. The amplitude of the calculated record is smaller than the actual release, indicating that the assumed expansion factor of 30 was too small.

The release records (Fig. 1 *C*) calculated for the shorter pulses show relatively large negative rates of release at the cesssation of the pulse. This is because the faster sites have an appreciable delay in equilibration, as shown by Dr. Stephenson, whereas we assume in our calculation that the fast sites are in instantaneous equilibration. We therefore overestimated the magnitude of the negative dCa_F/dt during the rapid phase of decay of Ca^{2+} after the shorter pulses. It is important to note that we have never obtained such negative "tails" in release records calculated for short pulses in any real muscle fiber, presumably indicating that the decay of Ca^{2+} in fibers is sufficiently slow that the fast sites are close to equilibrium throughout the decay of Ca^{2+}.

In Fig. 1 *D* we compare the time courses of the actual and calculated release records for the longest pulse, with both records scaled to the same peak value. The time courses are seen to be quite similar, clearly demonstrating that our procedure could extract a fairly accurate temporal wave form of the rate of calcium release despite the fact that the binding site properties used to generate the simulated calcium transients were quite different from the model we use to carry out the release calculation.

The scale factor, but not the time course of the calculated release, depends on the value assumed for the fast expansion. In Fig. 1 *E* we superimpose the release record for E_I = 30 with another record obtained by repeating the entire release analysis on the same simulated calcium transients, but with E_I now assumed to equal 5. When scaled to the same peak value the two records are virtually identical. Thus our determination of the release wave form is independent of the value used for E_I.

In conclusion, we find Dr. Stephenson's objections to our procedure ill founded in principle and believe that we have demonstrated, using the calcium binding properties selected by

him, that our procedure can indeed extract the actual temporal wave form of the rate of calcium release from the SR.

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REFERENCES

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1983. Sarcoplasmic reticulum calcium release in frog skeletal muscle fibres estimated from arsenazo III calcium transients. *J. Physiol. (Lond.)*. 344:625–666.

Melzer, W., E. Rios, and M. F. Schneider. 1986. The removal of myoplasmic free calcium following calcium release in frog skeletal muscle. *J. Physiol. (Lond.)*. 372:261–292.

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

Rios, E. 1984. Off gating of calcium release in skeletal muscle. *J. Gen. Physiol.* 84:40a (Abstr.).

Stephenson, D. G. 1987. Calcium release from the sarcoplasmic reticulum. *Biophys. J.* 51:1009–1010.

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