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INACTIVATION OF CALCIUM RELEASE FROM THE SARCOPLASMIC RETICULUM IN FROG SKELETAL MUSCLE

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SUMMARY

1. The rate of calcium release (R_{rel}) from the sarcoplasmic reticulum (SR) in voltage clamped segments of frog skeletal muscle fibres was calculated from myoplasmic free calcium transients ($\Delta[Ca^{2+}]$) measured with the calcium indicator dye Antipyrylazo III.

2. During a 100–200 ms depolarizing pulse R_{rel} reached an early peak and then declined markedly. The time course and extent of decline of R_{rel} were independent of membrane potential over a range of potentials where release activation varied severalfold.

3. For test pulses applied shortly after relatively large or long conditioning pulses R_{rel} completely lacked the early peak. The peak gradually recovered as the interval between the conditioning and test pulses was increased to 1 s.

4. A latency was often observed before the start of recovery of the peak in R_{rel}. The latency appeared to be correlated with the time for $\Delta[Ca^{2+}]$ to fall below a certain level, indicating that recovery of the peak might represent reversal of a calcium-dependent process. It was therefore proposed that the rapid decline in R_{rel} during a pulse was due to calcium-dependent inactivation of the SR calcium release channels.

5. Inactivation continued to develop during the interval between a relatively large 20 ms conditioning pulse and a test pulse applied 20 ms later. This was as expected for calcium-dependent inactivation of SR calcium release because of the elevated $[Ca^{2+}]$ between the conditioning and test pulses. It was not as expected for external membrane potential-dependent inactivation.

6. Small steady elevations in $[Ca^{2+}]$ due to relatively small 200 ms conditioning pulses produced marked inactivation of R_{rel}, indicating an apparent dissociation constant for calcium-dependent inactivation only slightly above resting $[Ca^{2+}]$.

7. All observations could be well simulated by a two-step model for inactivation in which myoplasmic free calcium equilibrates rapidly with a high-affinity calcium

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receptor on the release channel and then the calcium–receptor complex undergoes a slower conformational change to the inactivated state of the channel.

8. An alternative model in which calcium binds to a soluble receptor (e.g. free calmodulin) and then the calcium–receptor complex binds to and directly inactivates the channel was shown to be formally identical to the preceding model. Either model could closely simulate all observations.

INTRODUCTION

Depolarization of the transverse (T-) tubular membrane in a skeletal muscle fibre leads to release of calcium from the terminal cysternae of the adjacent but electrically and structurally distinct sarcoplasmic reticulum (SR). Under physiological conditions the T-tubular depolarization that triggers calcium release occurs as a result of an action potential spreading longitudinally along the fibre surface membrane and radially into the fibre along each T-system lattice. If a fibre is stimulated to produce a repetitive train of action potentials at a relatively high frequency, the calcium released from the SR by each action potential appears to decline during the first few action potentials in the train, even though the depolarization during each action potential is the same. The decline in release is indicated by a decline in the increment of myoplasmic free calcium produced by successive action potentials (Blinks, Rudel & Taylor, 1978; Miledi, Parker & Zhu, 1983; Baylor, Chandler & Marshall, 1983) and is seen directly in records of the rate of release of calcium from the SR (R_{rel}) calculated for each calcium transient in the train (Baylor *et al.* 1983).

Controlled electrical depolarization of the fibre also activates release of calcium from the SR and provides a convenient means of investigating the basis of the decline in release during repetitive stimulation. With this approach a marked decline in R_{rel} is seen routinely during 100–200 ms depolarizing voltage clamp pulses applied to electrically polarized fibres (Baylor *et al.* 1983; Melzer, Rios & Schneider, 1984, 1987; Rios & Brum, 1987). Examination of the time and voltage dependence of the release waveform indicated that the decline in R_{rel} during a 100–200 ms pulse was unlikely to be due to depletion of calcium from the SR but instead represented a true inactivation of the SR calcium release system (Melzer *et al.* 1984). Subsequent investigation confirmed that depletion gave rise to only a relatively small and slow component of decline in release and that the predominant relatively fast decline in R_{rel} most likely represented a true 'fast' inactivation of the SR calcium release channels (Schneider, Simon & Szucs, 1987*b*).

Activation of SR calcium release appears to be controlled by voltage sensors in the T-tubule membrane (Schneider & Chandler, 1973; Horowicz & Schneider, 1981; Melzer, Schneider, Simon & Szucs, 1986*b*; Rios & Brum, 1987). However, a 100–200 ms depolarization that produces near maximal 'fast' inactivation of release causes no obvious modification in the properties of the intramembrane charge movement generated by the voltage sensors since the On and Off charge movements for such pulses are essentially equal. The fact that the fast inactivation of the SR calcium channels occurs in the absence of noticeable immobilization of the T-tubule charge movement indicates that the fast inactivation process may not occur at the level of the T-tubule voltage sensor but may instead occur at a step beyond the

voltage sensor. A possible mechanism could be a calcium-dependent modulation of the SR calcium channel itself (Baylor *et al.* 1983).

Calcium channels in a variety of cells have been shown to exhibit inactivation mechanisms that depend on cytosolic free calcium and not on membrane potential *per se* (Eckert & Chad, 1984, review). A calcium-dependent inactivation of SR calcium release has been demonstrated in both SR vesicles prepared from skeletal muscle (Meissner, 1986) and in skinned skeletal muscle fibres (Kwok & Best, 1987). The present results are consistent with the hypothesis that a calcium-dependent mechanism may also be responsible for the decline in calcium release that develops during 100–200 ms depolarizations of skeletal muscle fibres with intact T-system and SR. Abstracts of some aspects of this work have been presented previously (Simon, Schneider & Szucs, 1985; Simon & Schneider, 1987).

METHODS

Experiments were carried out on cut segments of single muscle fibres isolated from the semitendinosus or ileofibularis muscles of frogs (*Rana pipiens*, northern variety) maintained at room temperature and fed on crickets. The cut segments were mounted in a double-Vaseline-gap chamber and exposed at both cut ends to a solution containing the calcium indicator dye Antipyrylazo III. The chamber was positioned and connected so as to provide for electrical recording of fibre membrane current and voltage and for optical recording of resting fibre light absorbance and of small absorbance changes during depolarization.

Details of all methods were exactly as reported in the preceding paper from this laboratory (Schneider *et al.* 1987*b*). These include the procedures for preparing and mounting the fibres, for voltage clamping, for simultaneous monitoring of fibre light absorbance signals at 700 and 850 nm, for converting the absorbance signals to myoplasmic free calcium transients ($\Delta[Ca^{2+}]$) and for analysing the calcium transients to calculate records of rate of calcium release from the SR. The 'external' solution applied to the intact region of the fibre in the middle pool and the 'internal' solution applied to the cut ends of the fibre in the end pools were as previously described (Schneider *et al.* 1987*b*). Fibres were notched just beyond the gaps in both end pools (Kovacs, Rios & Schneider, 1983) to minimize both the electrical impedance and the diffusion distance from the end-pool solution to the fibre interior. Experiments were carried out at a holding potential of -100 mV and at 6–10 °C.

Data were collected, stored and analysed as previously described (Schneider *et al.* 1987*b*) using a PDP8-E computer (Digital Equipment Corp, Maynard, MA, U.S.A.) running programs written in FORTRAN IV. Sampling intervals for the present records were 1, 2 or 6 ms/point.

The general procedure used for calculating R_{rel} from measured calcium transients was that developed by Melzer *et al.* (1984, 1987) and Schneider, Rios & Melzer (1985). In the present case we first characterized the calcium removal capability of each fibre by fitting a calcium removal model (Melzer, Rios & Schneider, 1986*a*) to the decay of $\Delta[Ca^{2+}]$ after pulses of several different amplitudes and durations (method 3 of Melzer *et al.* 1987). This characterization of the calcium removal system in each fibre was then used to calculate records of R_{rel} in that fibre. The nature of the model used to characterize removal has no effect on the calculated release provided the removal model accurately describes the decay of $\Delta[Ca^{2+}]$ following the pulses (Melzer *et al.* 1987). The calcium bound to the rapidly equilibrating calcium binding sites intrinsic to each fibre was assumed to be an instantaneous linear function of $\Delta[Ca^{2+}]$ (Melzer *et al.* 1984, 1987; Schneider *et al.* 1985). No attempt was made to quantitate the amount of intrinsic rapid calcium binding in each fibre. Rather, an arbitrary value was simply assumed for the 'intrinsic expansion' E_I arising from calcium binding to the rapidly equilibrating calcium binding sites intrinsic to each fibre (Kovacs *et al.* 1983; Melzer *et al.* 1986*a*). It should be noted that the size assumed for E_I has negligible effect on the time course calculated for R_{rel} but does influence the release amplitude (Schneider, Rios & Melzer, 1987*a*). Therefore, the absolute scale of the release records presented here is rather arbitrary. In contrast, the waveforms of the release records, which are the subject of the present study of inactivation of release, should be accurate (Schneider *et al.* 1987*a*).

RESULTS

Inactivation during a single pulse

Figure 1 shows calcium transients ($\Delta[Ca^{2+}]$), left panel, and the corresponding R_{rel} records, right panel, for changes in membrane potential from a holding potential of -100 mV to the indicated potentials. R_{rel} rose to a peak in about 20 ms and then decayed exponentially to a final level. As the test potential was made more positive

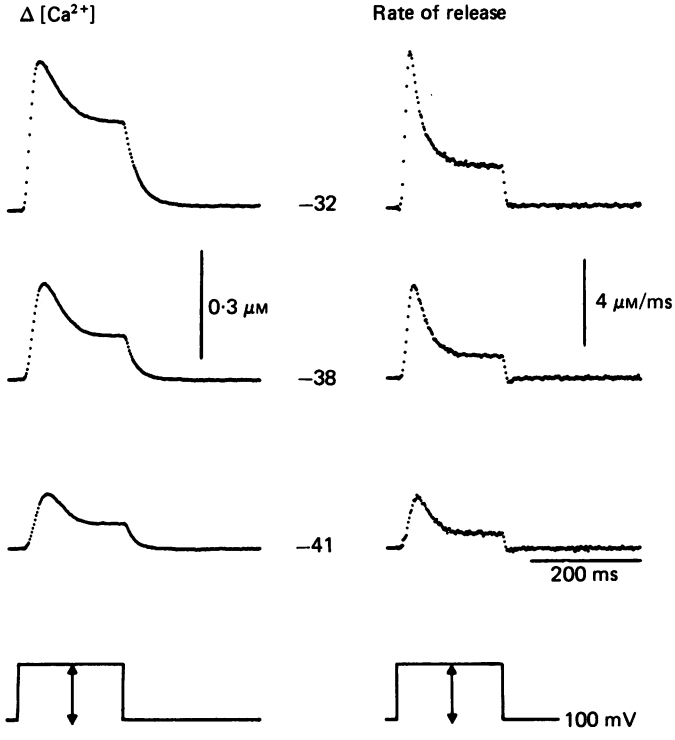

Fig. 1. Time course of the rate of calcium release from the sarcoplasmic reticulum. Calcium transients (left column) and the corresponding rate of release (R_{rel}) records (right column) are shown for 200 ms depolarizing steps to the indicated membrane potentials (mV) from a holding potential of -100 mV. The R_{rel} records rise to an early peak and then decline exponentially to a final steady level. Increasing the amplitude of the depolarizing step increases the amplitude of the R_{rel} but has little effect on the time course or extent of decline during release. Fibre B227, 3·2 μm per sarcomere, 883–1200 μM-Antipyrylazo III (10 °C). R_{rel} calculated assuming $E_I = 20$.

from -41 to -32 mV the peak R_{rel} increased about threefold. In agreement with previous observations (Melzer *et al.* 1984), the R_{rel} time course was similar at each of the three membrane potentials; the time constant and extent of the decline of R_{rel} were independent of membrane potential. The time constants (τ_{dec}) obtained by fitting a single exponential plus a constant to the decay phases of the R_{rel} records for the pulses to -32, -38 and -41 mV in Fig. 1 were 24·4, 24·2 and 24·0 ms, respectively. Table 1 presents values of τ_{dec} obtained in each of ten fibres by fitting

two to five records for pulses to one to three different voltages. Except for the value from the last fibre, which was from a later series of experiments using a different population of frogs, the decay time constants were quite consistent. Excluding the fibre from the later series, the mean ± S.E.M. value of τ_{dec} was 32·3 ± 1·9 ms.

We have previously shown that only a small fraction of the rapid decline in R_{rel} is due to depletion of calcium from the SR and that the majority of the decline represents a true inactivation of the calcium release channel (Simon, Schneider & Szucs, 1986; Schneider *et al.* 1987*b*). If depletion were a major cause of the decline

TABLE 1. Time constants of decay (τ_{dec}) and recovery (τ_{rec}) of peak rate of release

Fibre	τ_{dec} (ms)	τ_{rec} (ms)
217	35	105
218	34	85
222	36	63
223	30	111
224	40	135
227	24	—
229	33	64
232	31	44
236	24	94
310	7*	109
Mean ± S.E.M.	32 ± 2	90 ± 10

* This value not included in the mean.

then the largest release in Fig. 1 (at −32 mV) should have declined faster than the smaller release at −41 mV. This was not observed. The constant degree and rate of decline of R_{rel} for pulses of varying $\Delta[Ca^{2+}]$ originally also seemed to argue against a calcium-dependent inactivation mechanism (Melzer *et al.* 1984). However, it is now recognized that a two-transition model for calcium-dependent inactivation can reproduce this observation (Simon *et al.* 1985 and below).

Recovery from inactivation

To investigate the origin of the decline of R_{rel} during a long depolarizing pulse a double-pulse recovery experiment was performed in which a pre-pulse was followed after a variable recovery time by a constant test pulse. Figure 2 shows the result of such an experiment for a short pre-pulse (30 ms, left column) and a longer pre-pulse (120 ms, right column). The recovery times ranged from 30 to 1200 ms in each case. The two pre-pulses and the test pulse were all the same amplitude (to −20 mV, bottom). The top row in Fig. 2 presents $\Delta[Ca^{2+}]$ records and the middle row presents the corresponding R_{rel} records calculated from each $\Delta[Ca^{2+}]$ record. For short recovery times the early peak was completely absent in the test R_{rel} records. As the recovery time was increased the peak gradually reappeared. For a recovery time of about 1 s the test release was similar in waveform to a test release without any pre-pulse, but was only scaled down in amplitude. For recovery times of 1 s to 1 min, the test pulse R_{rel} followed the control waveform and gradually returned to its control amplitude. This slow recovery represents recovery from depletion (Schneider *et al.* 1987*b*) while the fast recovery shown here is recovery from inactivation.

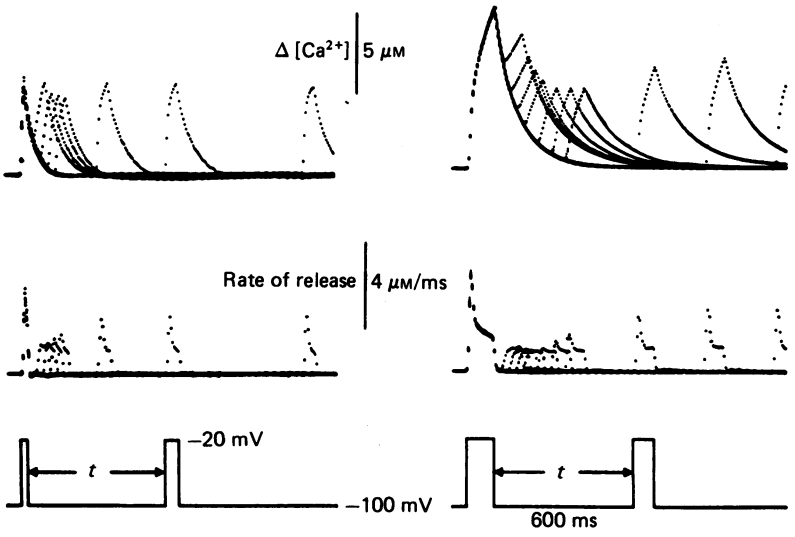

Fig. 2. Calcium transients and calcium release records from a double-pulse experiment studying the recovery of calcium release following a conditioning pre-pulse. A fixed test pulse of 60 ms duration to −20 mV followed a pre-pulse of the same amplitude after a variable recovery time, *t*. The pre-pulse duration was 30 ms on the left and 120 ms on the right. The recovery times varied from 30 to 1200 ms. Calcium transients (top) and the corresponding rate of calcium release (middle) are shown for each pulse protocol (bottom). Note the absence of the early peak in the rate of release records for short recovery times. Fibre B310, 4·0 μm per sarcomere, 270–540 μM-Antipyrylazo III (6·5 °C). R_{rel} calculated assuming $E_I = 5$.

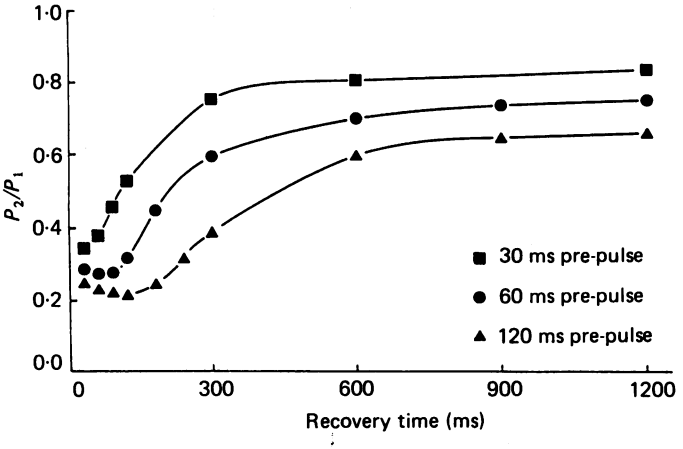

Fig. 3. Recovery time course of the peak rate of release in the double-pulse recovery experiment. The ratio of the peak rate of release (P_2) during the test pulse to the peak rate of release (P_1) during the pre-pulse is plotted as a function of the recovery time for pre-pulse durations of 30 ms (■), 60 ms (●) and 120 ms (▲) duration. Same fibre and conditions as in Fig. 2. The records for the 60 ms pre-pulse were not shown in Fig. 2.

Figure 3 presents the recovery time courses for the 30 and 120 ms pre-pulses in Fig. 2 and for a 60 ms pre-pulse in the same fibre. The ratio of the peak R_{rel} during the test pulse to the peak R_{rel} during the pre-pulse (P_2/P_1) is plotted as a function of the recovery time. Increasing the pre-pulse duration resulted in a greater depression of the test R_{rel} at a recovery time of 1200 ms, corresponding to a greater depletion of calcium from the SR during the longer pre-pulse. A more striking and unexpected effect of increasing the pre-pulse duration was to introduce a delay before the peak began to reappear in the test R_{rel} record. For the 30 ms pre-pulse there was virtually no delay in recovery, for the 60 ms pre-pulse there was a delay of about 100 ms before the start of recovery and for the 120 ms pre-pulse there was even a slight decline in release for short recovery times followed by a delay of about 200 ms before recovery began. A comparison of the calcium transients for the short and long pre-pulses in this experiment (Fig. 2) showed that calcium was elevated for a considerably longer period of time following the 120 ms pre-pulse than following the 30 ms pre-pulse. Following the 60 ms pre-pulse (not shown in Fig. 2) calcium was elevated for an intermediate period. These observations suggest that recovery may not take place until there is a significant decline in $\Delta[Ca^{2+}]$ prior to the test pulse, which would be consistent with a calcium-dependent mechanism of inactivation. Values of the recovery time constant obtained from nine fibres by fitting a single exponential plus a constant to the fast phase of recovery after the initial delay are presented in Table 1. The mean value of the recovery time constant was 90 ± 10 ms.

Inactivation by short pre-pulses

The effects of elevating $[Ca^{2+}]$ prior to a test pulse were further investigated in the experiment shown in Fig. 4. A 20 ms depolarizing pre-pulse of variable amplitude was followed after a short fixed period at -100 mV by a constant 200 ms test pulse to -38 mV (Fig. 4, bottom). In this experiment the recovery time between pre- and test pulses was set at only 20 ms, which would minimize recovery and thus provide conditions for detecting possible continued development of inactivation after a pre-pulse (Fig. 3). The records on the left in Fig. 4 are the $[Ca^{2+}]$ transients for no pre-pulse (control, bottom) or for pre-pulses to potentials varying from -40 to -10 mV as indicated. Increasing the pre-pulse amplitude increased the pre-pulse $\Delta[Ca^{2+}]$. The elevated $[Ca^{2+}]$ produced by the pre-pulse declined only partially during the 20 ms interval between pre- and test pulses. Thus, for the larger pre-pulses $[Ca^{2+}]$ remained elevated from early in the pre-pulse to the end of the test pulse (Fig. 4, top left).

The records on the right of Fig. 4 are the R_{rel} records calculated from the corresponding $\Delta[Ca^{2+}]$ records on the left. Increasing the amplitude of the pre-pulse introduced an increasingly large early spike of release during the pre-pulse but progressively decreased the peak of release during the test pulse. The largest pre-pulse (to -10 mV) completely eliminated the peak in R_{rel} during the following test pulse (upper right), even though the duration of the pre-pulse was only 20 ms. Longer pulses to -10 mV were not applied to the fibre in Fig. 4. However, release records from other fibres for longer pulses to -10 mV uniformally showed that R_{rel} had not appreciably declined by 20 ms after the start of a pulse to -10 mV. Thus, since the peak in test pulse release in Fig. 4 was completely eliminated by a 20 ms pre-pulse to -10 mV, inactivation must have continued to develop after repolarization at the

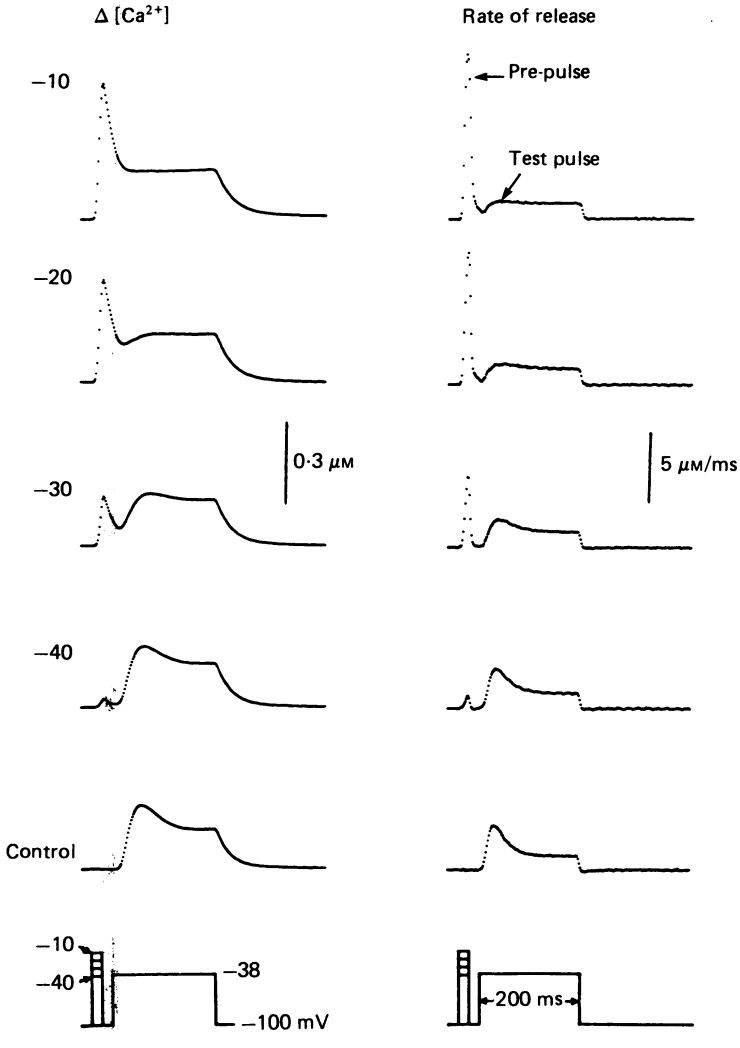

Fig. 4. The effects of brief pre-pulses on calcium release during a fixed test pulse. A 20 ms pre-pulse of variable amplitude was followed after a 20 ms separation by a fixed test pulse to −38 mV. Left panel: $\Delta[Ca^{2+}]$ records for pre-pulse amplitudes from −40 to −10 mV as indicated to the left of each record (mV). Right panel: the corresponding rate-of-release records. The pre-pulse to −10 mV (top right) completely suppressed the peak in the test release record. Same fibre and conditions as in Fig. 1.

end of the 20 ms pre-pulse to −10 mV. This result is consistent with the hypothesis that the elevation of $\Delta[Ca^{2+}]$ following the pre-pulse contributed to the inactivation of peak R_{rel} observed during the subsequent test pulse.

The test pulse release records with and without pre-pulses from Fig. 4 are further examined in Fig. 5. In each panel the R_{rel} record for the test pulse following a pre-pulse to the indicated potential is superimposed on the R_{rel} record for the same test pulse without pre-pulse. In each case only the early peak of the release record was decreased following the pre-pulse, with little or no change in the rate of release at the

end of the 200 ms pulse. The inactivation was thus specific for the early transient phase of release and did not suppress release late in the test pulse. It should be noted that the 20 ms interval at −100 mV between the pre- and test pulses should have been sufficiently long to allow virtually all of the intramembrane charge displaced by

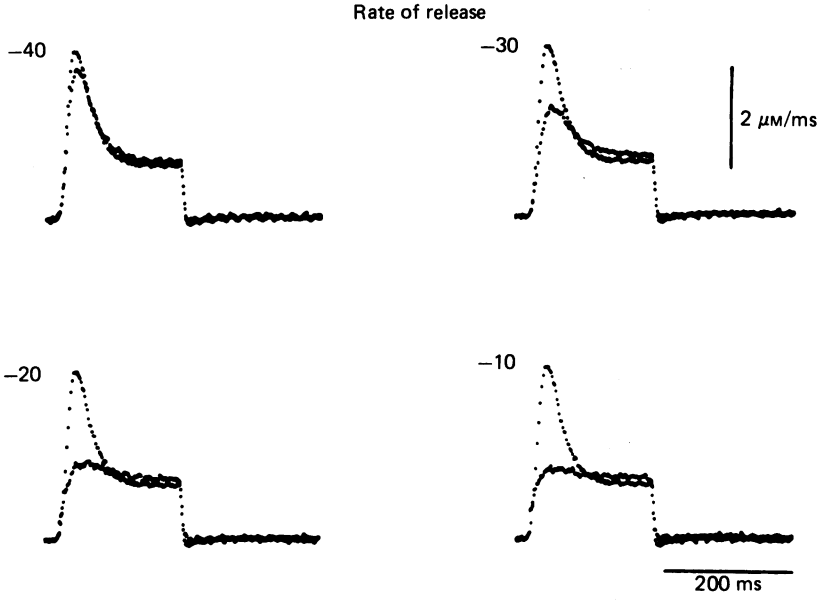

Fig. 5. A comparison of the test rates of release with and without brief pre-pulses. The rate-of-release records for the test pulses in Fig. 4 are replotted with each release record after a pre-pulse superimposed on the release record for the same test pulse without the pre-pulse. The release during the pre-pulse has been omitted in order to display the test release records at a higher gain than that used in Fig. 4. The pre-pulse potential is indicated to the upper left of each record (mV). Same fibre and conditions as in Fig. 4.

the pre-pulse to return to its resting distribution in the fibre membrane (Melzer *et al.* 1986*b*). Thus charge movement should have been essentially the same during all test pulses in Fig. 5, whether preceded by a pre-pulse or not, so that the altered test pulse R_{rel} waveforms following the pre-pulses cannot be attributed to modification of charge movement.

Inactivation by long pre-pulses

A final series of experiments examined the effects of long pre-pulses on the time course of calcium release during a constant test pulse. Results from one such experiment are shown in Fig. 6. A 200 ms pre-pulse to a membrane potential which was varied from −45 to −35 mV was followed after a set 20 ms interval by a test pulse to −32 mV as indicated by the pulse diagrams at the bottom of Fig. 6. A control test pulse was also applied without any pre-pulse (top). The records on the left in Fig. 6 are the $\Delta[Ca^{2+}]$ records and those on the right are the corresponding R_{rel} records for pre-pulses to the indicated potentials. Increasing the pre-pulse

amplitude increased the steady level of $\Delta[Ca^{2+}]$ at the end of the pre-pulse and also increased the degree of depression of the peak R_{rel} during the closely following test pulse, again consistent with a calcium-dependent inactivation mechanism.

An inactivation model based on calcium binding

In order to interpret the present results in terms of calcium-dependent inactivation of the SR calcium release channel several specific calcium-dependent mechanisms

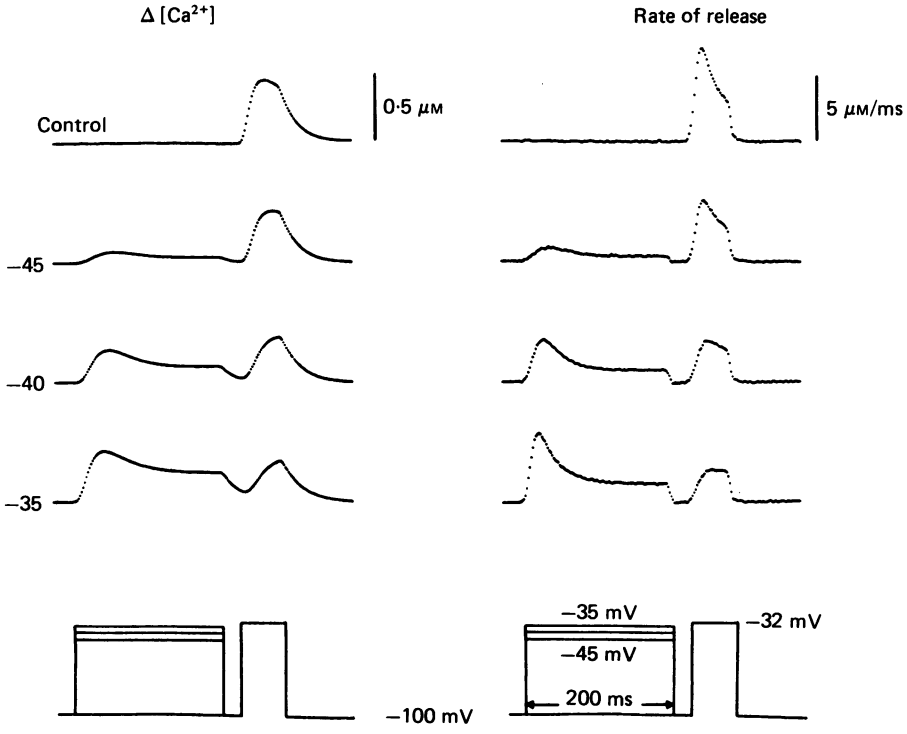

Fig. 6. The effects of long pre-pulses on the time course of calcium release during a fixed test pulse. A 200 ms pre-pulse of variable amplitude was followed after a fixed 20 ms interval by a test pulse to −32 mV. Left: calcium transients for pre-pulses to the membrane potentials indicated at the left of each record (mV). For the control record (top) there was no pre-pulse. Right: the corresponding rate-of-release records. Same fibre and conditions as in Figs 1, 4 and 5.

were considered and mathematical models derived for them. We will consider first one particularly simple model that appears to account for all of the present observations. The present experiments do not, however, rule out other models for inactivation which might require completely different mechanisms. As shown in the Discussion, other more complicated calcium-dependent models can give the same mathematical equations as the model presented below and thus provide equally good fits to the present data.

Consider the sequential, three-state model:

$$Ca^{2+} + R \underset{K}{\rightleftharpoons} CaR \underset{k_r}{\overset{k_f}{\rightleftharpoons}} CaR^* \quad (1)$$

(Simon *et al.* 1985), where R is a receptor on the SR calcium release channel which binds myoplasmic calcium (Ca^{2+}) with dissociation constant K. Calcium binding is assumed to be a relatively fast step so that calcium is always in equilibrium with R (see Discussion). Once calcium is bound, the channel can then make transitions between state CaR, which is not inactivated, and an inactivated state CaR*, with forward and reverse rate constants k_f and k_r. This whole process occurs in parallel with whatever mechanism opens the channel. Thus, if the receptor is in state CaR* the channel must be closed due to inactivation, whereas if the receptor is in states R or CaR then the channel is not inactivated and can be either open or closed depending on the condition of the activation mechanism. The differential equation describing the time course of the inactivated state CaR* is given by

$$dCaR^*/dt + CaR^*(k_r + k_f/(1 + K/[Ca^{2+}])) = k_f/(1 + K/[Ca^{2+}]), \quad (2)$$

where $[Ca^{2+}]$ is the calcium concentration in the vicinity of the inactivating site and CaR* is the fraction of channels in the inactivated state. The relationship between $[Ca^{2+}]$ and the degree of inactivation, in the steady state, can be obtained by setting $dCaR^*/dt$ in eqn (2) to 0 and solving for CaR*. The $[Ca^{2+}]$ at which the channel is half-inactivated in the steady state is given by $K' = K/(k_f/k_r + 1)$.

A crucial point in developing a calcium-dependent inactivation model consistent with the data was to include the first binding step which by itself produces no inactivation. A model in which calcium binds and inactivates in a single step predicts that the time course of R_{rel} records which have larger calcium transients should inactivate faster and to a greater extent. In the present two-step model, once $[Ca^{2+}]$ is significantly greater than K the time course of inactivation becomes a single exponential with a time constant given by $(k_f + k_r)^{-1}$. Thus, at high $[Ca^{2+}]$ inactivation no longer depends on $[Ca^{2+}]$, consistent with the results of Fig. 1. From the mean value of 32 ms for the R_{rel} decay time constants during the pulses in Table 1 the value of $k_f + k_r$ would be 31 s^{-1}.

The initial phase of recovery from inactivation in the model will depend on the time course of the decline of $\Delta[Ca^{2+}]$ after the pre-pulse. Significant recovery will not occur until $[Ca^{2+}]$ falls below K'. This allows the model to reproduce the delay seen in recovery in Figs 2 and 3 for the 120 ms pre-pulse. The 120 ms pre-pulse raised $[Ca^{2+}]$ to a greater extent than the 30 ms pre-pulse so that $[Ca^{2+}]$ took a longer time to decay below K' for the 120 ms pre-pulse than for the 30 ms pre-pulse. Once $[Ca^{2+}]$ is well below K', the recovery from inactivation will occur with a time constant equal to $1/k_r$. For the mean recovery time constant of 90 ms from Table 1 the value of k_r would be 11 s^{-1}. However, this value is probably a lower limit on k_r since $[Ca^{2+}]$ was still declining during the fast recovery.

Simulations of experimental results by the calcium binding model

The results in Fig. 2 could be simulated quite well using this model for inactivation. Figure 7 shows the same set of R_{rel} records as in Fig. 2 (top row) and the corresponding simulated records (middle row) generated from the model. The time course of the activation process for SR calcium release was assumed to follow

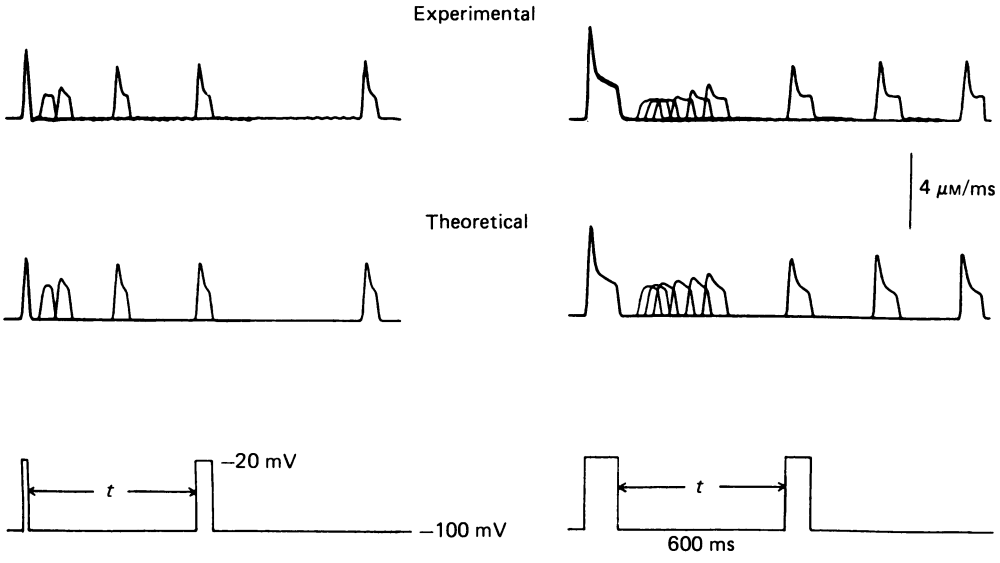

Fig. 7. Simulations of the double-pulse recovery experiment. The release records shown in Fig. 2 are replotted (top row) with the corresponding simulated records (middle row) generated from the inactivation model. The pre-pulse duration was 30 ms (left) or 120 ms (right) and recovery, t, times varied from 30 to 1200 ms. In the simulations the rate-of-release time course was obtained by multiplying the probability of a channel not being inactivated, obtained by solving eqn (2), by an activation function chosen to give appropriate-looking rising phases (see text for details). The values of the parameters which define the inactivation time course were varied to give a good visual simulation: $k_f = 200\ s^{-1}$; $k_r = 30\ s^{-1}$; $K = 3\ \mu M$. The initial SR calcium content was assumed to correspond to a myoplasmic concentration of 700 μM if the total content were free in the myoplasm. The assumed parameter values for activation were $\tau_Q = 15$ ms and final charge movement = 73% of the total charge.

$Q(t) - Q_{th}$ for $Q(t) > Q_{th}$, where Q_{th} is a constant amount of threshold charge which must move before activation can occur (Melzer *et al.* 1986*b*). Q_{th} was assumed to equal 30% of the total available charge. $Q(t)$ was assumed to follow a single exponential time course with time constant τ_Q chosen to give appropriate-looking rising phases in the simulated R_{rel} records. Depletion of calcium from the SR was included in the simulations by setting the initial SR calcium content so as to give the necessary amount of depression of the test R_{rel} after 1200 ms of recovery (Schneider *et al.* 1987*b*). This SR content also produced the appropriate amount of depletion to reproduce the slow phase of decline of R_{rel} seen during the 120 ms conditioning pulses (right side of Fig. 7). The calcium flux was assumed to be proportional to the SR

calcium content. Assuming negligible recycling of released calcium, the SR calcium content was calculated as a function of time during each record as the initial content minus the integral of R_{rel}. Figure 7 shows that the model was able to nicely reproduce the time course of R_{rel} during the pre-pulse and test pulses and to account for the difference in the delay in recovery with the different-duration pre-pulses.

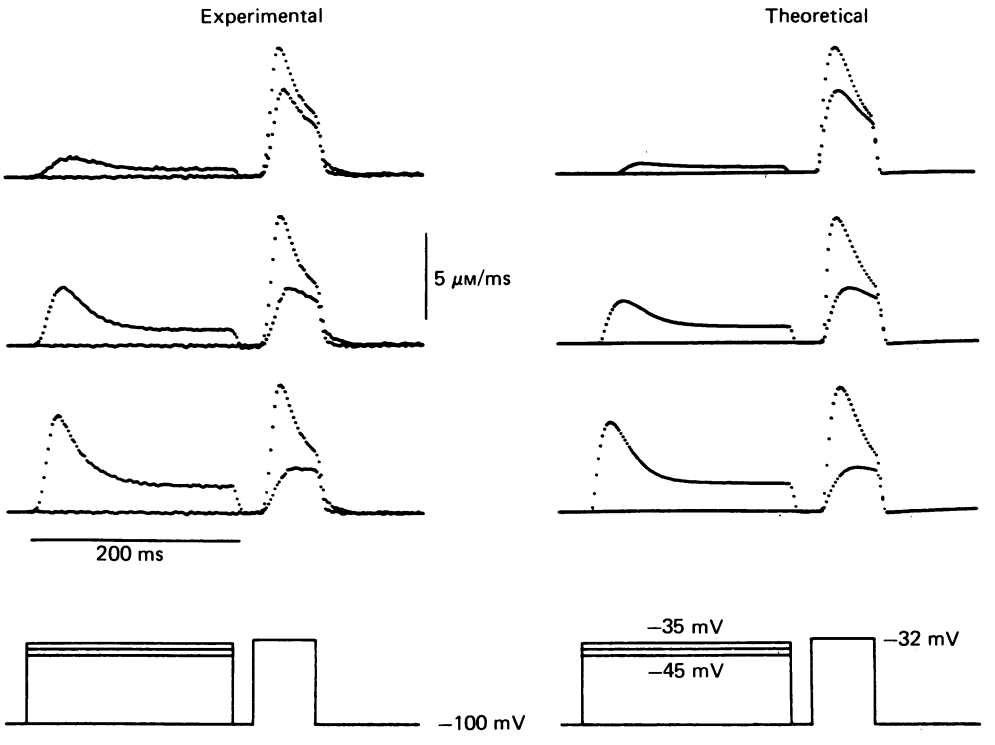

Fig. 8. Simulations of the long pre-pulse experiment. The release records shown in Fig. 6 are replotted (left) with the corresponding simulated release records (right). The release record for the control test pulse without a pre-pulse is superimposed on each release record with a pre-pulse. The parameters which define the inactivation model were chosen to give a good visual simulation of the experimental results: $k_f = 70\ s^{-1}$; $k_r = 7\ s^{-1}$; $K = 0{\cdot}3\ \mu M$. Depletion of calcium from the SR was assumed to be negligible. The parameter values assumed for activation were $\tau_Q = 16$–17 ms and final charge movement = 34–67% of the total charge at −45 to −32 mV.

Figure 8 shows the same R_{rel} records as presented in Fig. 6 for the long pre-pulse experiment together with simulations of the results by the inactivation model. The rationale for this experiment was to use a pre-pulse to fix $\Delta[Ca^{2+}]$ for a long enough period of time so that the CaR–CaR* transition would be in equilibrium prior to the test pulse. The R_{rel} records determined experimentally using 200 ms pre-pulses to −35, −40 and −45 mV are on the left and the corresponding simulated R_{rel} records are on the right. The record of R_{rel} for the control test pulse without a pre-pulse is superimposed on each release record with pre-pulse to directly illustrate the effect of the pre-pulse on calcium release during the test pulse. Figure 8 shows that the model

with a single set of parameter values could simulate quite well both the time course of R_{rel} during the pre-pulse and the effect of the pre-pulse on the test R_{rel}. In the simulations in Fig. 8 depletion of calcium from the SR was negligible, as indicated by the absence of a slow phase in the decline of R_{rel} during the conditioning pulses.

It should be noted that for purposes of the simulations in Figs 7 and 8 resting $[Ca^{2+}]$ was assumed to be zero. In this case resting inactivation would also be zero. If, however, resting $[Ca^{2+}]$ were not zero but comparable to the apparent dissociation constant for inactivation, $K/(k_f/k_r+1)$, the value of K would have to be higher.

DISCUSSION

Fast inactivation of SR calcium release

In the present paper we have investigated the 'fast' inactivation of calcium release from the sarcoplasmic reticulum using a skeletal muscle fibre preparation in which release is activated by voltage clamp depolarization of the external fibre membranes. Despite maintained depolarization, the activated release declines markedly but not completely during the first 100–200 ms of depolarization. Since this relatively rapid decline in release does not appear to be due to depletion of calcium from the SR (Schneider *et al.* 1987*b*), we refer to it as an inactivation process. We use the term fast inactivation to distinguish this process from the more slowly developing mechanically refractory state (Hodgkin & Horowicz, 1960), which requires seconds or tens of seconds to develop and which is associated with marked alteration in the properties of intramembrane charge movement (Chandler, Rakowski & Schneider, 1976; Adrian, Chandler & Rakowski, 1976). In contrast, minimal alteration in the charge movement properties should occur during a 100 ms pulse that does produce considerable fast inactivation of calcium release. The present results demonstrate further that fast inactivation can even be produced during and after a pulse lasting only 20 ms, which certainly should have had negligible effect on the charge movement system. The fast inactivation process thus does not appear to occur as a result of an alteration in the properties of the voltage sensor for activation of SR calcium release. Rather it appears to operate in parallel with and independently of the voltage sensor for activation.

It has been consistently observed in both the present and previous studies that fast inactivation of SR calcium release is incomplete (Melzer *et al.* 1984, 1987; Rios & Brum, 1987). In general, the steady level of release during a pulse is about 20–30% of its peak value during that pulse. Incomplete inactivation could occur as a result of partial steady-state inactivation of a single class of SR calcium release channels. Alternatively, it could be due to the presence of two types of SR calcium channels, one completely inactivating and the other non-inactivating. The two-channel hypothesis has some precedent since calcium channels of large and small conductance have been observed when SR vesicles are incorporated into lipid bilayers (Smith, Coronado & Meissner, 1986; Suarez-Isla, Orozco, Heller & Froehlich, 1986).

Fast inactivation due to complete inactivation of one type of channel would be difficult to reconcile with present observations using simple models of channel control. We have observed that the time constant of inactivation is independent of

pulse voltage and have explained this on the basis of a rapid initial step followed by a slower step that actually produces inactivation (eqn (1)). If inactivation of one type of channel were essentially complete, $k_f \gg k_r$ and the observed rate constant of inactivation would equal k_f. However, the lower limit on k_r obtained from the time course of recovery from inactivation (Table 1) is similar in magnitude to k_f. Thus, k_r would have to increase under conditions of recovery in order to explain present results on the basis of two populations of channels, one completely inactivating and the other non-inactivating. We have not pursued this possibility.

Calcium-dependent inactivation of SR calcium release

The present results indicate that fast inactivation of SR calcium release does not appear to be directly controlled by the membrane potential across the surface or T-tubular membranes but could be regulated by $[Ca^{2+}]$. The short pre-pulse experiments demonstrate that fast inactivation can continue to develop during the repolarization that follows a short, large pre-pulse. Since free calcium remains elevated following such a pulse, elevated $[Ca^{2+}]$ is a good candidate for the maintained signal that causes inactivation to continue to develop following a short pulse. This would be consistent with the finding that following a conditioning pulse there is a delay before the start of recovery from fast inactivation and that this delay is correlated with the time for $\Delta[Ca^{2+}]$ to fall into a certain range. Furthermore, several calcium-dependent inactivation models (above and below) can reproduce the present observed properties of fast inactivation. Finally, the existence of calcium-dependent inactivation is consistent with a variety of studies of calcium release from the SR in more disrupted preparations of skeletal muscle (Palade, Mitchell & Fleisher, 1983; Ikemoto, Antoniu & Meszaros, 1985; Meissner, 1986; Kwok & Best, 1987).

The molecular mechanism underlying a possible calcium-dependent inactivation of SR calcium release in intact skeletal fibres is not established. However, several calcium-dependent mechanisms have been proposed to account for inactivation of SR calcium channels in SR vesicles and in skinned fibres or for inactivation of calcium channels from the surface membranes of several cell types. These mechanisms include: (i) Direct binding of calcium to an inhibitory site on or near the channel (Standen & Stanfield, 1982; Simon *et al.* 1985; Chad & Ekert, 1986). Recovery from inactivation would occur as $[Ca^{2+}]$ declines and Ca^{2+} dissociates from the site. (ii) Binding of calcium–calmodulin complex to an inhibitory site on the channel (Meissner, 1986; Meissner & Henderson, 1987). Recovery would occur as $[Ca^{2+}]$ declines and Ca^{2+} dissociates from the calcium–calmodulin complex. (iii) Phosphorylation of a regulatory site on the channel by a calcium–calmodulin-dependent protein kinase (Tuana & MacLennan, 1984). Recovery would occur via an endogenous phosphatase. (iv) Dephosphorylation of a regulatory site on the channel by a calcium-dependent phosphatase (Chad & Eckert, 1986). Recovery would occur by the action of a protein kinase.

A calcium–calmodulin-dependent inactivation model

The mechanism assumed for eqn (2) and for the simulations in Figs 7 and 8 was inactivation by direct binding of calcium to a site on the release channel ((i) above).

However, other models with fundamentally different underlying molecular mechanisms could give a differential equation that is formally identical to eqn (2). We consider here one alternative model, based on the work of Meissner (1986), in which binding of the calcium–calmodulin complex to an inactivation site is assumed to decrease the probability that the channel will be open ((ii) above). Meissner (1986) found a partial, two- to threefold, calmodulin-dependent inhibition of calcium release in SR vesicles. The degree of inactivation observed by Meissner (1986) was similar to the degree of partial inactivation we have observed for long depolarizing pulses. A calcium–calmodulin-dependent inactivation model can also account for the present results as shown below.

Let free calcium (represented here as Ca) bind to free calmodulin (CaM) with dissociation constant K and assume that binding is rapid so that Ca is always in equilibrium with CaM:

$$\mathrm{Ca} + \mathrm{CaM} \rightleftharpoons \mathrm{CaMCa}, \quad K = \mathrm{Ca(CaM)/CaMCa}. \tag{3}$$

The calcium–calmodulin complex (CaMCa) binds to an inactivating site (R) on the channel to form an inactivated state RCaMCa:

$$\mathrm{CaMCa} + \mathrm{R} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{RCaMCa}. \tag{4}$$

The kinetics of the inactivated state are given by the differential equation:

$$\mathrm{dRCaMCa}/\mathrm{d}t = -k_r(\mathrm{RCaMCa}) + k_f(\mathrm{R_T} - \mathrm{RCaMCa})\,\mathrm{CaMCa}, \tag{5}$$

where $\mathrm{R_T} = \mathrm{R} + \mathrm{RCaMCa}$ is the total concentration of receptor. The total calmodulin concentration is given by:

$$\mathrm{CaM_T} = \mathrm{CaM} + \mathrm{CaMCa} + \mathrm{RCaMCa}. \tag{6}$$

Combining eqns (3), (5) and (6) we obtain a differential equation for RCaMCa in terms of Ca, $\mathrm{R_T}$ and $\mathrm{CaM_T}$:

$$\mathrm{dRCaMCa}/\mathrm{d}t + k_r(\mathrm{RCaMCa}) = k_f(\mathrm{R_T} - \mathrm{RCaMCa})\,((\mathrm{CaM_T} - \mathrm{RCaMCa})/(1 + K/\mathrm{Ca})). \tag{7}$$

If we now assume that $\mathrm{CaM_T} \gg \mathrm{R_T}$ we obtain:

$$\mathrm{dRCaMCa}/\mathrm{d}t + \mathrm{RCaMCa}(k_r + k_f(\mathrm{CaM_T})/(1 + K/\mathrm{Ca})) = k_f(\mathrm{R_T})\,(\mathrm{CaM_T})/(1 + K/\mathrm{Ca}). \tag{8}$$

Equation (8) is formally identical to the equation for the calcium-dependent inactivation model based on direct binding of calcium to a site on the channel (eqn (2)). This can be seen by normalizing eqn (8) by dividing by $\mathrm{R_T}$ to convert RCaMCa to fractional inactivation and then equating CaR*, k_f, k_r and K of eqn (2) with RCaMCa, $k_f(\mathrm{CaM_T})$, k_r and K respectively of the normalized eqn (8). The only difference in the two equations is that the value of the apparent forward rate constant $k_f(\mathrm{CaM_T})$ for the inactivating step in the calmodulin-dependent model will increase with the total concentration of calmodulin. Thus, in the calmodulin-dependent model, the effective time constant of inactivation and the degree of

steady-state inactivation for any given $[Ca^{2+}]$ will depend on the total calmodulin concentration (eqn (8)).

The assumption that $CaM_T \gg R_T$ can be checked by comparing the concentration of release sites with the calmodulin concentration measured in skeletal muscle (2 μM, Yagi, Yazawa, Kakiuchi, Oshima & Uenishi, 1978). Estimating R_T from the density of dihydropyridine binding sites (Schwartz, McClesky & Almers, 1985), feet structures (Franzini-Armstrong, 1970) or charge movement molecules (Schneider & Chandler, 1973) gives a range of R_T from 0·1 to 0·3 μM (230–700 sites/μm^2 of T-tubule membrane), roughly ten times less than the calmodulin concentration. Even if this assumption does not hold the properties of eqns (2) and (7) are still essentially the same. The major difference is that the inactivation time course using eqn (7) will not be strictly exponential when $[Ca^{2+}] \gg K$.

A second assumption in both inactivation models presented in this paper is that the calcium-binding step is so rapid that it is always in equilibrium. Assuming a dissociation constant of 1 μM and a diffusion limited on rate constant (10^8 M^{-1} s^{-1}), the off rate constant is calculated as 100 s^{-1}. At low $\Delta[Ca^{2+}]$ the binding reaction has an upper-limit time constant equal to the reciprocal of the off rate constant, which in this case would be 10 ms. However, the time constant of decline of $\Delta[Ca^{2+}]$ after a pulse is several times greater than this so that the actual rate-limiting step in the equilibration of $[Ca^{2+}]$ with the sites is the time course of $\Delta[Ca^{2+}]$. Thus, in practice the binding step can be treated as always in equilibrium, even at low $\Delta[Ca^{2+}]$.

Calcium binding at multiple sites in calcium-dependent inactivation

A limitation in fitting any calcium-dependent inactivation model to the data obtained with the experimental protocol described in this paper is the unknown resting $[Ca^{2+}]$, and the unknown degree of inactivation at rest. We have recently addressed these uncertainties by simultaneously measuring resting $[Ca^{2+}]$ with the high-affinity fluorescence indicator Fura-2 and $\Delta[Ca^{2+}]$ with Antipyrylazo III (Klein, Simon, Szucs & Schneider, 1988). In a series of preliminary experiments (Simon, Klein & Schneider, 1988) similar to that shown in Fig. 6 we determined the steady-state degree of inactivation of the peak R_{rel} of a test pulse as a function of the absolute $[Ca^{2+}]$ prior to the test pulse. These data could be better fitted with a calcium binding model with two calcium ions binding co-operatively (Hill coefficient = 2) rather than the single-site model described here. Inactivation due to calcium binding at multiple sites would be consistent with a calcium–calmodulin-dependent process (Manalan & Klee, 1984; Cox, Comte, Malnoe, Burger & Stein, 1984), but could also be incorporated into a direct binding model. In fact, the small discrepancy between the experimental and simulated data shown in Fig. 7 might be reduced by including multiple, co-operative calcium binding sites in the inactivation model. During recovery from the 120 ms pre-pulse the test R_{rel} in the experimental data shows a delay in recovery of 200 ms during which time the peak R_{rel} was totally absent. The simulated data shows a similar depression of the peak R_{rel} but there is a gradual increase in the peak of the simulated records during this same 200 ms. Co-operativity (Simon *et al.* 1988) would tend to decrease this creeping up of the simulated release by increasing the steepness of the dependence of inactivation on $[Ca^{2+}]$. Results from additional studies using Fura-2 and Antipyrylazo III

simultaneously may provide further information regarding the mechanism underlying calcium-dependent inactivation.

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