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CONTROL OF CALCIUM RELEASE AND THE EFFECT OF RYANODINE IN SKINNED MUSCLE FIBRES OF THE TOAD

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SUMMARY

1. Skinned muscle fibres from the toad were used to investigate the roles of T-system membrane potential and Ca^{2+} in controlling the calcium release channels of the sarcoplasmic reticulum (SR).

2. Replacement of K^+ in the bathing solution with Na^+ produced a large contraction which could last for 30 s or more under certain circumstances. This prolonged contraction could be quickly and completely terminated by repolarizing the fibre in the K^+ solution and then immediately re-initiated by returning to the Na^+ solution. These data indicate that the membrane potential tightly controlled the substantial and prolonged release of calcium.

3. T-system depolarization in the presence of 10 mM-free EGTA (pCa > 9) markedly depleted the SR of Ca^{2+}. This implies that depolarization of the T-system can still trigger substantial release of Ca^{2+} from the SR even when the myoplasmic $[Ca^{2+}]$ is very low and very heavily buffered by EGTA.

4. When the SR was heavily loaded with Ca^{2+}, substitution of a weakly buffered high $[Ca^{2+}]$ solution (pCa 5·4, 50 μM-EGTA) could produce a small to moderate, transient contraction taking between 3 and 12 s to reach a peak and lasting 30 s or more.

5. This contraction may be produced at least partly by 'calcium-induced calcium release' as ruthenium red (2 μM) completely blocked the responses. Moreover, repeated substitutions produced successively smaller responses in parallel with the 'run-down' of the depolarization-induced contractions.

6. Depolarization could always produce an additional large and fast response at any stage during a 'Ca^{2+}-induced' response.

7. In the presence of 25 μM-ryanodine, the rapid contraction produced by T-system depolarization was prolonged and could not be stopped by repolarization. During and after this contraction no depolarizing stimulus could induce a further contraction, even though in some fibres addition of 30 mM-caffeine produced a maximum response which indicated that there was still a substantial amount of calcium in the SR.

8. At pCa 6·4, 25 μM-ryanodine could itself induce a substantial slow contracture in a *normally polarized* fibre within 30–60 s, after which little or no response could be induced by T-system depolarization. At higher concentrations (25 μM) ryanodine produced a near-maximum contraction in only a few seconds. Ryanodine did not

produce such contractures in the presence of low myoplasmic [Ca^{2+}] (pCa > 8, 0·5 mM-EGTA) and the drug could be washed out without subsequent effects, indicating that it had not bound.

9. Thus, it appears that the major Ca^{2+} release channels involved in depolarization-induced contractions *can* be opened by Ca^{2+} and that ryanodine initially keeps the channels blocked open, thereby potentiating calcium-induced calcium release and producing a contracture, but then blocks them permanently shut.

10. Ryanodine could still induce a contracture, even after the rapid depolarization-induced responses had been abolished by inactivating the voltage sensor by (a) a prolonged depolarization, (b) D600 or (c) soaking the fibre in low [Ca^{2+}] before skinning. Similarly, these procedures also did not noticeably affect the responses to 1 mM-caffeine.

11. Together with other data, these results suggest that in a functioning muscle fibre, depolarization induces Ca^{2+} release from the SR through a single type of release channel which is influenced primarily by the voltage sensor in the T-system membrane but which is also affected by myoplasmic Ca^{2+} and by caffeine and other agents.

INTRODUCTION

In skeletal muscle, depolarization of the transverse tubular (T-) system triggers the release of calcium from the adjacent sarcoplasmic reticulum (SR) and contraction (see review by Lüttgau & Stephenson, 1986). This excitation–contraction coupling must involve a voltage sensor in the T-system which detects any potential change, but it is not known how this sensor influences the calcium release channels in the SR or indeed if the whole sequence involves more than one type of calcium release channel. For example, the voltage sensor may have a direct physical connection to a type of release channel (Chandler, Rakowski & Schneider, 1976) or involve a 'second messenger' such as inositol trisphosphate (IP_3) (Vergara, Tsien & Delay, 1985) or Ca^{2+} itself (Frank, 1982). Ca^{2+} can open calcium release channels in lipid bilayers (Smith, Coronado & Meissner, 1986) and trigger calcium release in SR vesicles (Meissner, Darling & Eveleth, 1986) and in skinned fibres under certain conditions (see reviews by Stephenson (1981) and Endo (1985)). This raises the possibility that the major release of Ca^{2+} in normal excitation–contraction coupling involves Ca^{2+}-induced Ca^{2+} release through such specialized channels, either by Ca^{2+} directly linking the sensor to a single type of release channel or by the sensor more directly controlling another type of calcium release channel and with the Ca^{2+} from that channel then triggering the major release from the Ca^{2+}-activated channel (Stephenson, 1981, 1985).

This study uses a skinned fibre preparation (Lamb & Stephenson, 1990) as it allows rapid application and removal of particular agents and influence over the myoplasmic [Ca^{2+}]. This technique permits investigation of whether myoplasmic Ca^{2+} can directly open Ca^{2+} release channels (Endo, 1985; Ohta, Endo, Nakano, Morohoshi, Wanikawa & Ohga, 1989) in a preparation with physiological intracellular pH and [Mg^{2+}], and in which the normal depolarization-controlled mechanism of calcium release is not only functional but also can be activated simultaneously with

any calcium-induced calcium release. Further information about the properties of the Ca^{2+} release channels involved in normal excitation–contraction coupling can also be obtained by application of ryanodine, an alkaloid which can bind to and affect at least one type of Ca^{2+} release channel (Pessah, Waterhouse & Casida, 1985; Meissner, 1986; Imagawa, Smith, Coronado & Campbell, 1987). The data from this paper and the results of other experiments on intact fibres, 'triad' preparations and single calcium release channels can be drawn together to show what type of release channels are involved in normal excitation–contraction coupling and what controls them.

METHODS

These experiments used the skinned-fibre preparation and solutions described in the preceding paper (Lamb & Stephenson, 1990). Briefly, toads (*Bufo marinus*) were stunned by a blow to the head and then pithed. The iliofibularis muscles were removed and bathed in a Ringer solution (mM: NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; $MgCl_2$, 1; Na_2HPO_4, 2·2; NaH_2PO_4, 1; pH 7·15). A segment of a single fibre was skinned under paraffin oil and attached to a force transducer (AME875) to measure isometric contractions, which were recorded on a chart recorder (Linear). The resting length (L) and diameter (D) of the fibre were measured in oil and then the fibre was stretched a further 20% (final sarcomere length 2·6–2·8 μm). The skinned fibre was then placed in a 2 ml Perspex bath filled with potassium HDTA solution for 2 min before being stimulated by rapidly removing the bath and replacing it with another filled with an appropriate solution. The potassium HDTA solution contained (mM): K^+, 117; Na^+, 36; $HDTA^{2-}$, 50; ATP (total), 8; free Mg^{2+}, 1; creatine phosphate, 10; EGTA (total), 0·05; HEPES buffer, 60; NaN_3, 1; with pH 7·10 ± 0·01 and pCa 6·7. (The azide (NaN_3) was present in order to prevent the mitochondria from interfering with the calcium movements.) The sodium HDTA solution had the same composition as the potassium solution except that all K^+ (117 mM) was replaced by Na^+. The choline chloride solution contained (mM): choline chloride, 103; Na^+, 42; free Mg^{2+}, 1; ATP (total), 8; creatine phosphate, 10; HEPES buffer, 20; EGTA (total), 0·05; pCa 6·7 and pH 7·10 ± 0·01; this solution was isosmotic with the potassium and sodium HDTA solutions (255 mosmol/kg). The 'heavily buffered' pCa 6·0 solution (only used in experiments described in Fig. 4) and the maximum-activating solution (pCa 4) were similar to the standard K^+ solution but contained 50 mM-total EGTA instead of HDTA. All other solutions with elevated [Ca^{2+}] ('weakly buffered' at pCa 6·4, 6·3, 6·0, 5·7 or 5·4) were made from the standard K^+ solution by adding an appropriate amount of $CaCl_2$.

Gallopamil (D600), a generous gift from Knoll (Australia), was added as required from a freshly made 20 mM stock in ethanol. Ryanodine from two different sources was used: ryanodine (Penick) was made into a 1 mM stock in double-distilled water and ryanodine (Agri Systems) was made into a 5, 25 or 100 mM stock in ethanol, as it was relatively insoluble in water. The two types of ryanodine gave very similar results in all experiments. When examining the effect of substances dissolved in ethanol, all other solutions used to bathe the fibre also contained the same final concentration of ethanol. Control experiments showed no effect of ethanol at these concentrations (0·05–0·25%). All experiments were performed at room temperature (24 ± 3 °C).

In all figures the fibre was bathed in the standard potassium HDTA solution unless specifically indicated otherwise.

RESULTS

Voltage control of Ca^{2+} release

Repolarization during a prolonged response

Substitution of the standard K^+ solution bathing a skinned fibre with the choline chloride solution depolarizes the T-system and as a result produces a large and rapid contraction lasting about 2–4 s (e.g. Fig. 1*A*). Na^+ substitution produces a similar

effect though in many fibres the initial substitutions produced little or no response and subsequent substitutions progressively produced larger responses until reaching a maximum similar to the response produced by choline chloride substitution (see Lamb & Stephenson, 1990). This progressive change was almost certainly the result of the initial Na^+ substitutions only causing a small depolarization of the T-system, possibly because of the high concentration of Cl^- in the T-system.

As the rate of onset of voltage-dependent inactivation should be slower with small depolarizations (Lamb & Stephenson, 1990), this phenomenon could be used to examine the effect of *repolarization* on a prolonged response. Fibres which showed only a small response to the initial Na^+ substitutions were heavily loaded with Ca^{2+} by exposure to a solution weakly buffered to a raised $[Ca^{2+}]$ (pCa 6·0, 50 μM-EGTA). Substitution with Na^+ then produced a large prolonged response (Fig. 1*A* and *B*) which could be terminated rapidly and completely by repolarizing the fibre again in the K^+ solution. When the fibre was depolarized again soon afterwards by returning to the Na^+ solution, the tension rapidly increased to approximately the same level as just before the fibre was repolarized (Fig. 1*A* and *B*). The response then decreased and when it had declined almost completely, repolarization again terminated the response and depolarization returned it to close to the level immediately before this second repolarization. Similar responses were seen in every fibre examined under these conditions. The broad envelope describing the tension during a sequence of repolarization–depolarization cycles (e.g. Fig. 1*B*) appeared to be basically the same as the time course of the tension when it was not interrupted by repolarizing the fibre. Hence, such an envelope presumably is also determined by the same factors which dictate the decline of tension during a continuous depolarization, which probably in this case is the development of voltage-dependent inactivation. (Presumably, even during a brief repolarization, a fibre would recover to some extent from any inactivation that has occurred, which probably explains the 'overshoot' upon the depolarization following the second repolarization period in Fig. 1*B*.) In these loaded fibres, the duration of the response to continuous depolarization by Na^+ substitution (or the envelope enclosing a sequence of interrupted responses) became progressively shorter on repeated substitutions (Fig. 1*A*). This is to be expected because voltage-dependent inactivation should set in more quickly, if the successive substitutions produced greater depolarizations (Caputo & Bolanos, 1979; and see Fig. 4 in Lamb & Stephenson, 1990). The proposal, that the duration of the response is primarily determined by the onset of voltage-dependent inactivation, is also consistent with the observation that the strongly depolarizing choline chloride solution only produced a very small response when the response to Na^+ substitution had declined almost completely (Fig. 1*B*).

Regardless of the factors determining the eventual decline of the prolonged response, the important point here is that repolarization *completely terminates* the response, even when the fibre is heavily loaded with calcium. The rapid decline of the responses upon repolarization shows not only that the Ca^{2+} release is promptly terminated but also that the Ca^{2+} is quickly taken up within the preparation. This is particularly clear in the data shown in Fig. 1*B* where the fibre was repolarized in a K^+ solution with raised $[Ca^{2+}]$. In this case during the repolarization the fibre must not only have taken up most of the Ca^{2+} on and between the myofilaments but also

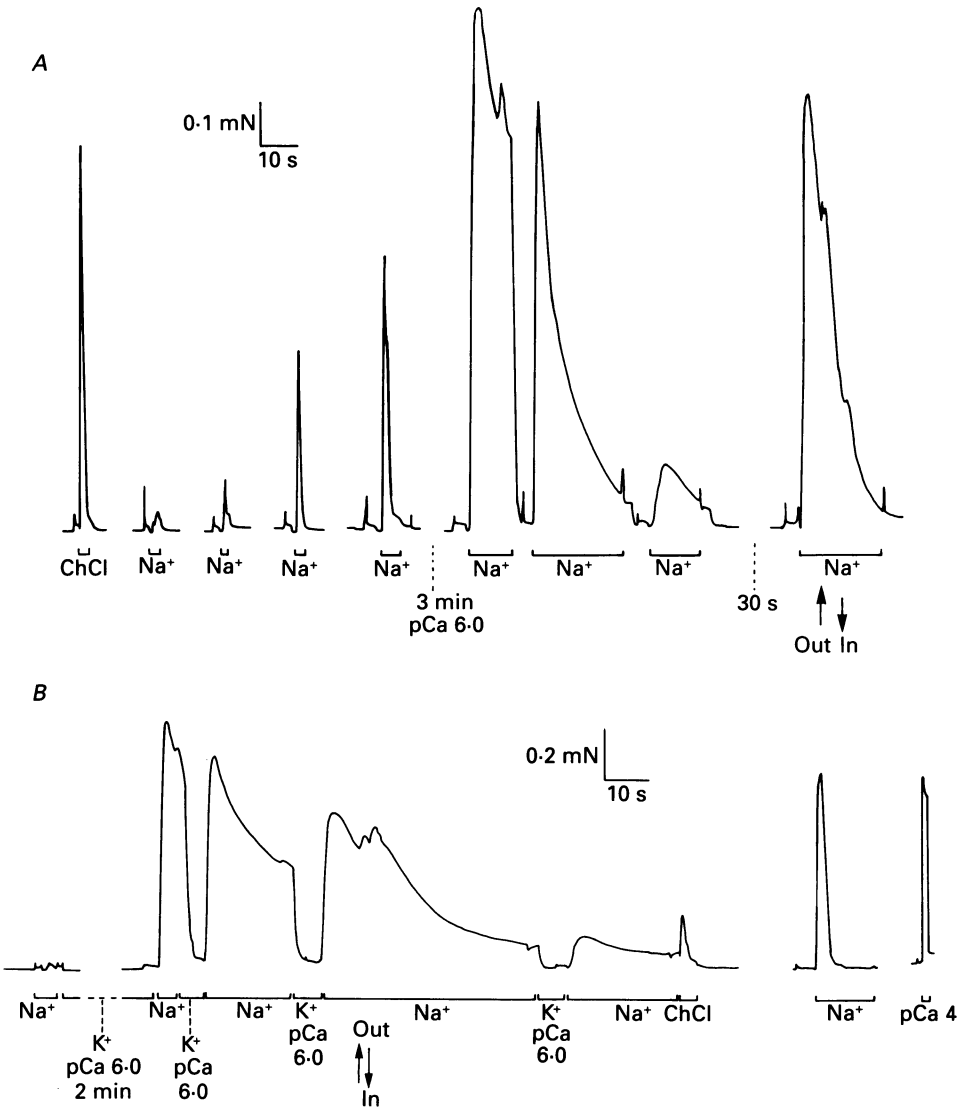

Fig. 1. Termination of Ca^{2+} by repolarization. *A*, weak to moderate depolarizations of the T-system induced by successive Na^+ substitutions. The responses became prolonged after Ca^{2+} loading of the SR (3 min pCa 6·0, 50 μM-EGTA). Returning the fibre to the (polarizing) K^+ solution stopped the response completely and rapid re-substitution of the Na^+ solution produced approximately the *same tension* as just before repolarization. (*D*, 50 μm; *L*, 1·9 mm.) *B*, similar termination of prolonged release in another fibre even when the repolarizing K^+ solution had a higher [Ca^{2+}] (pCa 6·0, 50 μM-EGTA) than the Na^+ solution (pCa 6·7, 50 μM-EGTA). (*D*, 58 μm; *L*, 2·1 mm.) The arrows indicate where the fibre was moved out of solution (into air) and back in again. Fibres bathed in standard K^+ solution except where otherwise indicated by horizontal bars. pCa 4 solution had 50 mM-EGTA and all others had 50 μM-EGTA. ChCl, choline chloride.

any Ca^{2+} diffusing into the fibre. The rapid rise in tension upon depolarizing the fibre again (Fig. 1*A* and *B*) indicates that the rate of Ca^{2+} release is very considerable and this further emphasizes the effectiveness of the repolarization mechanism in terminating Ca^{2+} release. The response was not terminated simply by moving the fibre out of the Na^+ solution and then back in again without entering the K^+ solution (Fig. 1*B*, arrows). As described in the preceding paper (Lamb & Stephenson, 1990), the entire response to Na^+ substitution was completely blocked by 2 μM-ruthenium red, even when the SR was heavily loaded with Ca^{2+}.

Depolarization-induced release in high EGTA

The preceding data and those in Lamb & Stephenson (1990) show that depolarization of the T-system can activate Ca^{2+} release when the myoplasmic [Ca^{2+}] is only weakly buffered by 50 μM-EGTA. Stephenson (1985) used choline chloride stimulations in skinned fibres heavily loaded with Ca^{2+} and reported that the great majority of Ca^{2+} release was triggered by raised [Ca^{2+}] because most of the release was blocked if the myoplasmic [Ca^{2+}] was buffered to low levels by 5 mM-EGTA. It is not clear how much of this Ca^{2+} release was due to true depolarization-induced release and how much to a direct effect of choline chloride on the SR (see Lamb & Stephenson, 1990). Experiments like that shown in Fig. 2 imply that depolarization of the T-system can indeed trigger very substantial release of Ca^{2+} even in the presence of 10 mM-free EGTA (EGTA isosmotically replacing HDTA, with 1 mM-free Mg^{2+}). After three depolarizations in 10 mM-EGTA, each of seven fibres gave no response when subsequently depolarized under the standard conditions (pCa 6·7, 50 μM-EGTA), and the response to 30 mM-caffeine was only about half-maximum (e.g. Fig. 2) indicating that the SR was substantially depleted of Ca^{2+}. In a normally responsive fibre addition of 30 mM-caffeine invariably produced a maximum response and so this relatively small response indicates a substantial depletion of the releasable Ca^{2+} in the SR. Experiments with fibres in which the SR had been destroyed by Triton X-100 showed a change in sensitivity of the contractile proteins in the presence of 30 mM-caffeine corresponding to a shift of the Ca^{2+} activation curve by about 0·2 pCa units to higher pCa values. Therefore the ionized Ca^{2+} at the peak of the caffeine response is less by a factor of 1·5 than the ionized Ca^{2+} required to produce the same response in the absence of caffeine.

In control experiments, fibres responded normally to depolarizations or caffeine after exactly the same period in the 10 mM-EGTA without any depolarizations (not shown). In these control experiments the fibres were moved in and out of the K^+ solution with 10 mM-EGTA six times instead of being transferred to the Na^+ solution, and then likewise rinsed for 1 min in 0·5 mM-EGTA to lower the [EGTA] without permitting substantial reloading of Ca^{2+}. In two cases it was also shown that this control treatment did not cause substantial depletion in a fibre which had been reloaded with Ca^+ after having previously failed to respond to depolarizations following activation in 10 mM-EGTA. Moreover, if a fibre was left to recover in the normal pCa 6·7 solution for about 5 min after depolarizations in the high EGTA solution, the depolarization-induced response usually recovered completely. This recovery could be accelerated by exposure to solutions with higher [Ca^{2+}] and prevented by placing the fibre in paraffin oil after only 1 min in the standard pCa 6·7 solution.

This phenomenon depended on release of Ca^{2+} and not on inactivation of the voltage sensor in the high-EGTA solution because it was not observed if a fibre was kept depolarized in a Na^+ solution before and during 1 min in a high-EGTA solution. Furthermore, activation of calcium release in high EGTA by addition of caffeine also

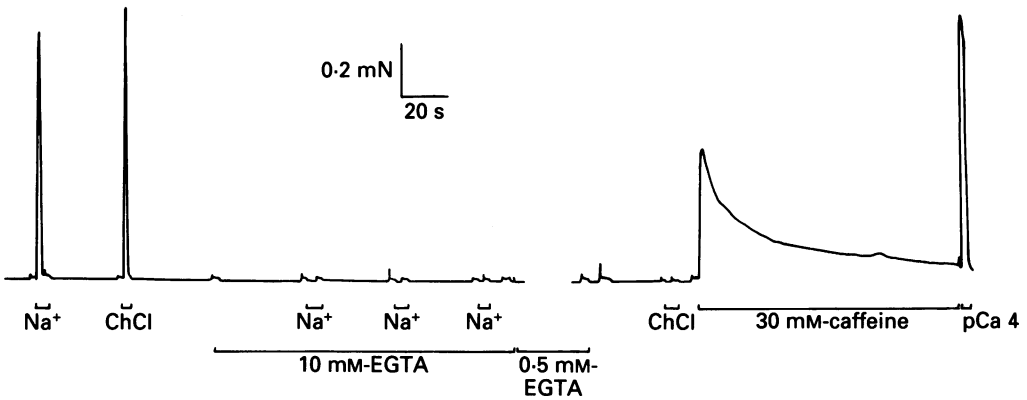

Fig. 2. Depolarization-induced release of Ca^{2+} in high EGTA. Following three Na^+ depolarizations in 10 mM-EGTA (K^+ solution and Na^+ solution both pCa > 9) and a 1 min rinse in the 0·5 mM-EGTA solution (pCa > 8), choline chloride (ChCl) substitution did not elicit a response after 30 s in the standard K^+ solution (pCa 6·7). Addition of 30 mM-caffeine produced only a half-maximum response indicating substantial depletion of releasable Ca^{2+}. (*D*, 50 μm; *L*, 1·6 mm.) In contrast, fibres subjected to the same EGTA protocol, without any depolarizations, responded normally to choline chloride substitution or caffeine afterwards.

eliminated subsequent depolarization-induced release in the normal solutions for several minutes. All together these experiments provide strong evidence that depolarization can induce very substantial release of Ca^{2+} even in the presence of 10 mM-EGTA, because it is clear that the SR is substantially depleted after only three brief depolarizations.

Direct activation of calcium release

The control of Ca^{2+} release was further investigated by examining the effects of agents reported to directly activate or affect Ca^{2+} release, namely caffeine, Ca^{2+} itself and ryanodine.

Responses to caffeine and the effect of voltage sensor inactivation

Lüttgau & Oetliker (1968) have shown previously that caffeine at high concentrations (6–10 mM) can activate Ca^{2+} release even in a depolarized fibre where the voltage sensor in the T-system is inactivated. Nevertheless it is possible that inactivation of the voltage sensor inhibits the response to caffeine to some degree but that this is only apparent at low concentrations of caffeine close to the threshold for activation. In the skinned-fibre experiments described here, 0·5 mM-caffeine does not reliably produce a response but 1 mM-caffeine elicits a large prolonged contraction lasting 20 s or more which can be rapidly ended by wash-out of the drug. There was no marked difference in the response to 1 mM-caffeine between the cases where a fibre (a) was normally polarized (in K^+ solution) and able to be activated by a

depolarization and (b) had been depolarized for a prolonged period (in the 100% Na solution) and was completely unresponsive to a further depolarizing stimulu (choline chloride) (Fig. 3*A*). (It should be noted that the myofilaments are slightly less sensitive to Ca^{2+} in solutions in which the predominant cation is Na^+ rather thai K^+ (Fink, Stephenson & Williams, 1986).) The responses to 1 mM-caffeine were completely suppressed by addition of 2 μM-ruthenium red (not shown).

Caffeine-induced Ca^{2+} release was studied also in the presence of D600 (gallopamil) a drug known to prolong inactivation of intact skeletal muscle fibres after a single depolarization-induced response (Eisenberg, McCarthy & Milton, 1983), even at 20 °C (Siebler & Schmidt, 1987). The response to 1 mM-caffeine appeared to be unaffected by the D600, even when the depolarization-induced responses had been completely abolished (Fig. 3*B*). In these skinned fibres a single depolarization-induced response (by either Na^+ or choline chloride substitution) in the presence of 10 μM-D600, abolished all response to subsequent depolarizations for some time (Fig. 3*B*). No responses were observed if the time between successive depolarizations was 1 min or less though substantial reactivation was usually observed after about 5 min (not shown). The recovery from this inactivation was not studied in detail but the results seem in general agreement with the prolongation of inactivation observed at a slightly lower temperature in intact fibres (Siebler & Schmidt, 1987). Fill & Best (1989) also have reported that D600 interferes with recovery from inactivation in skinned muscle fibres.

In contrast to the effect of a single depolarization, a large and comparatively prolonged contraction produced by 1 mM-caffeine in the presence of D600 did not affect a subsequent depolarization-induced release, that is it did not induce any prolongation of inactivation (Fig. 3*B*). This implies that caffeine does not activate the voltage sensor but instead activates Ca^{2+} release by a more direct action.

In summary, the response to 1 mM-caffeine does not seem to be affected by inactivation of the voltage sensor in either the absence or presence of D600.

Calcium-induced calcium release

Under some circumstances, such as at low $[Mg^{2+}]$, addition of Ca^{2+} can elicit substantial Ca^{2+} release from the SR of skinned fibres (see review by Stephenson (1981) and Endo (1985)). This 'calcium-induced calcium release' phenomenon has been studied in mechanically skinned fibres and in chemically skinned fibres in which the saponin treatment should also have disrupted and depolarized the T-system. In most cases the calcium-induced calcium release was determined indirectly by stimulating release with a heavily buffered high $[Ca^{2+}]$ solution and subsequently observing the amount of Ca^{2+} which could be released by application of caffeine (Endo, 1985; Ohta *et al.* 1988). The existence of calcium-induced calcium release was investigated here in skinned fibres with physiological pH and $[Mg^{2+}]$ and in which the normal depolarization-induced response is totally functional.

If a fibre was bathed in a solution weakly buffered (50 μM-EGTA) to a higher $[Ca^{2+}]$ (e.g. pCa 5·7) for several minutes no contraction was observed (Fig. 4). If the fibre was then transferred to a solution weakly buffered to a higher $[Ca^{2+}]$ (e.g. pCa 5·4) a small to moderate, transient contraction was usually observed. The contraction typically took between about 8 to 15 s to reach a peak (Figs 4 and 5), though in a few

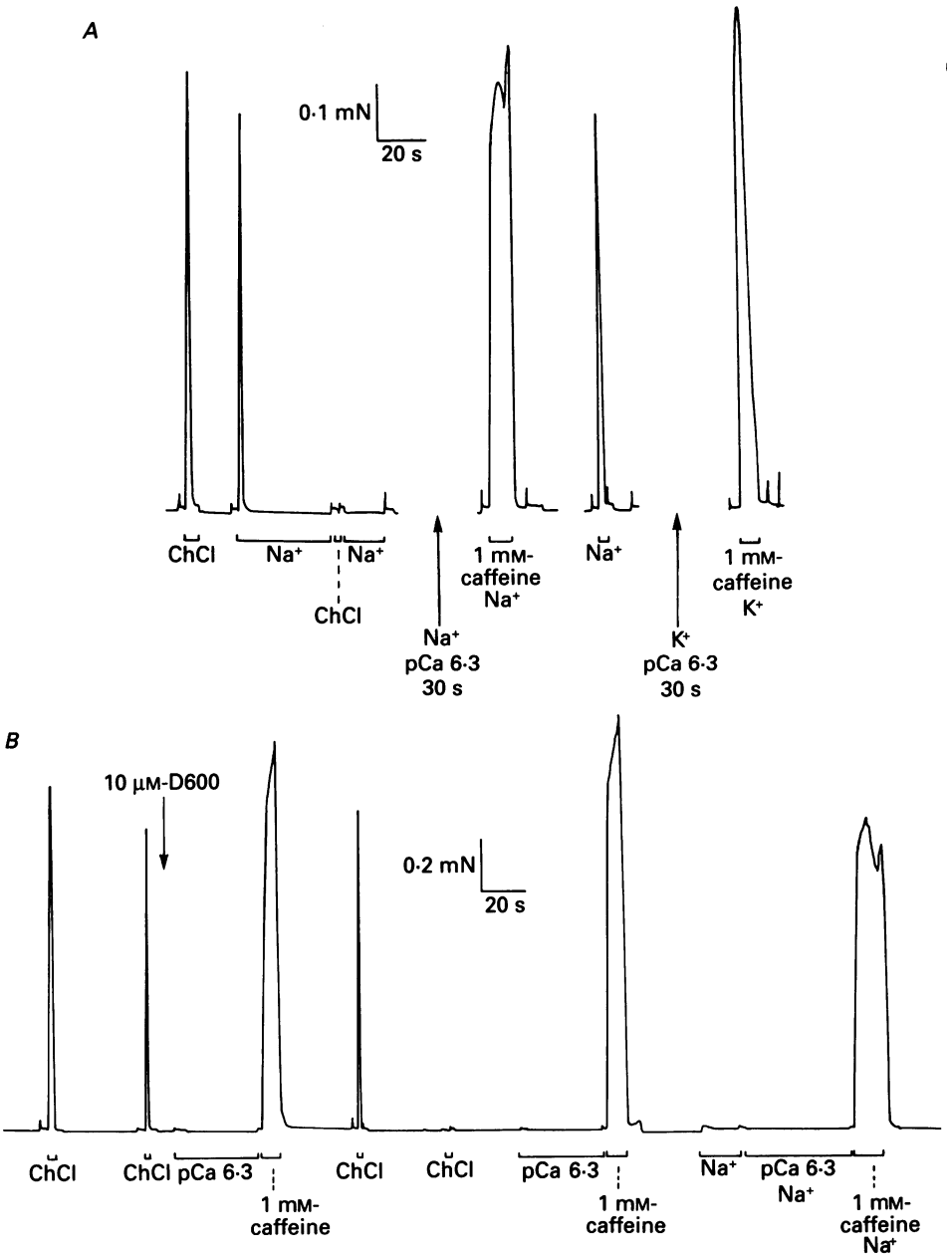

Fig. 3. Response to 1 mM-caffeine when the voltage sensors are activatable (*A* and *B*) and when they have been inactivated by a prolonged depolarization in the Na^+ solution (*A*) or by choline chloride (ChCl) stimulation in the presence of D600 (gallopamil) (*B*). *A*, the last response to Na^+ substitution shows that the fibre had been reprimed again before exposure to caffeine. (*D*, 49 μm; *L*, 2·8 mm.) *B*, after addition of 10 μM-D600 (in all solutions after arrow) a large response to caffeine had no effect on the response to depolarization with choline chloride, but after that single choline chloride response a subsequent choline chloride substitution gave no response. Subsequent responses to 1 mM-caffeine in either K^+ or Na^+ solutions appeared to be unaffected. (*D*, 50 μm; *L* 2·3 mm.) Before each caffeine response each fibre was bathed for 30 s in a solution at pCa 6·3 (50 μM-EGTA) with Na^+ or K^+ as appropriate, and after each response had peaked the caffeine was washed out.

cases it took as little as 3 s, and declined completely after about 20–40 s. This type of contraction often could not be induced by transferring a fibre straight from the standard solution (pCa 6·7) into the pCa 5·4 solution, and no response could be produced unless the SR was heavily loaded with Ca^{2+}. When the SR was destroyed

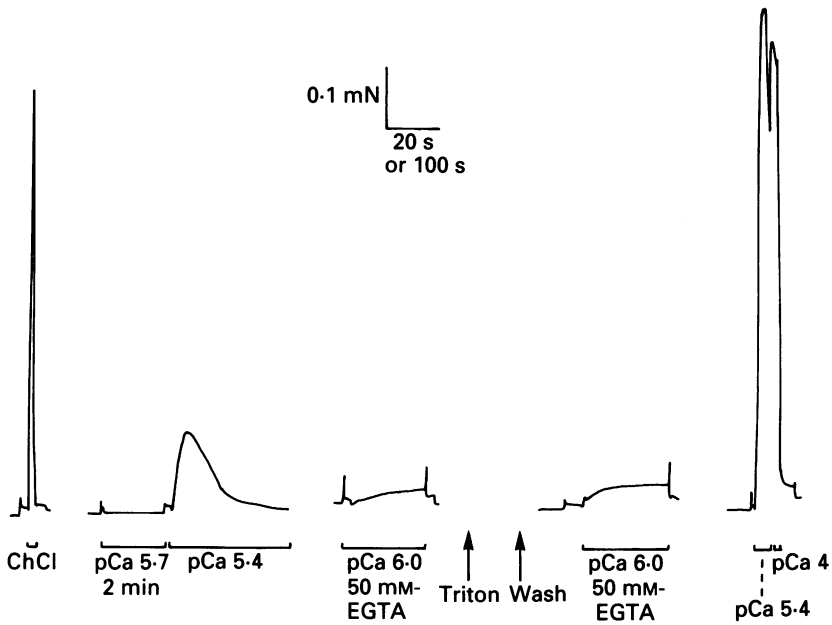

Fig. 4. Responses produced by exposure to high $[Ca^{2+}]$ solutions (standard $[K^+]$), before and after the SR had been destroyed by treatment with Triton X-100 (2%, 5 min). The Ca^{2+} was weakly buffered (50 μM-total EGTA, pCa 5·4 and 5·7) or strongly buffered (50 mM-EGTA, pCa 6·0). The pCa 4 and Triton solutions contained 50 mM-total EGTA. Time scale: 20 s, except in last segment (pCa 5·4 and pCa 4) where it is 100 s. (*D*, 55 μm; *L*, 2·6 mm.) ChCl, choline chloride.

by treatment with Triton X-100 (2%, 5 min) and the Triton was washed out, the same weakly buffered solution (pCa 5·4) produced maximum tension (Fig. 4). (The pCa 5·7 solution produced more than 70% maximum tension after Triton treatment, not shown.) The force produced by a heavily buffered solution (pCa 6·0, 50 mM-EGTA) was unchanged after Triton treatment, showing that the sensitivity of the myofilaments had not been affected. Clearly, when the fibre was bathed in the weakly buffered solutions (pCa 5·7 and 5·4) before Triton treatment, the SR must have been keeping the $[Ca^{2+}]$ near the myofilaments below the threshold for development of force (about pCa 6·3 in these experiments). Considering the $[Ca^{2+}]$ in the solutions, it might be proposed that the transient contraction produced by changing from the pCa 5·7 solution to the pCa 5·4 solution was simply the result of the heavily loaded SR initially not being able to remove the extra Ca^{2+} diffusing into the fibre.

Nevertheless, the following results suggest that at least some part of the response was produced by Ca^{2+} released from the fibre, or in other words that Ca^{2+} might have triggered further Ca^{2+} release (calcium-induced calcium release). Repeated exposures to the high $[Ca^{2+}]$ solution (pCa 5·4, 50 μM-EGTA) produced successively smaller responses roughly in parallel with the 'run-down' of the depolarization-induced

release (Fig. 5*A*) (see Lamb & Stephenson, 1990). This resembles the loss of calcium-induced calcium release in barnacle muscle fibres after several repetitions (Lea & Ashley, 1989). Interestingly, the 'run-down' of the depolarization-induced releases was greatly accelerated when a fibre was stimulated a number of times with the high [Ca^{2+}] solutions (in the five fibres examined in this way). This could be the result of a progressive depletion of a common Ca^{2+} store or a progressive 'inactivation' or disruption of common Ca^{2+} release channels.

Furthermore, addition of 2 μM-ruthenium red completely blocked the responses to both depolarization and raised [Ca^{2+}] within 30 s (Fig. 5*B*). Similar results were found in four other fibres. This suggests that the response to the pCa 5·4 solution is produced at least partly by *release* of Ca^{2+}, unless the effect of ruthenium red is entirely due to the drug stimulating uptake of Ca^{2+} by the SR (Meszaros & Ikemoto, 1985) rather than blocking Ca^{2+} release channels (Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988). Further evidence for the existence of calcium-induced calcium release in these fibres was provided by the actions of ryanodine (see later).

Similar 'calcium-induced calcium release' responses could be elicited when the normal excitation–contraction coupling mechanism was inactivated by keeping the fibre continuously depolarized in Na^+ solutions. As in the polarized fibres, the SR had to be heavily loaded with Ca^{2+} before exposure to a high [Ca^{2+}] solution would induce a response.

Depolarization-induced responses during 'calcium-induced calcium release'

In all these experiments it was possible to show that when a fibre was normally polarized it could still produce large contractions via the normal excitation–contraction coupling mechanism involving the voltage sensor in the T-system. This was possible not only between 'Ca^{2+}-induced' responses but during such responses as well (Fig. 5*B*). In this case, depolarization by either choline chloride or Na^+ substitution still produced a large fast contraction in addition to the 'Ca^{2+}-induced' response, both at early times while the response was still rising as well as at later times when the response had largely declined. This was possible irrespective of whether the 'Ca^{2+}-induced' response reached a peak quickly (4–5 s) or more slowly.

The action of ryanodine on calcium release

Ryanodine depresses twitches and produces an irreversible contracture in intact skeletal muscle fibres (see data and references in Fryer, Lamb & Neering, 1989). It binds under certain conditions to at least one type of calcium release channel in the SR (Pessah *et al.* 1985; Imagawa *et al.* 1987) and blocks the channel open or shut, depending on its concentration and perhaps other factors (Meissner, 1986; Smith *et al.* 1988; McGrew, Wolleben, Siegl, Inui & Fleischer, 1989). The skinned-fibre preparation enables application and wash-out of ryanodine, at various myoplasmic [Ca^{2+}], when the voltage sensors in the T-system are in the resting, activated or inactivated states.

Effect of ryanodine on depolarization-induced responses

If a fibre was depolarized in the presence of 25 μM-ryanodine (pCa 6·7), the first response was similar to but slightly more prolonged than the contractions in the

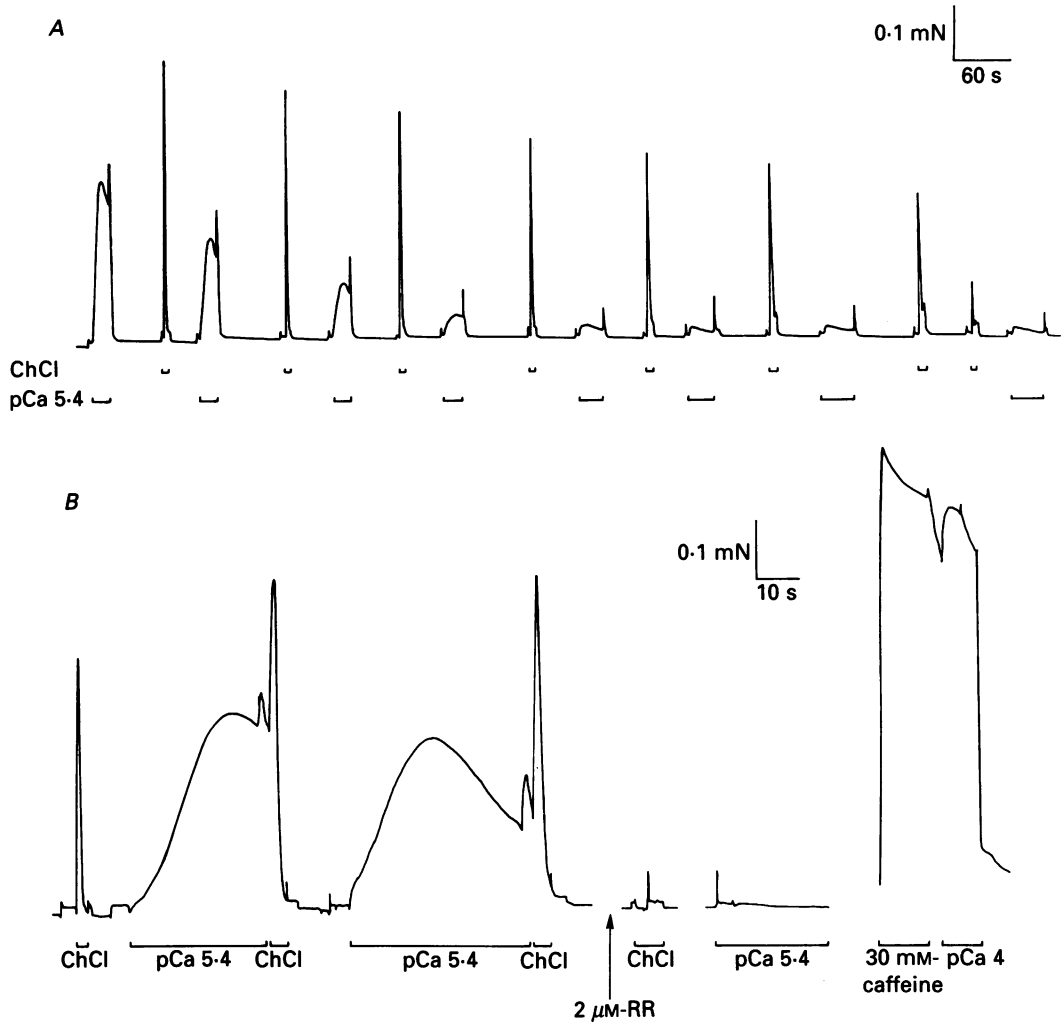

Fig. 5. Responses to repeated substitutions with a weakly buffered pCa 5·4 solution. *A*, the contractions produced by the pCa 5·4 solution (lower horizontal bars) became progressively smaller, roughly in parallel with the 'run-down' of the depolarization-induced responses (choline chloride (ChCl) substitution, upper horizontal bars). (*D*, 50 μm; *L*, 2·3 mm.) *B*, ruthenium red (RR, 2 μM) blocked both the depolarization-induced response and the response to a weakly buffered pCa 5·4 solution. (Ruthenium red in all solutions after arrow except caffeine and pCa 4 solutions.) During the response to the high [Ca^{2+}] solution, depolarization by choline chloride substitution still produced a large response. (*D*, 67 μm; *L*, 2·1 mm.) Each fibre was bathed in the standard K^+ solution except where indicated by the bars. All solutions except choline chloride have standard [K^+] and all solutions have 50 μM-EGTA except solution at pCa 4 (50 mM-EGTA).

absence of ryanodine (Fig. 6), and then no subsequent depolarization could elicit another response, even after 5 min in the standard K^+ solution. It is expected that straight after the depolarization most of the Ca^{2+} released is taken back up again by the SR (Lamb & Stephenson, 1990), because the results of Su (1987) show that ryanodine does not prevent skinned fibres loading Ca^{2+}. Nevertheless, several

minutes after the depolarization-induced response, the response to 30 mM-caffeine was relatively small in many fibres (Figs 6 and 7*B*), though in others the response was near maximum (Fig. 7*A*). The small response to caffeine in some fibres was not due solely to ryanodine blocking the caffeine-induced response (Su, 1987) because

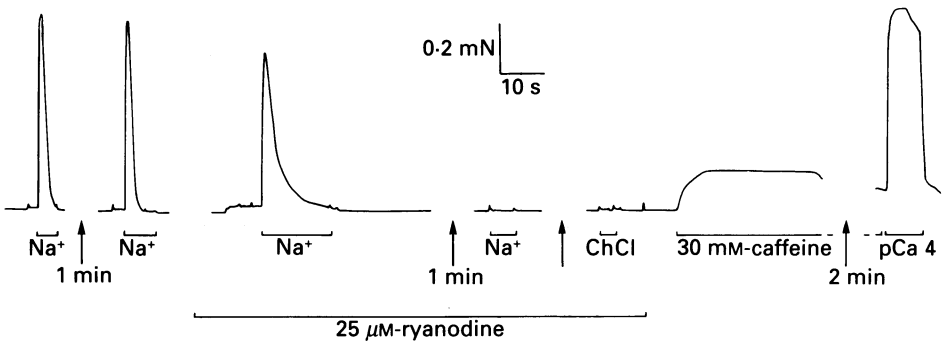

Fig. 6. Effect of ryanodine on depolarization-induced responses. After addition of 25 μM-ryanodine (in solutions indicated including Na^+ and choline chloride (ChCl) at pCa 6·7), the contraction triggered by Na^+ substitution was slightly prolonged. No subsequent depolarization could elicit a response and the response to 30 mM-caffeine was small. Note the small contracture immediately preceding the first depolarization in the presence of ryanodine. (*D*, 53 μm; *L*, 2·6 mm.)

disruption of the SR with Triton and caffeine together also produced relatively small responses (Fig. 7*B*). On the other hand, even taking account of the effect of 30 mM-caffeine on the Ca^{2+} sensitivity of the myofilaments (see earlier), the maximum or near-maximum response to caffeine in some fibres suggest that a substantial amount of Ca^{2+} could still be released. Therefore, assuming that depolarization releases Ca^{2+} from the same store as does 30 mM-caffeine, this implies that in some fibres the complete absence of the depolarization-induced responses is at least in part due to ryanodine blocking the release of Ca^{2+} rather than solely to the depletion of the releasable Ca^{2+}. Thus these results are consistent with ryanodine both blocking open some release channels, causing depletion of releasable Ca^{2+}, and blocking shut most of the channels, resulting in the complete absence of the depolarization-induced response. Clearly, the range of responses to caffeine found here may be accounted for by only a small difference in the proportion of channels blocked open, as the SR might be substantially depleted by only a small number of permanently opened channels.

The depolarization-induced responses also showed that ryanodine could block open some release channels at least transiently. When a fibre was exposed to 25 μM-ryanodine in a solution with higher [Ca^{2+}] (pCa 6·4), the first depolarization-induced contraction was markedly prolonged and could *not* be stopped by repolarizing the fibre, that is by returning to the K^+ solution (Fig. 7*A* and *B*). Similar results were obtained in four other fibres with 25 μM-ryanodine.

Ryanodine effects at low [Ca^{2+}]

The binding of ryanodine to SR vesicles is very dependent on [Ca^{2+}], there being virtually no binding at pCa > 6·5 (Pessah *et al.* 1985). This was confirmed here by

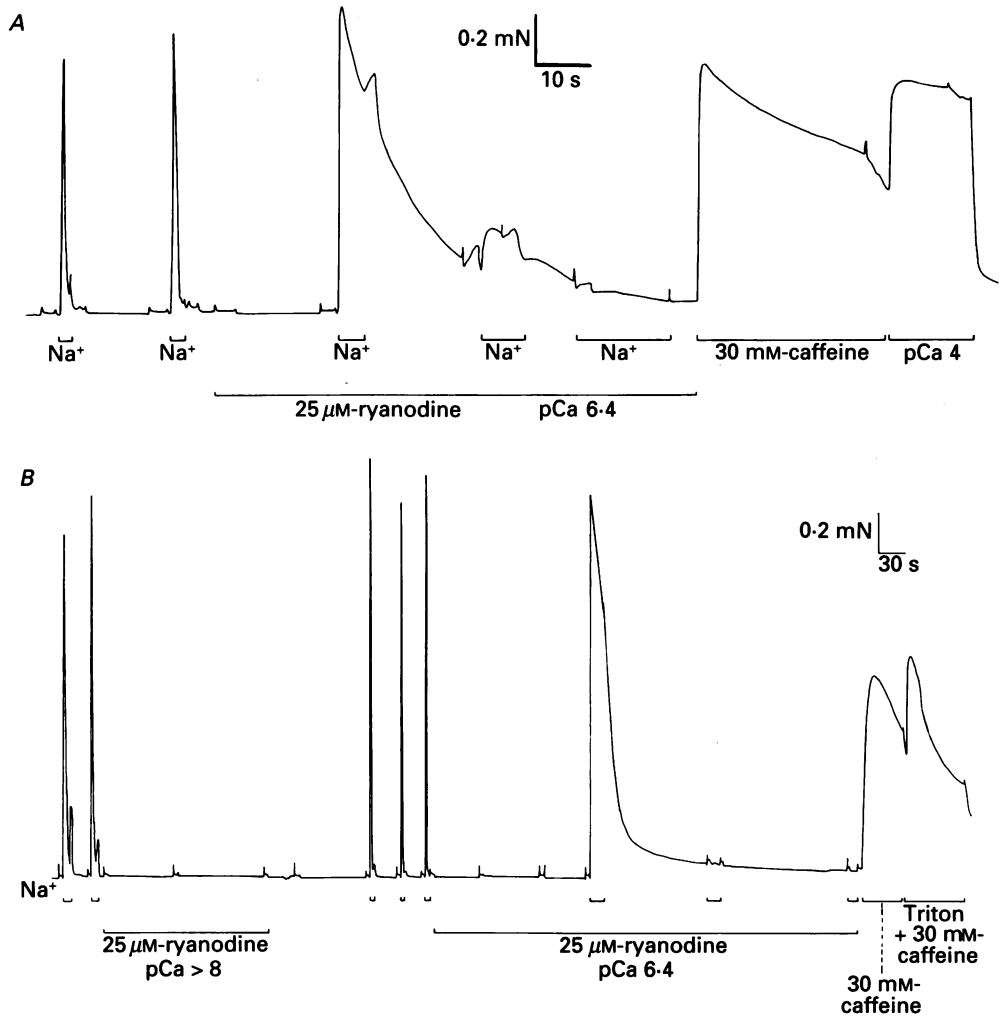

Fig. 7. Effect of ryanodine at different [Ca^{2+}]. *A*, after addition of 25 μM-ryanodine (pCa 6·4), the contraction triggered by Na^+ substitution was greatly prolonged and was not stopped by repolarizing the T-system, that is, by returning the fibre to the K^+ solution. Further depolarizations had virtually no effect, even though the calcium in the SR was not completely depleted as indicated by the large prolonged response produced by 30 mM-caffeine. (*D*, 53 μm; *L*, 3·0 mm.) *B*, when the myoplasmic [Ca^{2+}] was buffered to a low level (pCa > 8), 25 μm-ryanodine could be applied for 2 min and then washed out without subsequent effect on depolarization-induced responses in normal [Ca^{2+}] (pCa 6·7), indicating that the ryanodine had not bound. After further addition of ryanodine at pCa 6·4, the first depolarization-induced response was greatly prolonged and subsequent responses were abolished. (Unlabelled horizontal bars indicated Na^+ substitution.) (*D*, 68 μm; *L*, 2·1 mm.)

bathing a fibre for 3 min in a solution with 25 μM-ryanodine at pCa > 8 (0·5 mM-EGTA) and then washing out the ryanodine (Fig. 7*B*). This procedure had no effect on the responses to subsequent depolarizations indicating that the ryanodine did not bind to its receptor in the low [Ca^{2+}] solution if the fibre was not stimulated. Similar

results were obtained with two other fibres at pCa > 9 (10 mM free EGTA), as well as in two fibres when the ryanodine was applied for 1 min in a solution at pCa 6·7 (50 μM-EGTA) before being washed out. However, in other unstimulated fibres at pCa 6·7 a small contracture sometimes developed within 1 min (e.g. Fig. 6), in which case the subsequent responses were markedly affected. This was further investigated using higher $[Ca^{2+}]$.

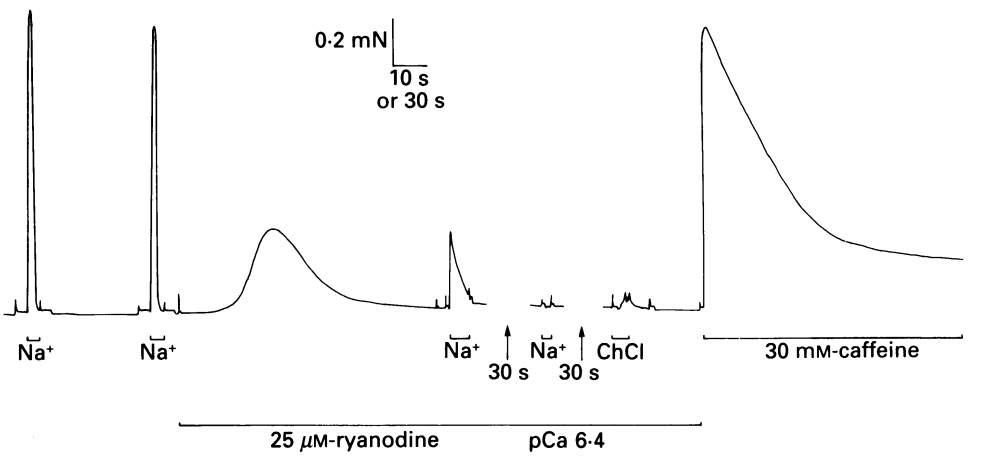

Fig. 8. Effect of ryanodine in a fibre with a normally polarized T-system. The presence of 25 μM-ryanodine at pCa 6·4 induced a slow contracture after about 45 s. After this contracture had declined almost completely, little or no response could be produced by depolarizing the fibre by Na^+ or choline chloride (ChCl) substitution, although 30 mM-caffeine could still elicit a maximal response. (*D*, 48 μm; *L*, 2·4 mm.) (Time scale: 10 s before and 30 s after addition of ryanodine.)

Ryanodine contracture in a polarized fibre

If a fibre was exposed to 25 μM-ryanodine at pCa 6·4 (in the K^+ solution with 50 μM-EGTA) a slowly rising contracture developed within about 15–60 s (Fig. 8), without the fibre being depolarized, in five of the nine fibres examined. This contracture peaked at between about 10 and 30 % of the maximum tension and then declined completely within 1 or 2 min, after which a depolarization elicited little if any response and subsequent depolarizations produced no response at all even if the fibre was left unstimulated for more than 5 min. It seems likely that at a critical $[Ca^{2+}]$ this ryanodine contracture starts as a self-reinforcing cycle of channel opening–ryanodine binding–increased $[Ca^{2+}]$ (see Discussion). This again would indicate that calcium-induced calcium release does exist in these fibres. A high concentration of ryanodine was used in these experiments to overcome the extremely slow binding of ryanodine to its receptor at low concentrations (of the order of the dissociation constant) and thus to produce an appreciable effect in a short time (see also Smith *et al.* 1988). Consistent with this, a much larger contracture was produced more quickly when an even higher ryanodine concentration (250 μM) was used (Fig. 10). The fact that depolarization-induced responses could not be elicited after such a ryanodine contracture, even though the caffeine response showed that there

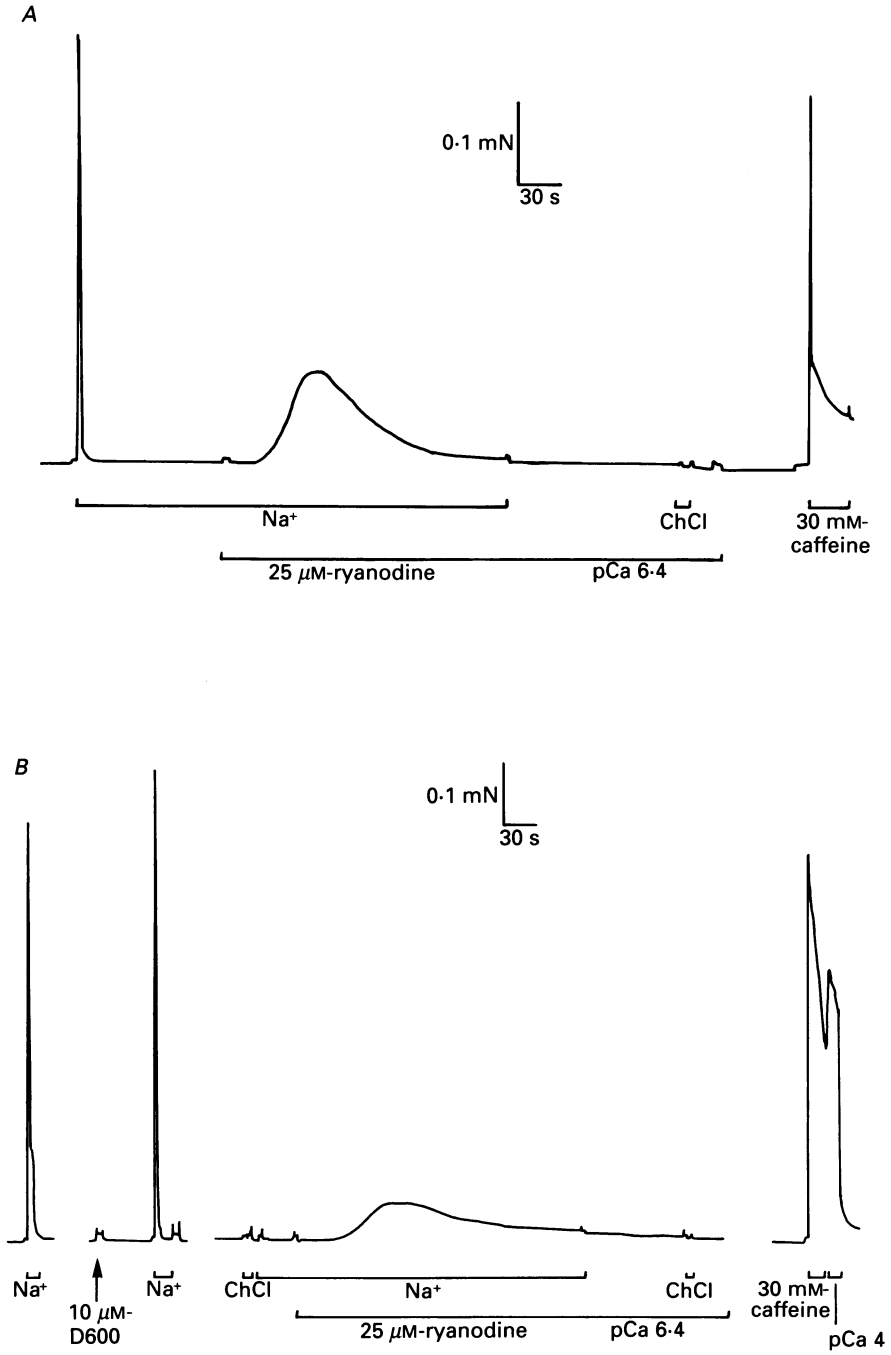

Fig. 9. Effect of ryanodine (25 μM) on fibres in which the voltage sensor had been inactivated by prolonged depolarization in the Na^+ solution (*A*) or by D600 (*B*). In *A*, following the ryanodine contracture, choline chloride (ChCl) substitution did not induce a response after the fibre had been repolarized; the fibre broke during the large contracture produced by 30 mM-caffeine. (*A*: *D*, 58 μm; *L*, 1·5 mm; *B*: *D*, 53 μm; *L*, 1·9 mm.) In *B*, D600 was present in all solutions after the arrow.

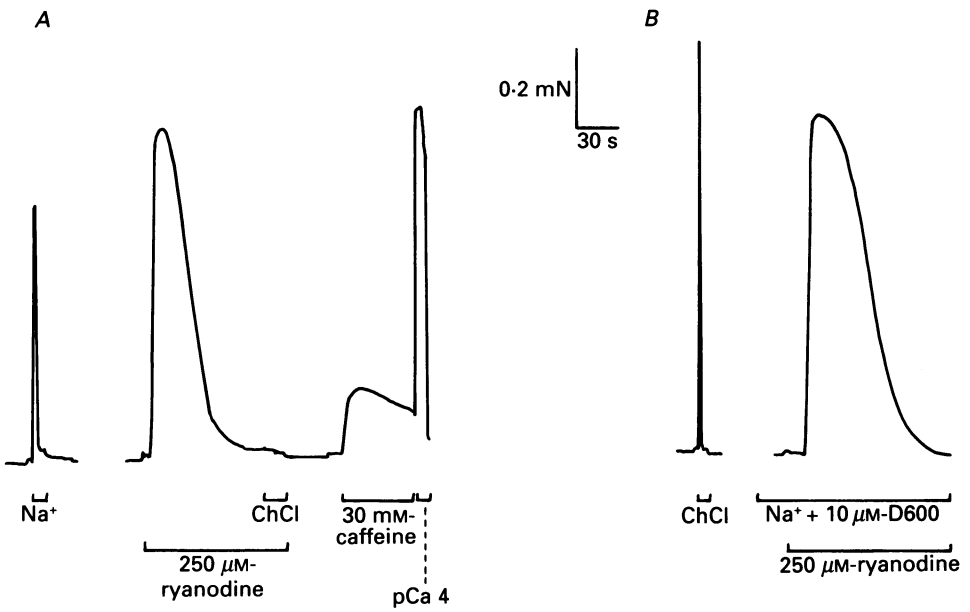

Fig. 10. Effect of high concentration of ryanodine. *A*, application of 250 μM-ryanodine rapidly produced a large contracture in a normally polarized fibre (*A*) and in another fibre (*B*) in which rapid contractions had been abolished by prolonged depolarization with Na^+ substitution in the presence of D600 (not shown). (All solutions pCa 6·7 with 50 μM-EGTA except pCa 4 solution.) (*A*: *D*, 58 μm; *L*, 2·1 mm; *B*: *D*, 52 μm; *L*, 1·7 mm.) ChCl, choline chloride.

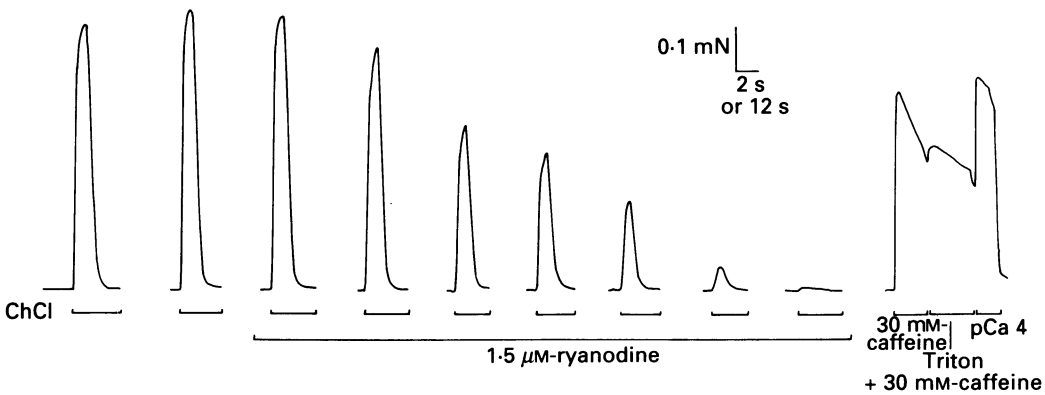

Fig. 11. Effect of low concentration of ryanodine. On successive depolarizations with choline chloride (ChCl) in the presence of 1·5 μM-ryanodine, the response became progressively smaller, without any change in the duration. After the response was abolished completely, 30 mM-caffeine could still produce a near-maximum contraction. (One minute between successive stimuli. All solutions pCa 6·7 with 50 μM-EGTA except pCa 4 solution. Time scale 2 s before and 12 s during and after application of caffeine.) (*D*, 50 μM; *L*, 1·4 mm.)

was an appreciable amount of Ca^{2+} in the SR (Fig. 8), implies that the channels producing most of the Ca^{2+} release in normal excitation–contraction coupling had been blocked shut.

Ryanodine contracture in a depolarized fibre

In order to examine whether inactivation of the voltage sensor in the T-system has any effect on the action of ryanodine, fibres were depolarized by Na^+ substitution for 1 min before addition of 25 μM-ryanodine in the Na^+ solution at pCa 6·4. In four out of the six fibres examined, a ryanodine contracture was induced within 1 min and when the fibre was repolarized in the K^+ solution after the contracture, no further depolarizations could elicit a response (Fig. 9*A*). These results show that 25 μM-ryanodine can still induce a contracture when the voltage sensors have been inactivated by prolonged depolarization. Similarly, a ryanodine contracture was elicited with 25 μM-ryanodine in five out of seven fibres in which the voltage sensors have been inactivated by prolonged depolarization in the presence of D600 (e.g. Fig. 9*B*) and in a skinned fibre obtained from a muscle bathed in solution with 5 mM-EGTA and no added Ca^{2+}. Before addition of ryanodine, depolarization did not elicit any response in this latter fibre, presumably because the voltage sensors had been inactivated in the presence of the low $[Ca^{2+}]$ in the T-system (see Lamb & Stephenson, 1990). As found in polarized fibres, the ryanodine contracture in a fibre inactivated by D600 was larger and occurred more rapidly when an even higher concentration of ryanodine (250 μM) was used (Fig. 10*B*).

Effect of a low concentration of ryanodine

Finally, the effects of low concentrations of ryanodine were examined to investigate whether the channels can be kept blocked open rather than shut, as might be inferred from reports in SR vesicles where 0·01–10 μM increases rather than decreases the calcium permeability (Meissner, 1986; Lattanzio, Schlatterer, Nicar, Campbell & Sutko, 1987). No contracture was observed in any of five fibres exposed to 1·5 μM-ryanodine (pCa 6·7) and the first depolarization-induced response after 5 min exposure produced the same tension as control responses in the absence of the drug. However, if the fibre was stimulated the responses to successive depolarizations progressively declined, without any increase in width, until no response could be elicited after about five or six depolarizations (Fig. 11). Application of 30 mM-caffeine then indicated that there was a substantial amount of Ca^{2+} in the SR. These data suggest that at this concentration ryanodine has little effect unless the channels are opened by the depolarization and that then most of the channels are not blocked opened but instead are blocked shut, though this may have occurred after a brief blocked open period.

DISCUSSION

Actions of ryanodine

The data in Fig. 7*A* provide direct evidence that ryanodine interferes with the ability of the voltage sensor to control Ca^{2+} release in a muscle fibre with functional excitation–contraction coupling. Once some Ca^{2+} release channels had been opened

by the depolarization in the presence of ryanodine, repolarization could not terminate the release. This is in marked contrast with the effect of repolarization in the absence of ryanodine (Fig. 1) and is consistent with ryanodine blocking at least some of the channels into a partially open state (Smith *et al.* 1988). The reduced response to caffeine observed in some fibres several minutes after a depolarization in the presence of ryanodine (Figs 6 and 7*B*) indicates that the SR was somewhat depleted of Ca^{2+} despite the long loading times, and this again is consistent with some of the channels being blocked open. The spontaneous contracture in ryanodine also indicates that the alkaloid blocks open some Ca^{2+} release channels (see later). Nevertheless, the absence of a depolarization-induced response at times when caffeine can release substantial amounts of Ca^{2+} from the SR (Figs 7*A*, 8 and 9*A*), implies that ryanodine eventually blocks shut most of the channels involved in normal excitation–contraction coupling, including those channels initially blocked open.

Calcium-induced calcium release

Clearly, Ca^{2+} can activate one type of Ca^{2+} release channel in lipid bilayers (Smith *et al.* 1986). Nevertheless, this does not necessarily imply that it can do so in functioning muscle fibre where the release channel may be controlled solely by some other mechanism, for example by a close physical arrangement with the voltage sensor in the T-system (Chandler *et al.* 1976). This close association might be altered in some skinned-fibre preparations, such as in chemically skinned fibres (Ohta *et al.* 1989) where saponin is used to disrupt the surface and T-system membranes. The results of this study, in a preparation where normal excitation–contraction coupling is intact (Lamb & Stephenson, 1990), suggest that Ca^{2+} can indeed stimulate Ca^{2+} release from the SR in a functional muscle fibre at physiological levels of Mg^{2+}, ATP and pH. If a fibre heavily loaded with Ca^{2+} was suddenly exposed to a high [Ca^{2+}] solution a substantial response could be elicited (Fig. 4), and the 'run-down' of this response upon repeated stimuli and its elimination by 2 μM-ruthenium red (Fig. 5) suggested that it involved Ca^{2+} release from the SR. It also seems similar to the calcium-induced calcium release observed in mechanically skinned fibres from barnacle muscle (Lea & Ashley, 1989).

Ryanodine contracture and calcium-induced calcium release

High concentrations of ryanodine could be applied and washed out without subsequent effects if the [Ca^{2+}] was buffered to very low levels (pCa > 8, 0·5 mM-EGTA) (Fig. 7*B*), showing that ryanodine had not bound at this low [Ca^{2+}]. The binding of ryanodine appears to depend on the channel being *opened* (by depolarization or Ca^{2+}), rather than on the presence of Ca^{2+} *per se*. This is suggested by (a) the similarity of the Ca^{2+} dependence of ryanodine binding (Pessah *et al.* 1985) and Ca^{2+} release rate in SR vesicles (Meissner *et al.* 1986), and (b) the fact that ryanodine binding and calcium release are similarly facilitated by ATP (Meissner *et al.* 1986; Imagawa *et al.* 1987). Thus, this would readily explain the initiation of the ryanodine contractures (Figs 8–10): when the [Ca^{2+}] is raised above a critical level (about pCa 6·5), some channels would be opened by the Ca^{2+} and then blocked open by the ryanodine, thereby further raising the [Ca^{2+}] and opening other channels and

so on. Thus it appears that ryanodine produces a contracture by potentiating calcium-induced calcium release.

Normal control of Ca^{2+} release

The fact that the depolarization-induced responses are blocked after a ryanodine contracture *without* any depolarization (Fig. 5), shows that normal excitation–contraction coupling must involve a type of release channel which *can* be opened by Ca^{2+} under some circumstances. Nevertheless, the following points strongly suggest that the channels involved in normal excitation–contraction coupling are *not* controlled simply by Ca^{2+}. (1) Neither high concentrations of EGTA (Fig. 2) nor Fura-2 (Baylor & Hollingworth, 1988) prevent very substantial release of Ca^{2+} by depolarization. This indicates that Ca^{2+} is not necessary for the opening of many if not all of the release channels. (2) When calcium-induced calcium release was produced it was considerably smaller and slower than the depolarization-induced responses recorded in the same fibres (Figs 4 and 5). Moreover, the fact that there was some tension generated clearly indicates that the $[Ca^{2+}]$ near the myofillaments was raised considerably (i.e. to about pCa 5·9–5·7) and that $[Ca^{2+}]$ near any channels releasing Ca^{2+} must have been considerably higher, but nevertheless this did not produce an 'explosive' response comparable with the depolarization-induced response. It might be proposed that the response to the exogenous Ca^{2+} was much smaller than the response to Ca^{2+} released close to other calcium release channels because the $[Ca^{2+}]$ rose so slowly that it 'inactivated' many of the Ca^{2+}-sensitive release channels without activating them. However, if this accounts for the small responses, it would imply that such channels could not be involved in depolarization-induced release because depolarization still produced a larger response even when the calcium-induced calcium release had almost completely declined (Fig. 5*B*). (3) Repolarization rapidly stops Ca^{2+} release (Fig. 1) even when the fibre is heavily loaded with Ca^{2+}, a condition which might be expected to promote calcium-induced calcium release through any Ca^{2+}-activated channels. This strongly indicates that many if not all of the release channels involved in normal excitation–contraction coupling remain tightly under control of the voltage sensor in the T-system and could not be activated primarily by Ca^{2+} as repolarization would not stop them releasing Ca^{2+} once they had opened.

It might be proposed that such Ca^{2+}-activated channels produced the fast initial Ca^{2+} release and then inactivate, leaving separate voltage-controlled channels to produce a sustained release. If this were the case, the actions of (a) ruthenium red in completely abolishing all parts of a prolonged response (Lamb & Stephenson, 1990) and (b) ryanodine, would imply that the two types of channel were pharmacologically similar. However, such a role of Ca^{2+}-activated channels seems unlikely in view of the arguments above and the fact that the response controlled directly by the voltage sensor is clearly very considerable (Fig. 1), making any amplification seem unnecessary.

Thus together these results indicate that normal excitation–contraction coupling does not primarily involve Ca^{2+} release from a channel opened *exclusively* by Ca^{2+}, either as the sole type of release channel or as an 'amplifier' to Ca^{2+} released from another type of channel more directly controlled by the depolarization (Stephenson,

1981). This is also consistent with voltage clamp and potassium contracture studies which show that a small depolarizing stimulus can produce a prolonged sub-maximum response (Hodgkin & Horowicz, 1960; Caputo, Bolanos & Gonzalez, 1984), which would seem difficult to achieve if many or most of the release channels were activated solely by Ca^{2+}. However, it is possible that Ca^{2+} might further activate or modulate a channel already opened by some other stimulus, such as voltage (see below).

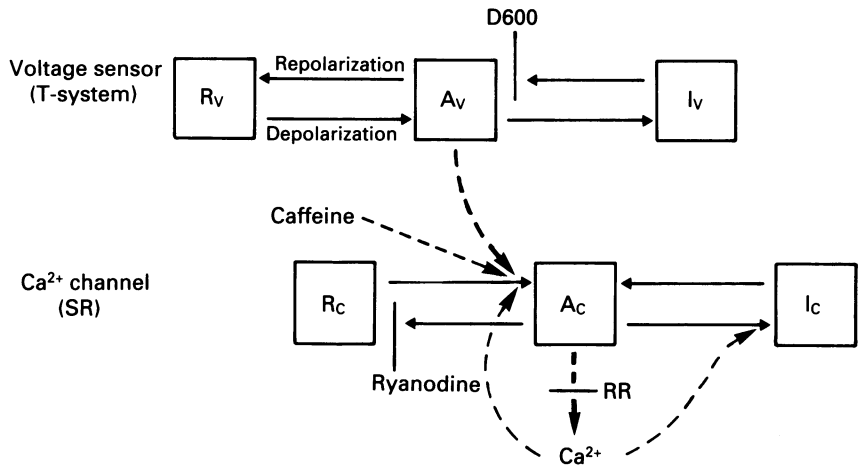

Fig. 12. Schematic diagram showing the effects of T-system potential and various agents on the state of the voltage sensor in the T-system and the Ca^{2+} release channel in the SR. The voltage sensor (subscript V) and release channel (subscript C) move between their respective resting (R), activated (A) and inactivated (I) states. D600 and ryanodine hinder certain transitions as indicated by a bar blocking an arrow and ruthenium red (RR) blocks Ca^{2+} release. Activation of the voltage sensor strongly favours activation of the release channel, as does caffeine. By itself, myoplasmic Ca^{2+} only weakly activates the release channel, but in conjunction with activation of the voltage sensor Ca^{2+} may further potentiate release. High myoplasmic $[Ca^{2+}]$ may also inhibit Ca^{2+} release by promoting inactivation of the release channel.

A single type of Ca^{2+} release channel

We propose that the results presented here and in the preceding paper (Lamb & Stephenson, 1990) can be accounted for by a single type of Ca^{2+} release channel which is normally activated primarily by the voltage sensor in the T-system but which also can be activated to some extent by Ca^{2+} in the myoplasm. Figure 12 is a schematic diagram of the sum of the effects of various factors on the voltage sensor and Ca^{2+} release channel, based on these results and the results of many other studies. Some of the key features are as follows. (1) Activation of the voltage sensor strongly promotes activation (i.e. opening) of the release channel. (2) De-activation and inactivation of the voltage sensor removes the positive stimulus to release allowing the channel to revert to its resting state (Fig. 1). (3) Myoplasmic Ca^{2+} can activate the release channel to a small degree under physiological conditions, which accounts for observations of calcium-induced calcium release (Figs 4 and 5). (4) Caffeine can directly stimulate the release channel without any action on the voltage sensor, and

inactivation of the voltage sensor does not affect the action of caffeine (Fig. 3*A*). Caffeine and activation of the voltage sensor together probably stimulate the release channel more strongly than either stimulus alone. (5) Two micromolar ruthenium red acts on the release channel and probably physically blocks Ca^{2+} release or alternatively ruthenium red may promote inactivation of the channel (Fig. 5). (6) Ryanodine binds to the open channel and prevents it from reverting to its resting state (Fig. 7*A*). (7) D600 prevents the voltage sensor from recovering from its inactivated state but does not prevent direct stimulation by caffeine or Ca^{2+} (Fig. 3*B*). (8) High myoplasmic Ca^{2+} might be able to inactivate the release channel (Lamb & Stephenson, 1989); this is 'calcium-dependent inactivation'.

In this scheme, myoplasmic Ca^{2+} is greatly limited in its ability to activate the release channel when the voltage sensor is in its resting or inactivated states. Nevertheless, when the channel has been at least partially activated by activation of the voltage sensor, the released Ca^{2+} may well act upon the channel to further increase the proportion of time the channel is activated. Such a mechanism has been proposed previously (Lüttgau & Stephenson, 1986). In any case, when the voltage sensor is deactivated, the Ca^{2+} is not sufficient to keep the channel activated and it again reverts to its resting state. Of course, if the Ca^{2+} release channel is not in its normal association with the voltage sensor, such as in a lipid bilayer experiment, Ca^{2+} may well activate the channel more strongly.

It is proposed that the coupling between the voltage sensor and the release channel is relatively direct, though this does not necessarily mean that the two molecules are physically linked to each other. For example, the sensor might exert its effect on the channel via some other molecule, so binding studies would not reveal the coupling. This intervening molecule might not form a physical link but instead be a 'second messenger'. We have already indicated that it is unlikely that Ca^{2+} could act in such a way, but it is possible IP_3 could (Vergara *et al.* 1985). Nevertheless, any second messenger must be continuously produced and removed for many seconds to explain the prolonged response which can be terminated at any time (Fig. 1).

There seems to be no evidence from these experiments that inactivation of the voltage sensor can *inhibit* calcium release. The response to 1 mM-caffeine seemed completely unaffected by inactivation of the voltage sensor, regardless of whether this was induced by prolonged depolarization or treatments with ouabain, saponin, D600 or low T-system $[Ca^{2+}]$ (Fig. 3 and see Lamb & Stephenson, 1990). Similarly, calcium-induced calcium release was not noticeably different when the voltage sensor was in its resting or in its inactivated state. The observation that 25 μM-ryanodine could still induce a contracture in a fibre when the voltage sensor had been inactivated by prolonged depolarization, D600 or low $[Ca^{2+}]$ in the T-system, seems to indicate that ryanodine can still bind to and affect the release channel. Thus it seems that the action of low external $[Ca^{2+}]$ in abolishing the ryanodine contracture in an intact fibre (Fryer *et al.* 1989) is not the result of inactivation of the voltage sensors in the T-system, but instead is the result of a direct lowering of the myoplasmic $[Ca^{2+}]$. Finally, we note that in contrast to Pessah *et al.* (1985), Valdivia & Coronado (1989) report that in a triad junction preparation high-affinity binding at low concentration of ryanodine is inhibited by the binding of verapamil. However, in the absence of exact details of how this experiment was performed it is difficult to

be certain that this inhibition was directly and solely associated with inactivation of the voltage sensor, though if it were, this would further support the type of model we propose.

In summary, the results of this and many other studies can be drawn together to suggest that normal excitation–contraction coupling in skeletal muscle involves the voltage sensor in the T-system activating a *single* type of calcium release channel in the SR in a relatively direct manner.

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