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THE ACTION OF RYANODINE ON RAT FAST AND SLOW INTACT SKELETAL MUSCLES

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SUMMARY

1. The action of ryanodine on force development of bundles dissected from rat extensor digitorum longus (EDL) and soleus muscles has been examined.

2. Ryanodine (100–5000 nM) irreversibly depressed twitch and tetanic tension of both muscle types in a dose-related manner.

3. At concentrations above 250 nM, ryanodine induced a slowly developing, dose-dependent contracture which could not be blocked by 5 mM-Co^{2+}. Increasing the stimulation rate or decreasing the oxygenation of the preparation accelerated the rate of contracture development while the total removal of extracellular Ca^{2+} was required to prevent it.

4. Following the relaxation of the initial contracture (IC) in Ca^{2+}-free solution, a second type of contracture (SC) could be induced by the readdition of Ca^{2+}. This contracture differed from IC in that it was dependent on Ca^{2+} in the millimolar range and was prevented by 5 mM-Co^{2+}. Both IC and SC were relaxed by perfusion with Ca^{2+}-free, EGTA-containing solution.

5. Subcontracture doses of ryanodine (100 nM) markedly potentiated caffeine contractures of both muscle types.

6. Asymmetric charge movement in EDL fibres was recorded with the Vaseline-gap technique. The amount of charge moved near threshold was virtually unaffected by the presence of 10 μM-ryanodine over the time examined.

7. The results are consistent with the suggestion that ryanodine locks the calcium release channels of the sarcoplasmic reticulum (SR) in an open subconductance state with reduced conductance. It appears that lowering the external calcium concentration might still inactivate the release channels after they have been blocked open by ryanodine, possibly by an effect on the T-tubular voltage sensor.

INTRODUCTION

Ryanodine is a plant alkaloid that has profound effects on skeletal, smooth and cardiac muscle function at concentrations in the nanomolar to micromolar range (for reviews see Jenden & Fairhurst, 1969; Sutko, Ito & Kenyon, 1985). In skeletal muscle, ryanodine depresses twitch tension and then causes a slow, irreversible

contracture (Edwards, Weiant, Slocombe & Roeder, 1948; Hadju, 1969). Such actions were observed as long as 40 years ago yet the mechanisms underlying these potent effects have not been clarified. Early studies showed that ryanodine had little effect on electrical excitability, contractile protein sensitivity or the maintenance of intracellular ATP levels (Edwards *et al.* 1948; Blum, Creese, Jenden & Scholes, 1957; Seraydarian, Jenden & Abbott, 1962). Since then it has been postulated that ryanodine's primary effect is to modify the release and/or the uptake of Ca^{2+} by the sarcoplasmic reticulum (SR). Supporting evidence for such an action has recently come from studies utilizing radioligand binding (Pessah, Waterhouse & Casida, 1985), Ca^{2+} fluxes in isolated SR vesicles (Meissner, 1986; Nelson, 1987), tension transients in skinned striated fibres (Su, 1987, 1988) and SR Ca^{2+} channels incorporated into lipid bilayers (Rousseau, Smith & Meissner, 1987). These studies suggested that ryanodine first binds with high affinity to the SR Ca^{2+} channel and then modifies both the gating and conductance of the channel depending on the prevailing ionic environment of the cytoplasm.

In this paper, we have systematically examined the action of ryanodine on contractile properties of intact, mammalian fast- and slow-twitch muscles, as well as asymmetric charge movement and have attempted to interpret the results in the light of the findings referred to above.

A preliminary account of some of the results described here was presented to the Australian Physiological and Pharmacological Society (Fryer & Neering, 1988).

METHODS

Contractile studies

Wistar rats of either sex (200–300 g) were killed by exsanguination under ether anaesthesia. Either a soleus or extensor digitorum longus (EDL) muscle was removed and a small bundle of 10–40 fibres dissected free. The bundle was then transferred to a small, temperature controlled (25 ± 0.2 °C) bath where it was perfused with normal Krebs solution and stimulated directly (0·5 ms duration, supramaximal voltage) by platinum electrodes set 5 mm apart. Bundle length was adjusted to give maximum tetanic tension. Force was measured isometrically using a semiconductor strain gauge (Ackers AME, Horten, Norway) and was recorded on both chart paper and FM tape. After digitization, the time course and amplitudes of force responses were analysed on an IBM-XT computer using ASYSTANT (Macmillan) software. The relaxation of both twitch and tetanic tension was well fitted by a single exponential between 70 and 10% of peak force ($r^2 > 0.97$ in all cases.).

The normal Krebs solution contained (in mM): NaCl, 118; KCl, 4·7; KH_2PO_4, 1·2; $MgSO_4$, 0·6; $NaHCO_3$, 25; glucose, 11; $CaCl_2$, 2·5.

Calcium-free EGTA (ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid) solution had the same composition as normal Krebs except that $CaCl_2$ was omitted and 1 mM-EGTA was added.

HEPES (*N*-2-hydroxy ethyl piperazine-*N*-2-ethanesulphonic acid) buffered solutions were used for solutions containing 5 mM-$CoCl_2$, composition (in mM): NaCl, 143; KCl, 4·7; $MgSO_4$, 0·6; glucose, 11; $CaCl_2$, 2·5; HEPES, 1·0. All solutions were equilibrated with 95% O_2–5% CO_2 and contained (+)−tubocurarine (15×10^{-3} mM). The pH of all solutions was 7·4 at 25 °C. Ryanodine (Penick) was dissolved in double distilled water and stored as a stock solution (1 mM) at 0–5 °C. Aliquots of stock were diluted to the required concentration in the appropriate buffer. Because ryanodine's action was irreversible with normal washing, only a single concentration was used in each dose–response experiment.

Charge movement

Asymmetric charge movement and calcium currents were recorded using the triple Vaseline-gap technique to voltage clamp a segment of a single EDL fibre, as described previously (Lamb, 1986;

Lamb & Walsh, 1987). Non-linear ionic currents were suppressed by ion replacement and the presence of specific channel blockers. The fibre was bathed internally with (in mM): sodium glutamate, 150; MgATP, 3; Tris cyclic AMP, 1; Tris phosphocreatine, 5; TES buffer (*N*-tris(hydroxy-methyl)-methyl-2-aminoethanesulphonic acid), 10; with the pH adjusted to 7·1 (with NaOH) and the osmolarity adjusted to 290 mosmol/l. In the experiments noted, 10 mM-EGTA replaced a similar amount of glutamate. The external solution contained (in mM): tetraethylammonium bromide, 150; $CaBr_2$, 2; $MgBr_2$, 0·2; TES buffer, 2; 2,4-dichlorophenoxyacetic acid, 2·5 (to block chloride channels); sucrose, 40; tetrodotoxin, 10^{-3}; with pH 7·3 and osmolarity 330 mosmol/l.

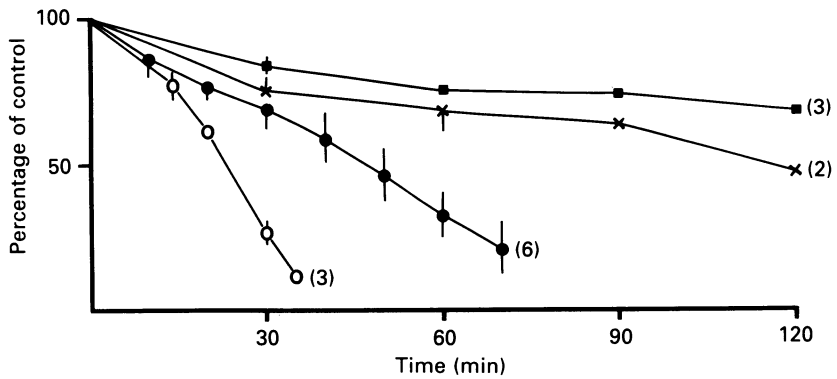

Fig. 1. Dose-related depression of twitch tension by ryanodine in EDL muscles. Stimulation frequency 0·033 Hz. Temperature 25 °C. Symbols: (■) 100 nM; (×) 250 nM; (●) 1000 nM; (○) 5000 nM-ryanodine. Points represent peak twitch tension in presence of ryanodine expressed as a percentage of control responses (± 1 standard error of the mean, (S.E.M.)). At some points S.E.M. is less than the symbol size. Only a single concentration of ryanodine was studied in each experiment. Number of experiments at each dose is shown in brackets.

From a holding potential of −90 mV, the fibre was subjected to a 20 mV hyperpolarizing 'control' step (35 ms long) followed 50 ms later by a 35 ms depolarizing 'test' step. Both the voltage and the current signals were filtered at 2 kHz by a 4-pole Bessel filter and sampled at 10 kHz by the DEC PDP 11/23 computer which generated the voltage command sequence. Records are the average of four control–test sequences, 2 s apart. Linear, ionic and capacitive currents were removed by both analog and digital means by scaling and subtracting the currents generated by the control step from that generated by the corresponding test step. In experiments with contracting fibres (no internal EGTA), the fibre was arranged in the gap with a considerable amount of slack, so that it could contract with some vigour without pulling itself out of the Vaseline; typically this was for potentials about 10 mV above the threshold for just visible contraction. All experiments were performed at 22 °C.

RESULTS

Effects of ryanodine on isometric twitch

Ryanodine (100–5000 nM) irreversibly depressed the magnitude of the twitch tension of skeletal muscles in a dose-related manner. This is shown in Fig. 1 which illustrates the results obtained from a number of EDL muscles in each of which a single concentration of ryanodine was studied at 25 °C. A similar relationship was found for tetanic tension except that the curves (not shown) had an upward shift of 5–10 % compared to those for the twitch. In these muscles the average twitch : tetanus

ratio was 0·2–0·3 for all muscles at room temperature. From Fig. 1 it can also be seen that the time course of twitch depression by ryanodine was also dose dependent. Concentrations of ryanodine higher than 250 nM additionally induced a slowly developing contracture.

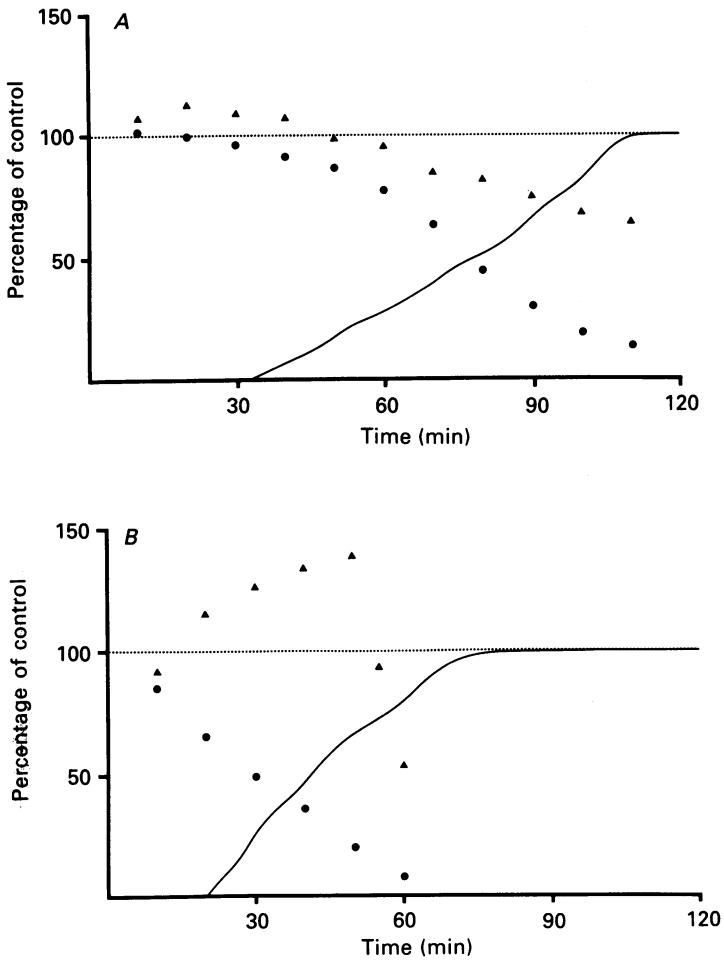

Fig. 2. Typical effects of ryanodine (1000 nM) on peak twitch tension (●), twitch relaxation rate constant (▲) and contracture development (continuous line) in single soleus (*A*) and EDL (*B*) muscles. Muscles were stimulated at 0·033 Hz. Ryanodine was added at time zero. Twitch values are expressed as a percentage of control while contracture is normalized so that 100% equals peak contracture tension.

The effects of 1 μM-ryanodine at 25 °C on soleus and EDL muscles stimulated to twitch at 0·033 Hz are shown in Fig. 2. This allows a comparison of changes in twitch tension, contracture development and twitch relaxation with time. The decline in twitch tension was more rapid in EDL than soleus muscles with 50% twitch depression occurring by 30 and 75 min respectively. There was considerably more

scatter in the grouped EDL results (1 μM, see Fig. 1) which had a mean value of 47 ± 7 min over six experiments.

An initial acceleration of the twitch relaxation rate was consistently observed in both muscle types, being particularly noticeable in the EDL. Eventually a slowing of relaxation occurred but this was only observed after the contracture had already developed.

Characterization of ryanodine contracture

After an initial latent period, ryanodine at concentrations above 250 nM induced a slowly developing contracture, the characteristics of which were dose dependent over the range studied (250–5000 nM). Figure 2 illustrates contracture development at a ryanodine concentration of 1000 nM with a stimulation frequency of 0·033 Hz. A comparison of panels *A* and *B* in the figure reveals that the contracture in the soleus muscle was slower to develop than in the EDL, with peak contractures being reached at 110 and 75 min, respectively. The latency of onset of a 1000 nM-ryanodine contracture in both muscles was between 20 and 40 min and was consistently longer for soleus than for EDL. The contracture's peak amplitude was around 30% of maximum tetanic tension (soleus, $30 \pm 6\%$, $n = 4$; EDL, $29 \pm 6\%$, $n = 6$).

A number of factors were found to accelerate the rate of contracture development. Increasing the stimulation rate or decreasing the flow of oxygenated Krebs solution to the muscle both had this effect (not shown). Ryanodine (5000 nM) induced a contracture even when the muscle was left unstimulated. Total removal of extracellular calcium ($[Ca^{2+}]_o$) prevented the development of this contracture while an 80% reduction of $[Ca^{2+}]_o$ to 0·5 mM had no effect on contracture development. Perfusion with a HEPES-buffered solution containing 5 mM-Co^{2+} also failed to affect this contracture.

The ryanodine contracture partially relaxed after reaching its peak but was essentially irreversible after washing out the drug with normal Krebs solution. In contrast, washing with Ca^{2+}-free Krebs (containing 1 mM-EGTA) completely relaxed the contracture within 15–30 min (Fig. 3*A*). Following the EGTA wash, there was no discernible response to electrical stimulation; however, the reintroduction of a normal Krebs solution (with or without ryanodine) brought about a rapid contracture (Fig. 3*B*), the properties of which differed from the original ryanodine-induced contracture.

Properties of the second contracture

This contracture did not require the presence of ryanodine in the normal Krebs (NK) solution. Its peak amplitude was highly variable but was always much greater in the soleus than the EDL. Soleus values ranged from 40 to 100% of maximum tetanic tension while for the EDL they were 5–20%. Peak tension was normally achieved within 10–20 min. If the preparation was perfused with Ca^{2+}-free Krebs solution containing 1 mM-EGTA at this point, the contracture could be rapidly relaxed (Fig. 3*B*). The most significant difference between this contracture and the original ryanodine-induced contracture was its sensitivity to variations in $[Ca^{2+}]_o$. Figure 4 illustrates the effect of grading $[Ca^{2+}]_o$ from 0·5 to 5·0 mM on the

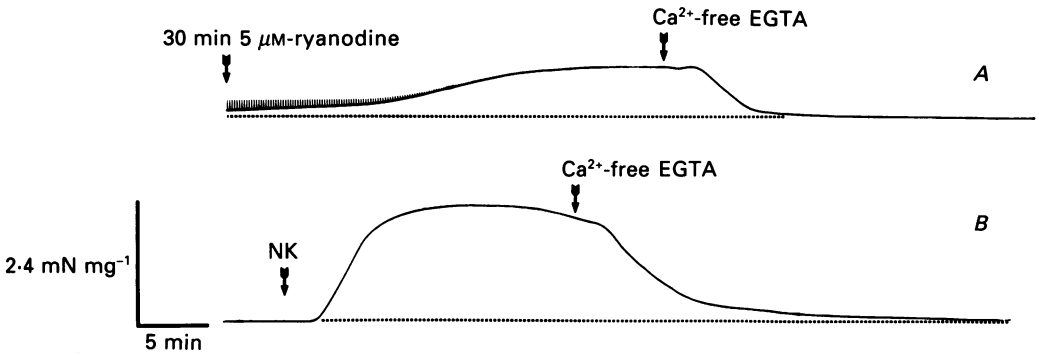

Fig. 3. Comparison of the initial contracture induced by ryanodine (*A*) and the second contracture (*B*) induced by readmission of normal Krebs solution after relaxing the first contracture with Ca^{2+}-free EGTA solution. Rat soleus, 25 °C. Trace *B* is a continuation of trace *A*. Before the start of (*A*) the muscle had been stimulated for 30 min in 5 μM-ryanodine at 0·033 Hz. At peak contracture the muscle was relaxed with Ca^{2+}-free solution containing 1 mM-EGTA. In trace (*B*) the muscle was reperfused with normal Krebs solution (NK) which induced a second contracture. This contracture was also relaxed by Ca^{2+}-free EGTA perfusion. Vertical calibration 2·4 mN (mg wet weight)$^{-1}$. Horizontal calibration 5 min.

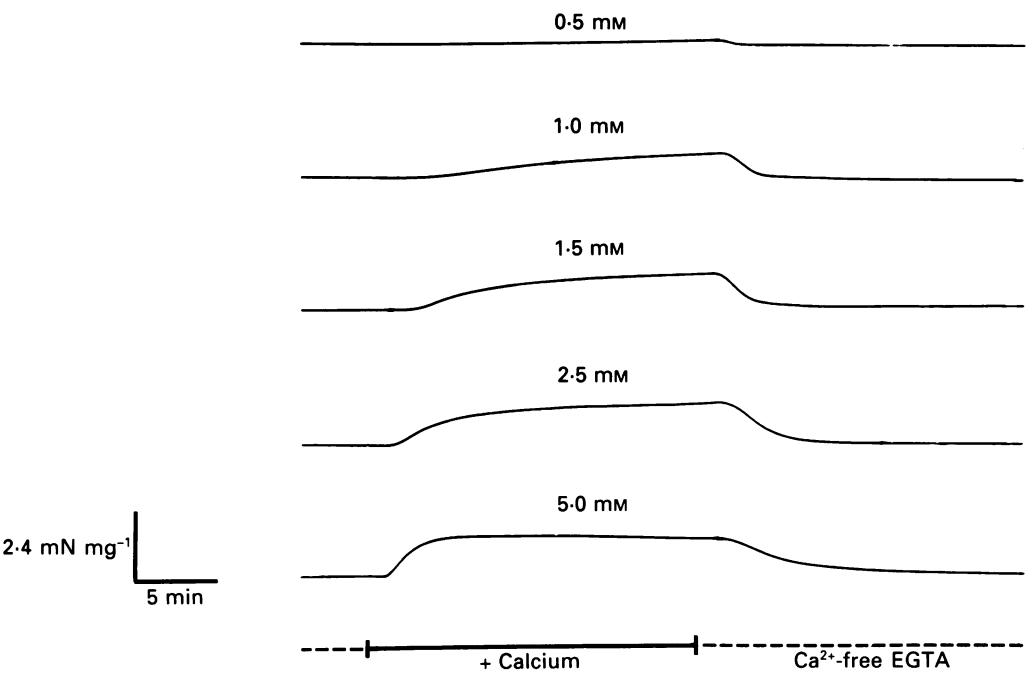

Fig. 4. Dependence of the peak amplitude and rate of rise of the second contracture on extracellular calcium. Rat soleus, 25 °C. Contracture was activated by perfusing the muscle with normal Krebs solution containing varying amounts of extracellular Ca^{2+} as shown, over the time interval indicated by the continuous line below the graph. Contracture was relaxed by perfusion with Ca^{2+}-free EGTA solution (dashed line below graph). Records were obtained in the order from top to bottom. Vertical calibration 2·4 mN (mg wet weight)$^{-1}$. Horizontal calibration 5 min.

development of this second contracture (SC) in a soleus muscle. Both the peak height and the rate of rise of force were clearly dependent on $[Ca^{2+}]_o$ over this concentration range. Pre-equilibration of preparations with 5 mM-Co^{2+} prevented the SC, presumably either by blocking I_{Ca} or antagonizing the effect of $[Ca^{2+}]_o$ on the voltage sensor (Fig. 5*B*). This effect was reversible upon wash-out of the Co^{2+} (Fig. 5*C*).

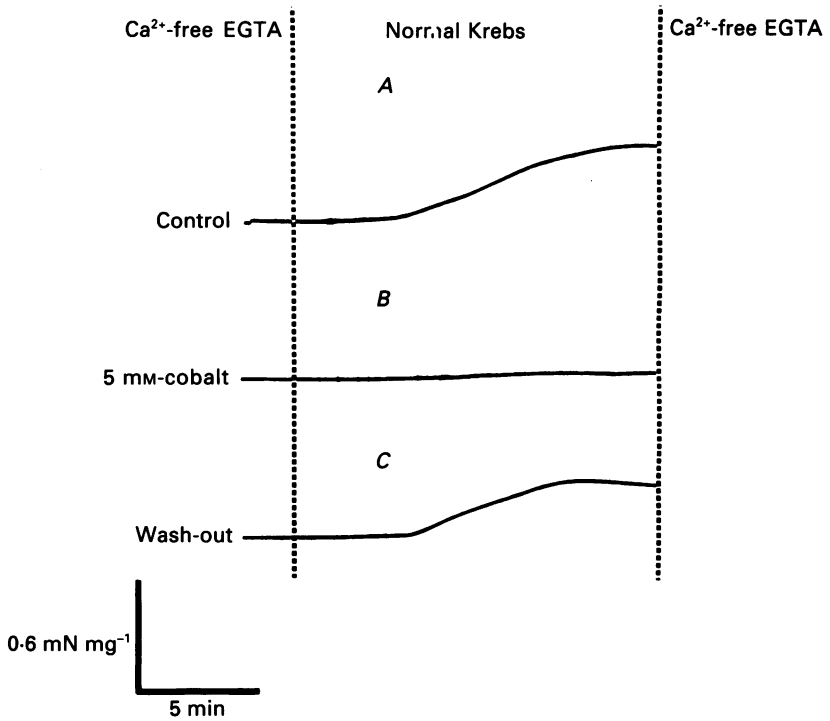

Fig. 5. Abolition of the second contracture by 5 mM-Co^{2+}. Rat soleus, 25 °C. On either side of the dotted line the muscle was relaxed with Ca^{2+}-free EGTA solution. Between the dotted lines the bundle was activated by perfusion with normal Krebs solution containing 2·5 mM-Ca^{2+}. *A*, control; *B*, contracture in the presence of 5 mM-Co^{2+} after 30 min Co^{2+} equilibration; *C*, contracture 30 min after Co^{2+} wash-out. Vertical calibration 0·6 mN (mg wet weight)$^{-1}$. Horizontal calibration 5 min.

From the observations above it could be argued that this contracture might be due to the destruction of the surface membrane in a manner analogous to the 'skinning' of muscles fibres with EGTA solutions (McLennan & Winegrad, 1978; Sutherland, Stephenson & Wendt, 1980). A number of observations make this possibility seem unlikely. Firstly, control experiments demonstrated that muscles exposed to Ca^{2+}-free, 1 mM-EGTA solutions for an hour or more did not develop contractures upon the readmission of normal Krebs solution. Moreover, high Mg^{2+} (10 mM), which has been used to reseal 'skinned' muscles, (McLennan & Winegrad, 1978; Sutherland *et al.* 1980) failed to abolish the calcium contracture. Figure 6 (top panel) shows a calcium contracture following ryanodine treatment and Ca^{2+}-free perfusion of an EDL muscle. Following another period in Ca^{2+}-free medium, the preparation was perfused with a Ca^{2+}-free Krebs solution containing 10 mM-Mg^{2+} for 20 min before being

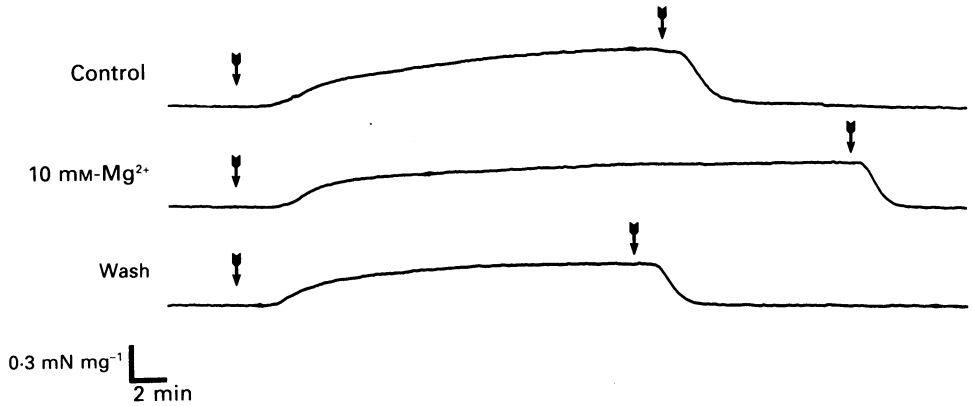

Fig. 6. High magnesium has little effect on the second contracture. Between the arrows in each trace, the bundle was activated with normal Krebs solution containing 2·5 mM-Ca^{2+}. Rat soleus at 25 °C. In the middle panel this solution also contained 10 mM-Mg^{2+}. Each contracture was relaxed with Ca^{2+}-free EGTA solution when it reached its peak. Between the first and second panels this solution also contained 10 mM-Mg^{2+}. Vertical calibration 0·3 mN (mg wet weight)$^{-1}$. Horizontal calibration 2 min.

exposed once more to Ca^{2+}-containing Krebs solution (middle panel). It can be seen that the Mg^{2+} caused only a small decrease in contracture amplitude although it did significantly slow the rate of rise of the contracture.

Effect of ryanodine on caffeine contractures

Ryanodine at subcontracture concentrations (100 nM) potentiated the ability of caffeine (10–50 mM) to produce contractures of both muscle types. After ryanodine treatment, the peak of the caffeine contracture was increased dramatically (soleus, $226 \pm 6\%$, $n = 3$, EDL, $321 \pm 51\%$, $n = 3$). This was reversible upon wash-out of caffeine.

Effect of ryanodine on asymmetric charge movement

Asymmetric charge movement is believed to reflect the movement of the voltage sensors in the transverse-tubular (T-) system which control calcium release from the SR (Schneider & Chandler, 1973), and these sensors appear to be closely related to the slow calcium channels (Lamb & Walsh, 1987; Rios & Brum, 1987; Tanabe, Beam, Powell & Numa, 1988). The preceding results suggest that ryanodine affects the control of myoplasmic calcium, and this raises questions about whether this results from, or is reflected in, any effect of ryanodine on charge movement or the inflow of calcium through the T-system calcium channels.

Internal EGTA

Addition of 5 or 10 μM-ryanodine to the external solution had virtually no effect over 30 min on either the calcium current or the asymmetric charge movement in the six fibres studied with 10 mM-EGTA inside the fibre. As the internal calcium concentration was buffered to very low levels in these fibres, even depolarizations to +20 mV did not elicit contractions, and thus the maximum amount of asymmetric

charge movement and the peak calcium current could be determined (see Lamb & Walsh, 1987). Over the period of ryanodine exposure the linear capacitance increased slightly in magnitude (mean 3%) and its time course was somewhat slowed, as observed previously in other fibres in the absence of ryanodine. The maximum charge (normalized by linear capacitance) decreased by 3 (±1·5)% (mean±S.E.M.) and the peak calcium current decreased by 4 (±4)%. As the slower linear capacitive current probably indicates that access resistance to the T-system had increased (Lamb, 1986), the small decrease in these parameters is to be expected and probably does not indicate any effect of ryanodine. There was also no consistent change in the membrane resistance of the fibres after addition of ryanodine; this showed that the fibres had not become more permeable to ions, in particular to calcium, at the holding potential. These results, showing no effect of ryanodine in fibres with low intracellular calcium, are consistent with the observation that ryanodine binding to intracellular sites is calcium dependent (Pessah *et al.* 1985).

Unbuffered calcium

The effect of ryanodine was studied in six fibres without internal EGTA, and thus the fibres were able to contract upon depolarization. If contractions were too vigorous, a fibre would pull itself out of the Vaseline, so depolarizations were restricted to about 10 mV past threshold. Consequently, the maximum charge and the size of the calcium current could not be recorded. (It was not possible to eliminate contraction by stretching the fibre, as this induced large non-linear ionic currents at the holding potential and upon depolarization.) Figure 7 shows the charge movement recorded in two EDL fibres for a depolarization to approximately 5 mV above threshold. Superimposed on each trace is the charge recorded at the same potential in the presence of 10 μM-ryanodine, just before each fibre contracted and destroyed the seal. The fibres were subjected every 3 min to sequences of depolarizations to between −60 mV and the potential shown. In every fibre examined, within 12–25 min after exposure to ryanodine the fibre took up the slack length in the gap and then within another 5 min swelled and pulled itself out of the Vaseline. In four cases, the contractions elicited by the depolarizations had become noticeably weaker or ceased altogether. Over this period the time course of the capacitive current appeared to slow more than in control experiments, probably because of the repeated contractions of the fibres, and this also resulted in a slowing of the charge movement signals as seen in Fig. 7 (see also Lamb, 1986). Nevertheless, despite this artifactual change in time course, the amount of charge moved at these potentials was virtually unaffected by the ryanodine; the mean amount of charge decreased by about 0·2 (±0·3) nC μF^{-1} or about 2·5%, which is not distinguishable from the control values, particularly considering the measurement errors. The mean capacitance dropped by 4 (±1)%. The presence of ryanodine appeared to affect myoplasmic calcium and contraction in these cut fibres in a way comparable to intact fibres. The loss of the fibre occurred on average 21 (±4) min after application of the ryanodine, whereas in control experiments with three other fibres without internal EGTA this occurred 46 (±5) min after addition of the same solution without ryanodine. In experiments with EGTA inside the fibre, fibres typically remained viable for 90–120 min. This suggests that the eventual swelling, increased leak and final contraction of the cut

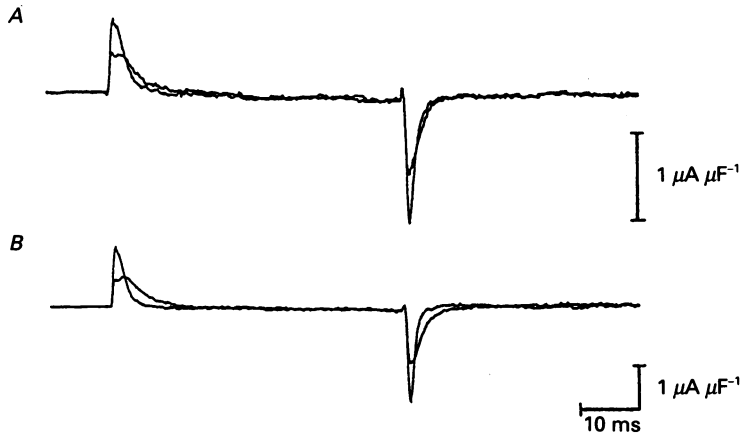

Fig. 7. Effect of ryanodine on asymmetric charge movement in 2 EDL fibres. *A*, superposition of charge movement traces produced by depolarizations to −45 mV just before (larger, faster trace) and 20 min after the addition of 10 μM-ryanodine. Over this period, the fibre ceased giving contractions and took up most of the slack, the capacitance (C_m) decreased by 4%, and the on and off charge (normalized by capacitance) dropped from 3·7 and 4·0 nC μF^{-1} to 3·6 and 3·7 nC μF^{-1} respectively. Note: the later trace has not been rescaled to take the decreased capacitance into account. *B*, superposition in another EDL fibre before and 16 min after addition of ryanodine. C_m unchanged, on and off charge increased from 5·9 and 6·0 nC μF^{-1} to 6·5 and 6·8 nC μF^{-1} respectively. Both fibres pulled themselves out of Vaseline within 2 min of records shown. The traces have not been corrected for any ionic currents.

fibres may be related to a rise in myoplasmic calcium, which is exacerbated in the presence of ryanodine.

DISCUSSION

The action of ryanodine in skeletal muscle is most probably intracellular. There was no indication that ryanodine could affect the charge movement, calcium current or membrane resistance by some action at an extracellular site. In the heavy SR fragments of skeletal muscle, Pessah *et al.* (1985) reported that [H^3]ryanodine bound to a single class of high-affinity receptors and suggested that these might be the SR Ca^{2+} release sites. It is thus probable that many of the effects of ryanodine illustrated in Figs 1–7 are a consequence of specific modifications of SR Ca^{2+} release sites. Recently, Rousseau, Smith & Meissner (1987) found that ryanodine affected SR Ca^{2+} channels by decreasing their conductance twofold and locking them in the open subconductance state.

Effects of ryanodine on isometric twitch

The potent depression of twitch tension by ryanodine is probably due to a combination of the two effects described above. In the early stages, SR Ca^{2+} release per twitch is likely to be decreased by ryanodine as a consequence of its effect on SR Ca^{2+} channel conductance. Twitch relaxation at this point is either unaffected or quite often faster than control indicating that SR Ca^{2+} uptake is not inhibited. The

second, delayed effect of ryanodine is to progressively lock SR Ca^{2+} channels in the open subconductance state, thereby allowing a passive Ca^{2+} leak from the SR to the cytoplasm and eventually causing a contracture. This enhances the twitch depression by decreasing the amount of SR Ca^{2+} available for release. At this stage, twitch relaxation becomes progressively slower suggesting that SR Ca^{2+} uptake is being impaired. It is important to note that this slowing of relaxation occurs only after the contracture is already well developed indicating that it may be a consequence rather than the cause of contracture.

The effects of ryanodine on speeding and slowing of twitch relaxation have not been previously described in skeletal muscle though an equivalent action has been observed in cardiac muscle (Sutko, Willerson, Templeton, Jones & Besch, 1979). Soleus muscles appeared to be more resistant to the effects of ryanodine than were EDL muscles. This observation is in agreement with the skinned fibre results of Su (1988) which showed that rabbit fast-twitch fibres were more sensitive to ryanodine-induced force depression than the slow-twitch fibres. Our results showed that (1) the onset of ryanodine contracture in the soleus was delayed and slower compared to the EDL and (2) the decrease in twitch tension as well as twitch relaxation rate was more gradual in the soleus. These results are consistent with those of Salviati & Volpe (1988) which showed that ryanodine had a greater affinity for SR from fast muscles than for SR from slower rabbit muscles. Another possibility is that the differences may reflect a basic variation between muscle types in the sensitivity and/or the open-state probability of individual SR Ca^{2+} channels.

Contractures induced by ryanodine

The slow contracture induced by ryanodine may be a result of the slow leakage of SR Ca^{2+} stores into the cytoplasm as discussed above. Evidence for a ryanodine-induced depletion of intracellular Ca^{2+} stores has previously been described in smooth (Hwang & Van Breemen, 1987), skeletal (Hwang, Saida & Van Breemen, 1987; Hwang, Godber, Lea, Ashley & Van Breemen, 1987) and cardiac (Bers, 1987; Hansford & Lakatta, 1987; Hilgemann, 1987) muscles.

The observation that increased stimulation rate and hypoxia accelerate contracture development is not a new one (Procita, 1956; Blum *et al.* 1957). It follows that if ($[Ca^{2+}]_i$) is further increased (faster stimulation) or its removal by the SR pump is slowed (hypoxia) then the conditions for contracture are enhanced. On the other hand if conditions favour extrusion of Ca^{2+} across the sarcolemma, the intracellular Ca^{2+} load would be diminished and a relaxation of the contracture would occur. This intepretation is supported by the observation that the contracture was completely relaxed by perfusion with Ca^{2+}-free EGTA solution (Fig. 4) though this relaxation could also be interpreted as an action of low Ca^{2+} on the T-system voltage sensor.

The second type of contracture (elicited by the reintroduction of normal Krebs solution after ryanodine and Ca^{2+}-free EGTA perfusion) was clearly different from the one described above. It has been previously described by Hadju (1969) though no systematic attempt was made to characterize it. Our results showed that unlike the initial contracture (IC), this second contracture (SC) was (1) dependent on extracellular Ca^{2+} in the millimolar range (Figs 3 and 4) and was prevented by 5 mM-

Co^{2+} (Fig. 5). Despite these differences both IC and SC were relaxed by perfusion with Ca^{2+}-free EGTA solution.

Effects of ryanodine on charge movement

It appears that ryanodine affects the control of myoplasmic calcium in cut fibres in the Vaseline gap and that this may be comparable with its effect in intact fibres, despite the different experimental conditions. Nevertheless, the charge movement in a cut fibre was virtually unaffected just prior to the fibre contracting irreversibly. This could imply that any action of ryanodine on the calcium release channel does not interfere with the movements of the proposed voltage sensor which normally controls that channel. Alternatively, it could be argued that because such a large amount of calcium might be released over several minutes through a small number of release channels, the fibre contracts and is lost even though only a few per cent of the sensors have been affected by the action of ryanodine. A third possibility is that perhaps even a relatively large proportion of voltage sensors has been permanently 'activated' by the ryanodine and hence does not contribute to the charge movement signal because the sensors no longer change conformation. As the maximum amount of charge movement could not be measured in these contracting fibres, this alternative cannot be eliminated.

Regardless of whether or how the charge movement is affected, it is known that when a mammalian muscle fibre is activated for a prolonged time in the presence of low extracellular calcium, subsequent contractions are eliminated (Graf & Shatzmann, 1984) because of a shift in the steady-state inactivation curve (Lüttgau & Spiecker, 1979). Nevertheless, calcium inflow from the extracellular solution is not necessary for E–C coupling as brief twitches can be produced for many minutes with very low extracellular calcium (Armstrong, Bezanilla & Horowicz, 1972). Thus, it appears that during long depolarizations, low extracellular calcium can directly inactivate calcium release from the SR, and this may involve an effect on the voltage sensor in the T-system (Graf & Shatzmann, 1984; Brum, Fitts, Pizarro & Rios, 1988; Brum, Rios & Stefani, 1988). If such inactivation depends on the prolonged activation of the SR channels or elevation of myoplasmic calcium, rather than on the prolonged depolarization *per se*, this would readily explain why low calcium abolishes both the initial and second ryanodine contractures, and why the size of the second contracture appears to be graded by the extracellular calcium concentration. If this is the case, the finding that cobalt prevented the occurrence of the second contracture implies that Co^{2+} interferes with the action of Ca^{2+} on the extracellular site controlling inactivation.

Effects of ryanodine on caffeine contractures

The marked potentiation of caffeine contractures by a low concentration of ryanodine might be explained by a number of possible mechanisms: (1) an increased Ca^{2+} uptake by the SR during ryanodine exposure; (2) a potentiation of SR Ca^{2+} channel opening or (3) an elevated $[Ca^{2+}]_i$ caused by Ca^{2+} leak from the SR that is subthreshold for contracture.

Evidence for (1) comes from Jones, Besch, Sutko & Willerson, (1979) who showed

that ryanodine could stimulate Ca^{2+} uptake in isolated SR vesicles from cardiac muscle.

The second hypothesis involves the locking open by subcontracture levels of ryanodine of the SR Ca^{2+} channels. The action of caffeine, by activating such channels, would thus be potentiated. This type of action of ryanodine has been seen in isolated SR Ca^{2+} channels incorporated into bilayers (Rousseau *et al.* 1987) and in isolated SR vesicles (Nelson, 1987). This hypothesis seems quite plausible in the light of experiments reported by Meissner, Rousseau & La Dine (1987) that caffeine acts on the SR Ca^{2+} release channel.

Finally, it is possible that even at low concentrations of ryanodine (100 nM) there is a small, subthreshold leak of Ca^{2+} from the SR to the cytoplasm. If this is so then more force would be generated for an equivalent amount of SR Ca^{2+} release induced by caffeine. Confirmation of such a mechanism can only be realized by direct measurement of $[Ca^{2+}]_i$ using suitable Ca^{2+} indicators (Wier, Yue & Marban, 1985).

Our results show that ryanodine is potent in depressing twitch tension and inducing contractures in rat fast- and slow-twitch skeletal muscles. These effects can largely be explained by considering ryanodine's complex actions on SR Ca^{2+} homeostasis. In particular it seems that ryanodine's actions are specific to the conditions under which it has been studied.

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