

Calcium-gated Calcium Channels in Sarcoplasmic Reticulum of Rabbit Skinned Skeletal Muscle Fibers

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ABSTRACT The action of ruthenium red (RR) on Ca^{2+} loading by and Ca^{2+} release from the sarcoplasmic reticulum (SR) of chemically skinned skeletal muscle fibers of the rabbit was investigated. Ca^{2+} loading, in the presence of the precipitating anion pyrophosphate, was monitored by a light-scattering method. Ca^{2+} release was indirectly measured by following tension development evoked by caffeine. Stimulation of the Ca^{2+} loading rate by 5 μM RR was dependent on free Ca^{2+} , being maximal at pCa 5.56. Isometric force development induced by 5 mM caffeine was reversibly antagonized by RR. IC_{50} for the rate of tension rise was 0.5 μM ; that for the extent of tension was 4 μM . RR slightly shifted the steady state isometric force/pCa curve toward lower pCa values. At 5 μM RR, the pCa required for half-maximal force was 0.2 log units lower than that of the control, and maximal force was depressed by ~16%. These results suggest that RR inhibited Ca^{2+} release from the SR and stimulated Ca^{2+} loading into the SR by closing Ca^{2+} -gated Ca^{2+} channels. Previous studies on isolated SR have indicated the selective presence of such channels in junctional terminal cisternae.

INTRODUCTION

In skeletal muscle fiber, contraction occurs when Ca^{2+} is released from the terminal cisternae (TC) of the sarcoplasmic reticulum (SR) (for a review, see Stephenson, 1981a). Excitation of the surface membrane (depolarization of the sarcolemma and of transverse tubules) is linked to contraction by an unknown coupling mechanism that takes place at the triadic junction. Among the several models put forward to explain transverse tubule-SR coupling and Ca^{2+} release from the SR (see Schneider and Chandler, 1973; Mathias et al., 1980; Stephenson, 1981a; Volpe et al., 1985), the one postulating a role for Ca^{2+} has recently gained strength (Stephenson, 1981a; Fabiato, 1982, 1983, 1985). Calcium might conceivably (a) mediate transmission at the transverse tubule-SR junction (i.e., Ca^{2+} is the messenger), (b) be involved in enzymatic reactions within the triadic

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junction, or (c) interact with SR Ca^{2+} -gated Ca^{2+} channels responsible for the massive Ca^{2+} release that activates contraction. These steps are not mutually exclusive.

Studies carried out on isolated SR have clearly indicated that only fractions mainly derived from TC exhibit Ca^{2+} -induced Ca^{2+} release at micromolar free Ca^{2+} (Miyamoto and Racker, 1982), and this implies the occurrence of Ca^{2+} -gated Ca^{2+} channels (Kim et al., 1983; Meissner, 1984; Chu et al., 1984). Furthermore, studies on isolated SR fractions have shown that ruthenium red (RR) modulates transmembrane Ca^{2+} fluxes by inhibiting selectively Ca^{2+} -dependent Ca^{2+} efflux (Kirino and Shimizu, 1982; Kim et al., 1983; Chu et al., 1984; Chamberlain et al., 1984). Under steady state conditions, RR does not affect the Ca^{2+} pump turnover and stimulates Ca^{2+} loading by reducing Ca^{2+} efflux of isolated TC (Kirino and Shimizu, 1982; Chu, A., P. Volpe, B. Costello, and S. Fleischer, unpublished).

In this study, we used RR, an inhibitor of Ca^{2+} -induced Ca^{2+} release and a Ca^{2+} channel blocker of isolated TC fractions (Antoniou et al., 1985), to document the occurrence of Ca^{2+} -gated Ca^{2+} channels in the SR of skinned skeletal muscle fibers. A preliminary account of this study has already appeared (Volpe and Salviati, 1985).

METHODS

Materials

RR was obtained from Sigma Chemical Co. (St. Louis, MO) and was not further purified. Since the commercial RR is ~40% pure, the actual concentration of RR was adjusted for the purity. Caffeine was obtained from both Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co., and antipyrylazo III was obtained from Sigma Chemical Co.

Skinned Fiber Experiments

Chemically skinned fibers were prepared from the fast-twitch adductor and gastrocnemius muscles of New Zealand White rabbits, as previously described in detail (Wood et al., 1975; Salviati et al., 1982). Briefly, bundles containing several hundred fibers were excised from the bulk of the muscle and each bundle was chemically skinned by exposure to a "skinning" solution containing 5 mM K_2EGTA , 0.17 M K propionate, 2.5 mM $\text{Na}_2\text{K}_2\text{ATP}$, 2.5 mM Mg propionate, and 10 mM imidazole propionate, pH 7.0. After 24 h at 0°C, the bundles were transferred to skinning solution made up in 50% glycerol, and stored at -20°C until used. This skinning procedure preserves the SR integrity and the architecture of contractile proteins (Eastwood et al., 1979).

For tension and Ca^{2+} loading measurements, segments of single skinned fibers (mean diameter, 50 μm) were dissected from the bundle, placed in a chamber containing relaxing (R) solution (5 mM K_2EGTA , 0.17 M K propionate, 5 mM $\text{K}_2\text{Na}_2\text{ATP}$, 2.5 mM Mg propionate, and 10 mM imidazole propionate, pH 7.0), and stretched to 130% of slack length between two clamps, one of them attached to a strain gauge (Brandt et al., 1980). All experiments were carried out at room temperature (22–24°C); the ionic strength was 0.2 M.

Ca^{2+} loading was measured by following the increase in light scattering after the addition of 10 mM K pyrophosphate (pH 7.0) to the Ca^{2+} loading solution. In the presence of pyrophosphate, a precipitating anion (Martonosi and Feretos, 1964), active transport of Ca^{2+} leads to the formation of Ca pyrophosphate crystals in the SR lumen (Sorenson

et al., 1980) and to a progressive increase in the fiber light scattering that is proportional to the increase in Ca^{2+} content (Sorenson et al., 1980). The calibration procedures for converting the light-scattering signal to fiber Ca^{2+} concentration were described in detail by Sorenson et al. (1980). The experimental setup was as described by Salviati et al. (1982). The Ca^{2+} loading solutions were modified solution R containing 10 mM K pyrophosphate, different total Ca^{2+} concentrations (1.60–4.89 mM), different total Mg^{2+} concentrations (2.44–2.40 mM), and a fixed EGTA concentration (5 mM). The concentration of MgATP was 0.65 mM and was adequate for saturating the SR Ca^{2+} pump, which, in isolated SR, is saturated at 0.1–0.5 mM MgATP (Vianna, 1975). Free Mg^{2+} was kept constant at 30 nM and the pCa ranged from 6.43 to 5.46. Apparent association constants were derived from Fabiato and Fabiato (1979) and Martell and Smith (1976). For pCa values lower than 6.4, fibers were first stretched to 180% of slack length to avoid interference in the light-scattering measurements caused by actin-myosin interactions (Katz et al., 1978).

For tension measurements, fibers were exposed to Ca^{2+} loading solution (pCa 6.40) for 1 min, washed twice in solution W (a modified solution R without EGTA), and then challenged with 5 mM caffeine. Free Mg^{2+} and MgATP were kept constant at 0.09 and 2.2 mM, respectively.

Steady state isometric force/pCa curves were obtained by exposing the fiber sequentially to solutions of different free Ca^{2+} (from pCa 6.6 to pCa 4.8). The isometric tension generated in each solution was continuously recorded, and the baseline tension was established as the steady state voltage output recorded with a fiber in solution R. At the end of each experiment, the fiber was again placed in solution R and the cycle was repeated in the presence of RR.

Ca²⁺ Loading by Isolated TC

Highly purified TC fractions were obtained by differential and density gradient centrifugation from rabbit fast-twitch skeletal muscles, as described by Saito et al. (1984). The protein concentration was estimated by the method of Lowry et al. (1951). Ca^{2+} loading was measured by following the differential absorbance change of the Ca^{2+} indicator antipyrilazo III at 710–790 nm. The assay was carried out at 25°C in a medium containing, in a final volume of 1 ml, 0.17 M K propionate, 2.5 mM Mg propionate, 5 mM Na_2ATP , 200 μM antipyrilazo III, 10 mM imidazole propionate, pH 7.0, 10 mM pyrophosphate, and 50 μg of TC protein. The reaction was started with a pulse of CaCl_2 (50–400 nmol).

Experiments on isolated TC fractions were performed to assess the pCa dependence of RR action in the presence of pyrophosphate. We found that RR stimulated the Ca^{2+} loading rate of TC fractions in a Ca^{2+} -dependent manner at 0.1 mM free Mg^{2+} , and produced a bell-shaped curve as a function of pCa. Fig. 1 shows that 5 μM RR increased sixfold the Ca loading rate at pCa 5.92, half-maximal stimulation being attained at pCa 6.26. The drug effect was maximal at ~5–10 μM (not shown). These results indicate that isolated TC fractions contain Ca^{2+} -gated Ca^{2+} channels (Kim et al., 1983; Meissner, 1984) and confirm the results of previous studies (Chu et al., 1984), in which phosphate was used as the precipitating anion. Phosphate could not be used for skinned fiber experiments, because it caused no increment in the fiber light scattering up to 50 mM (Sorenson et al., 1980).

RESULTS

The effect of RR on Ca^{2+} loading by the SR of chemically skinned fibers is shown in Figs. 2 and 3. The Ca^{2+} loading rate was measured by following the increase in light scattering upon addition of 10 mM pyrophosphate (Fig. 2). As

previously reported (Sorenson et al., 1980), active transport of Ca^{2+} leads to the formation of Ca pyrophosphate crystals in the SR lumen (both longitudinal and terminal cisternae SR), and to a progressive increase in the fiber light scattering. The effect of pyrophosphate on the Ca^{2+} loading rate was saturated at 10 mM (not shown). The action of RR was Ca^{2+} dependent. At pCa 6.06, the rate of Ca^{2+} loading did not appreciably change upon addition of 5 μM RR (Fig. 2A), whereas at pCa 5.56 it increased by a factor of 2.6: after converting the light-scattering signal to fiber Ca^{2+} concentration (Sorenson et al., 1980), it was estimated that RR increased the Ca^{2+} loading rate from 18 to 46.8 nmol $\text{Ca}^{2+}/$

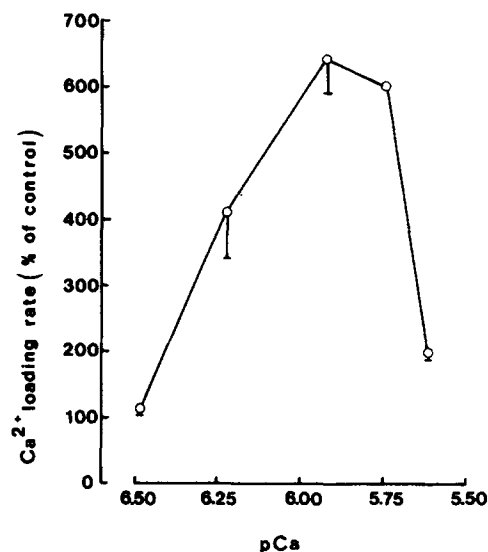


FIGURE 1. pCa dependence of RR effect (5 μM) on Ca^{2+} loading by isolated TC. Ca^{2+} loading was followed using the Ca^{2+} indicator antipyrylazo III (see Methods for details), and rates are expressed as mean percentages of controls for each pCa. The SD is given when at least three experiments were carried out on different TC preparations. At pCa 5.92, RR increased the Ca^{2+} loading rate from 0.13 ± 0.02 to $0.83 \pm 0.07 \mu\text{mol Ca}^{2+}/\text{mg protein} \cdot \text{min}$ ($n = 4$). A bell-shaped curve, peaking at pCa 6.22, was also obtained for control loading rates (not shown), and was comparable to that first reported by Weber et al. (1966).

μl of fiber volume $\cdot \text{min}$ (Fig. 2B). The effect of RR was reversible, because the Ca^{2+} loading rate slowed down to control values when RR was washed away (not shown). For pCa values above 6.0, RR was virtually without effect (Fig. 3). Below pCa 6.0, RR speeded up the Ca^{2+} loading rate, maximal stimulation being attained at pCa 5.56 (Fig. 3). RR stimulation of the Ca^{2+} loading rate of isolated TC was approximately threefold that of SR of skinned fibers (cf. Figs. 1 and 3). The apparent discrepancy can be explained by the fact that TC account for one-third of the SR structure (Mobley and Eisenberg, 1975) and that only isolated TC fractions are sensitive to RR (Miyamoto and Racker, 1982).

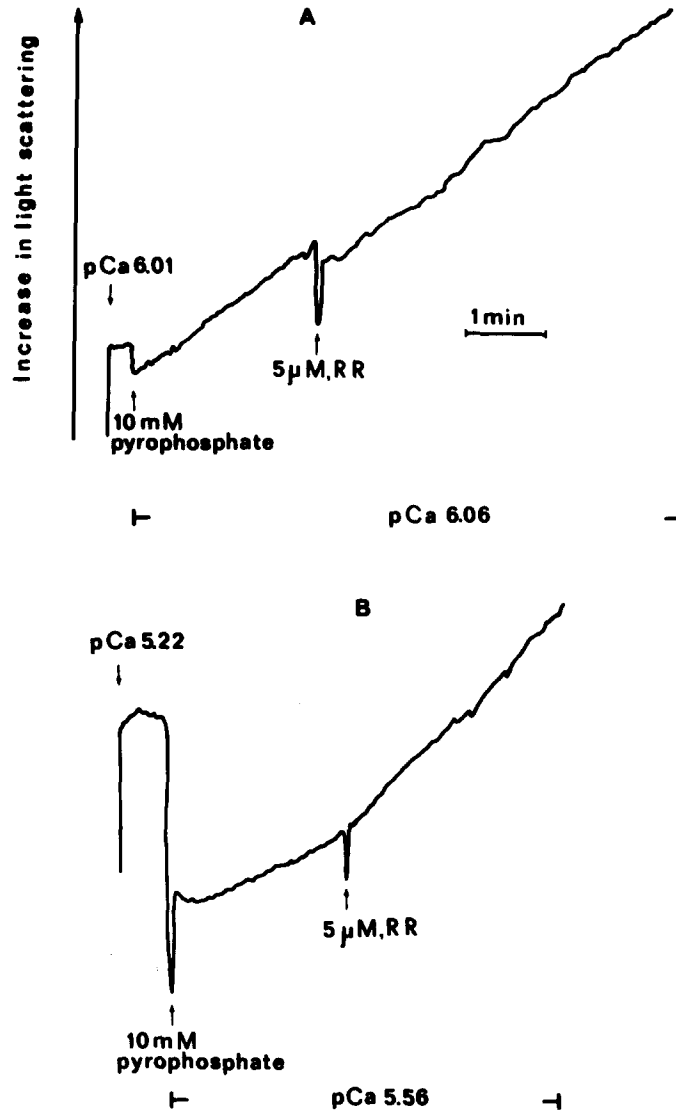


FIGURE 2. Effect of RR on Ca^{2+} loading by the SR of chemically skinned fibers. Typical tracings are shown. (A) A segment of a single fiber was stretched to 180% of slack length in solution R (not shown) and then exposed to Ca^{2+} uptake solution (pCa 6.01). Addition of 10 mM K pyrophosphate caused an immediate, small decrease in light scattering caused by actin-myosin dissociation (Katz et al., 1978) and an increase in pCa (6.06), followed by an increase in light scattering caused by Ca pyrophosphate precipitation into the SR lumen. 5 μM RR, added after ~ 2 min, did not change the Ca^{2+} loading rate. (B) Another single fiber was exposed to Ca^{2+} uptake solution (pCa 5.22). Pyrophosphate decreased light scattering and changed pCa (5.56). 5 μM RR increased the Ca^{2+} loading rate 2.6-fold. Downward deflections are addition artifacts.

Caffeine is thought to act directly on the SR and to cause Ca^{2+} release from TC. This evidence has been derived from experiments on intact fibers, in the absence of transverse tubule depolarization (Lüttgau and Oetliker, 1968), on skinned fibers (Wood et al., 1975; Stephenson, 1981*b*), and on isolated SR (Weber, 1968; Su and Hasselbach, 1984, and references therein). Fig. 4 shows that caffeine not only inhibited pyrophosphate-facilitated Ca^{2+} loading (cf. Sorenson et al., 1980), but also stimulated net Ca^{2+} release from the SR, as evidenced

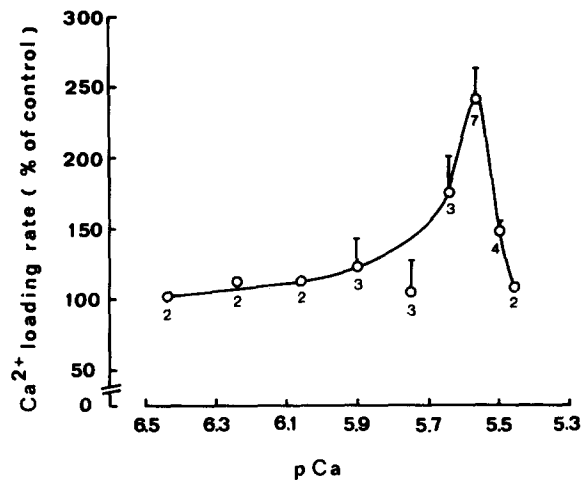


FIGURE 3. pCa dependence of the effect of RR ($5 \mu\text{M}$) on Ca^{2+} loading by the SR of chemically skinned fibers. Data are derived from several experiments, as shown in Fig. 2, and are expressed as mean percentages of control rates \pm SD (n). Each single fiber was stretched to 180% of slack length in solution R and exposed to an uptake solution of the specified pCa. Similar results were obtained using higher concentrations of RR up to $20 \mu\text{M}$ (not shown). Actual Ca^{2+} loading rates varied significantly depending on the fiber diameter and on the SR development of each skinned segment, as already noted by Sorenson et al. (1980). Since experiments were carried out on different fiber preparations for each pCa, actual rates were not directly comparable. Half-maximal activation of the Ca^{2+} loading rate, i.e., half-maximal activation of Ca^{2+} efflux (see Discussion), occurred in a range of pCa slightly lower than that obtained by Kim et al. (1983), Meissner (1984), and ourselves (Fig. 1) in isolated SR, and this was probably due to differences in free Mg^{2+} concentration.

by the decrease in fiber light scattering. $20 \mu\text{M}$ RR immediately antagonized caffeine action and fully restored Ca^{2+} loading, i.e., RR blocked Ca^{2+} release from the SR.

The effect of RR on caffeine-induced tension in skinned fibers is shown in Figs. 5 and 6. In Fig. 5A, a single fiber was allowed to accumulate Ca^{2+} from uptake solution (pCa 6.40) for 60 s and was then rinsed in solution W for 30 s. 5 mM caffeine evoked transient force development. Afterward, the same fiber was placed in solution R, loaded again with Ca^{2+} for 60 s, and rinsed in solution W supplemented by $0.1 \mu\text{M}$ RR. Caffeine elicited force development, but the

rate of tension rise was slower than with caffeine alone. At 5 μM RR (Fig. 5B), the rate of force development was only 7% of that obtained with caffeine alone. Eventually, 25 μM RR (Fig. 5C) completely abolished caffeine action, i.e., RR inhibited Ca^{2+} release from the SR. Inhibition by RR was found to be reversible: after rinsing the fiber three times in solution W, caffeine was again able to evoke tension (Fig. 5C).

Fig. 6 shows the inhibition of caffeine-induced tension as a function of RR concentration. IC_{50} for the rate of tension rise was 0.5 μM (Fig. 6A) and that for the extent of tension (relative force) was $\sim 4 \mu\text{M}$ (Fig. 6B). If the rate of force development ($dP/dt = k \Delta[\text{Ca}]$) depends upon the myoplasmic free Ca^{2+} (Ashley

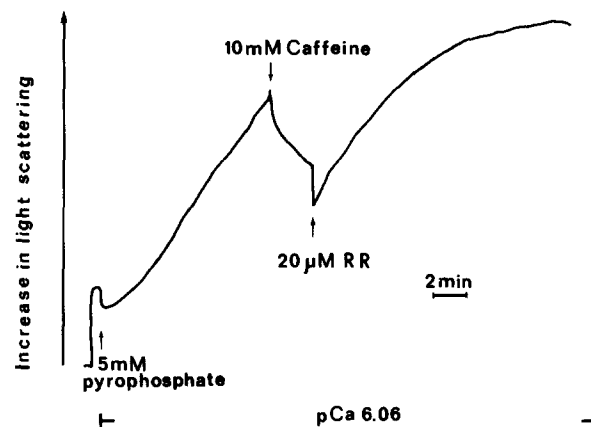


FIGURE 4. Effect of caffeine and RR on Ca^{2+} loading by the SR of chemically skinned fibers. A single fiber was stretched to 180% of slack length in solution R (not shown) and then exposed to Ca^{2+} uptake solution (pCa 6.06). 5 mM pyrophosphate stimulated Ca^{2+} loading and 10 mM caffeine promoted net Ca^{2+} release (decrease of light scattering). Calcium, although complexed to pyrophosphate within the SR lumen, is still available for release, as previously shown by Mitchell et al. (1984) and Palade (1985) in isolated SR vesicles. 20 μM RR again stimulated the Ca^{2+} loading rate. The downward deflection with RR is an addition artifact.

and Ridgway, 1970; Stephenson and Williams, 1981), and if isometric force (P) is directly related to the fractional occupancy of the regulatory Ca^{2+} binding sites on troponin C (TnC) (Godt and Lindley, 1982), then our data would suggest that, with a suboptimal concentration of RR, $\Delta[\text{Ca}]/\Delta t$ in the myofilament space is reduced, but a sizable proportion of TnC binding sites are finally being occupied.

Steady state isometric force/pCa relations were investigated in the presence of RR (Fig. 7) to detect possible effects on the contractile apparatus, which would bias the interpretation of the experiments reported in Figs. 5 and 6. Submaximal forces generated at each subsaturating pCa were transformed to percentages of the maximal tension generated at saturating pCa (4.8). The curves in Fig. 7 show the relative forces generated at a given pCa and reflect changes in the Ca^{2+} sensitivity of the contractile apparatus per se. 5 μM RR displaced the force/pCa

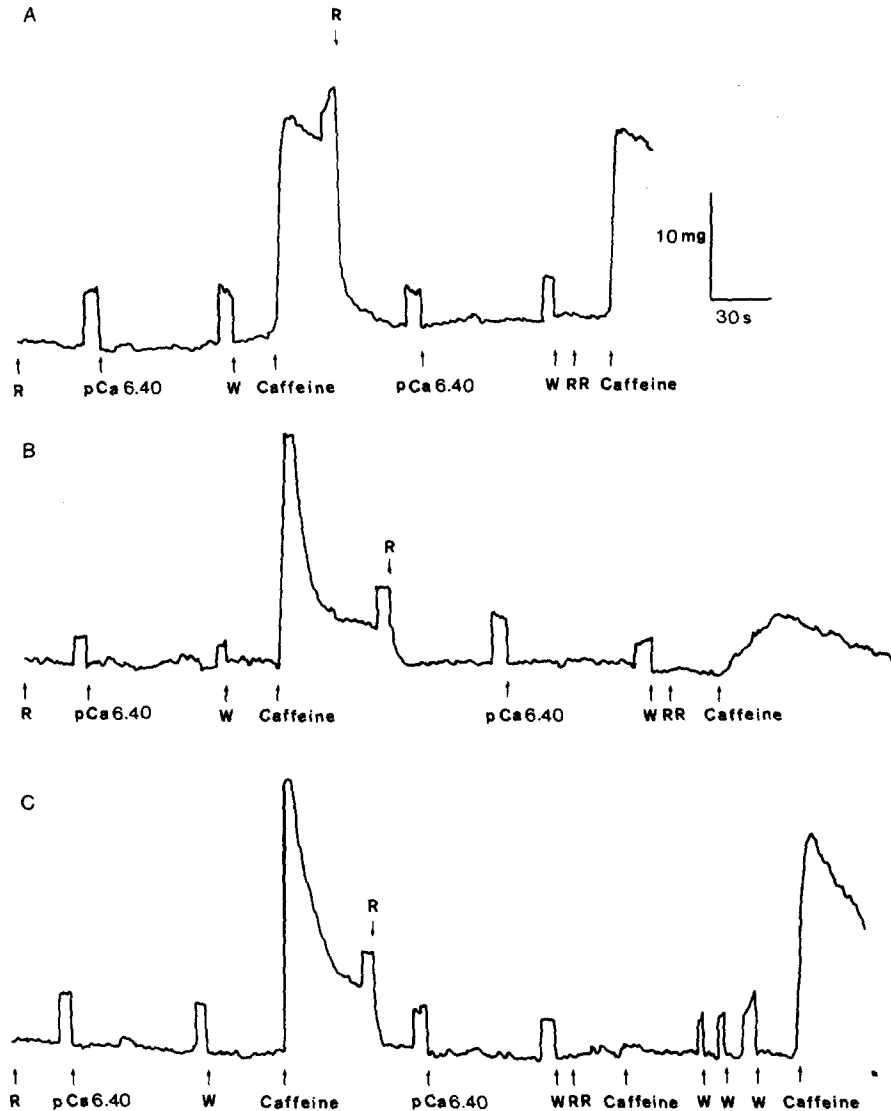


FIGURE 5. Effect of RR on caffeine-induced tension development. Typical tracings are shown. Each single fiber was stretched to 130% of slack length in solution R, exposed to Ca^{2+} uptake solution (pCa 6.4) for 60 s, rinsed in solution W for 30–45 s, and then challenged with 5 mM caffeine. Transient tension developed. The same fiber was relaxed in solution R and loaded again with Ca^{2+} (pCa 6.4) for 60 s. A second application of 5 mM caffeine was preceded by addition of RR to solution W: 0.1 (A), 5 (B), and 25 (C) μM . In the last tracing, since caffeine-induced tension was completely abolished, the fiber was rinsed three times in solution W and then challenged a third time with 5 mM caffeine. The rate of tension rise, in the presence of RR, was normalized against that obtained in the presence of caffeine alone. The low-amplitude square pulses are wash artifacts.

curve by 0.2 log units toward lower pCa values (midpoint from 6.02 to 5.82), which suggests a decreased affinity of TnC for Ca^{2+} . The steepness of the curves was essentially the same in the presence and absence of RR (Hill coefficient, 3.2 and 3.4, respectively), and this indicates that the degree of positive cooperativity for Ca^{2+} involved in force regulation was not affected by RR. Maximal force (P_o), measured at saturating pCa (4.8), was slightly depressed by RR (inset of Fig. 7). 5 μM RR decreased maximal force by $16.8\% \pm 2.3$ ($n = 4$). Taken together, these results indicate that RR had a small effect on both the affinity of TnC for Ca^{2+} and the number of attached cross-bridges.

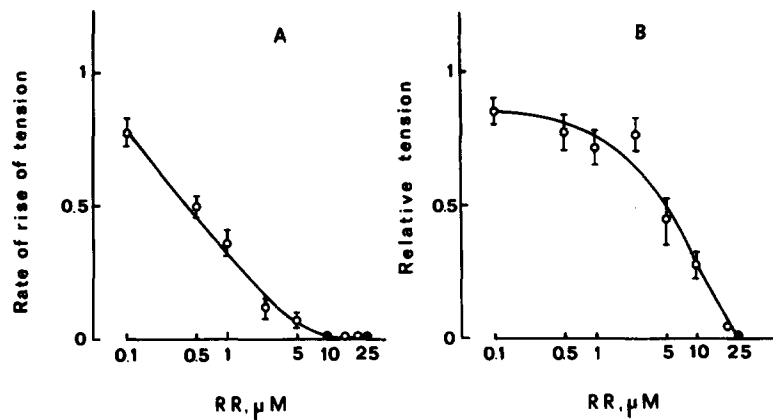


FIGURE 6. Inhibition by RR of caffeine-induced tension development. Data are derived from several experiments, as shown in Fig. 5, and are expressed as mean fractional values \pm SD ($n = 3$). The rate of tension rise (A) and the extent of tension (relative force; B), measured in the presence of specified concentrations of RR, were normalized against the rate and extent obtained with caffeine alone.

DISCUSSION

Ca²⁺-dependent Ca²⁺ Efflux from the SR of Skinned Fibers Is Blocked by RR

This paper provides evidence that Ca^{2+} -gated Ca^{2+} channels are localized in the SR of skinned skeletal muscle fibers, and supports similar conclusions inferred from flux experiments on isolated SR (Kirino and Shimizu, 1982; Kim et al., 1983; Meissner, 1984; Chu et al., 1984) and from single channel recording of "heavy" SR vesicles incorporated in planar lipid bilayer (Smith et al., 1985).

The main findings of this study are illustrated in Fig. 3: a plot of RR stimulation of Ca^{2+} loading rate vs. pCa produced a bell-shaped curve. Ca^{2+} loading by the SR is the net difference of Ca^{2+} influx, mediated by the Ca^{2+} pump, and Ca^{2+} efflux via Ca^{2+} channels. Since RR does not influence the Ca^{2+} pump turnover (Kirino and Shimizu, 1982; Chu et al., 1984), or increases Ca^{2+} influx very slightly (Meszaros and Ikemoto, 1985), its action is due to the inhibition of Ca^{2+} efflux. Interestingly, Smith et al. (1985) have reported that RR affects the SR Ca^{2+} channel by decreasing the probability of open channel events. At 3.1 μM

free Ca^{2+} (Fig. 3), SR Ca^{2+} channels (or a portion of them) were held in an open conformation, and RR, by closing them (cf. Antoniu et al., 1985), increased the Ca^{2+} loading rate. This interpretation is based on the fact that RR influences Ca^{2+} fluxes by inhibiting only Ca^{2+} -dependent Ca^{2+} efflux. Studies on isolated SR fractions derived largely from TC have shown that RR virtually abolishes

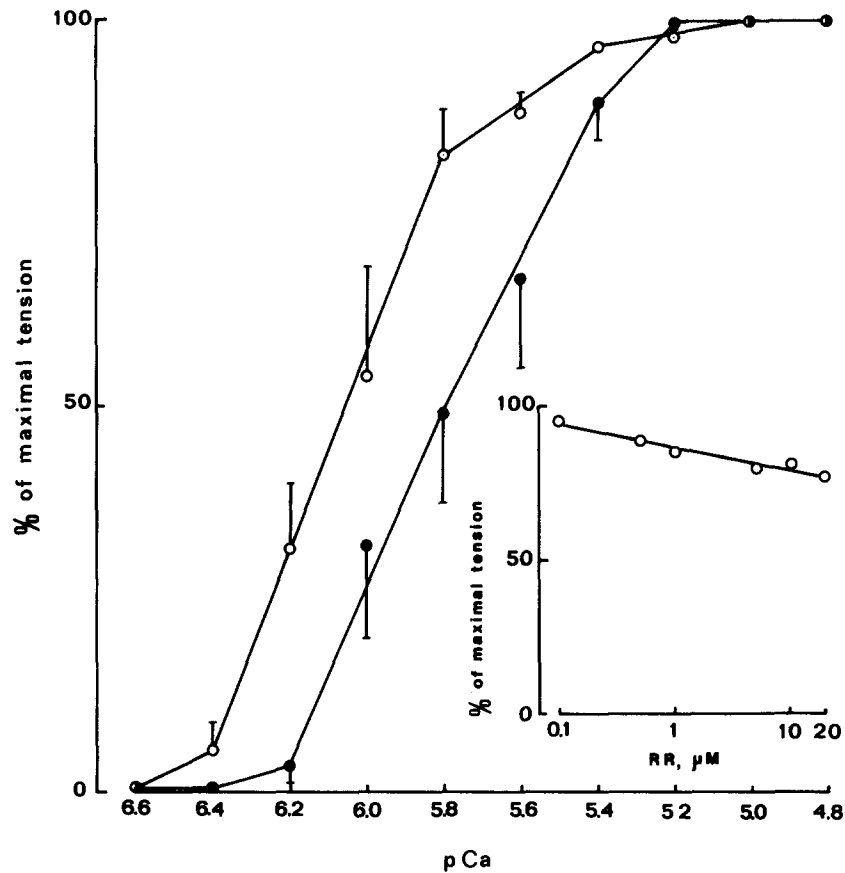


FIGURE 7. Effect of RR on steady state isometric force/pCa curve. Each single fiber was stretched to 130% of slack length in solution R and then sequentially exposed to solution of increasing free Ca^{2+} , in the absence (○) and presence (●) of $5 \mu\text{M}$ RR. Free Mg^{2+} and MgATP were kept constant at 0.09 and 2.2 mM, respectively. Submaximal forces generated at subsaturating pCa were converted to percentages of maximal force generated at saturating pCa (4.8), and are expressed as means \pm SD ($n = 3$). The Hill coefficient, determined as the slope of a straight-line fit to the data by the least-squares method, was 3.4 and 3.2 in the absence and presence of RR, respectively. Inset: effect of RR on maximal force (P_o) measured at saturating pCa (4.8). A single fiber was stretched to 130% of slack length in solution R and then placed in pCa 4.8 solution. When steady state force was attained, consecutive additions of specified concentrations of RR were made. Data are expressed as percentages of P_o .

Ca^{2+} -induced Ca^{2+} release (Kirino and Shimizu, 1982; Kim et al., 1983; Meissner, 1984; Antoniu et al., 1985), and stimulates Ca^{2+} loading in a Ca^{2+} -dependent manner (Fig. 1; Chu, A., P. Volpe, B. Costello, and S. Fleischer, unpublished; Kirino and Shimizu, 1982). Moreover, RR antagonizes the Ca^{2+} -releasing action of caffeine on the SR of skinned fibers (Figs. 4–6) and of isolated preparations (Miyamoto and Racker, 1982; Kim et al., 1983). RR was also found to inhibit Ca^{2+} release from the SR of skinned cardiac cells (Fabiato and Fabiato, 1973) and of isolated preparations (Chamberlain et al., 1984).

If RR blocks Ca^{2+} efflux from the SR of skinned fibers, the difference between the stimulated (with RR) and unstimulated Ca^{2+} loading rate at pCa 5.56 (Fig. 2B) gives a minimal estimate of the Ca^{2+} -dependent Ca^{2+} efflux rate, which is ~ 30 nmol $\text{Ca}^{2+}/\mu\text{l}$ fiber volume \cdot min. Assuming that 1 ml of fiber volume is equivalent to 1 g of muscle, and knowing that Ca^{2+} release is restricted to the TC (Huxley and Taylor, 1958; Winegrad, 1970), which represent one-third of the SR structure (Mobley and Eisenberg, 1975), we calculate a rate of $1.5 \mu\text{mol}$ Ca^{2+}/g muscle \cdot s, which is still 15–30 times lower than that estimated in vivo (see Meissner, 1984). Fabiato (1983, 1985) has shown that, in skinned skeletal muscle fibers, the rate of Ca^{2+} -induced Ca^{2+} release from the SR depends on the rate at which bath free Ca^{2+} is changed, i.e., $\Delta[\text{Ca}]/\Delta t$. Because (a) our experimental setup does not allow rapid solution changes, (b) there is not synchronous and instantaneous Ca^{2+} diffusion throughout the cross-section of a $50\text{-}\mu\text{m}$ -width fiber, and (c) our calculations rely on steady state flux rates, it is reasonable to suppose that initial efflux rates via Ca^{2+} -gated Ca^{2+} channels may be higher. On the other hand, an overestimate of efflux rate might derive from the very low free Mg^{2+} used (Figs. 2 and 3).

Fig. 3 also shows that the opening and closing of Ca^{2+} release channels are regulated by free Ca^{2+} . It seems that Ca^{2+} exerts a fine control over the conformational states of the channels, which opened at pCa values ranging from 5.8 to 5.4. Stimulation by RR was half-maximal at $2.2 \mu\text{M}$ free Ca^{2+} , which indicates an activating site for Ca^{2+} efflux with a relatively high affinity. The inhibiting site for Ca^{2+} efflux had an apparent lower affinity. These results, as well as those obtained on isolated SR (Kim et al., 1983; Meissner, 1984), suggest that Ca^{2+} binding at a lower-affinity site blocks Ca^{2+} efflux via the channels, and that Ca^{2+} release, in vivo, may be inactivated by the rising myoplasmic free Ca^{2+} , i.e., by a negative feedback of the released Ca^{2+} (see Fabiato, 1983).

Is RR a Specific Tool?

RR is a polyvalent cationic dye largely used by electron microscopists to stain anionic substances such as acid mucopolysaccharides, and has multiple pharmacological actions in a variety of tissues and several subcellular organelles. For instance, RR stains SR (Howell, 1974), junctional SR areas, and calsequestrin (Forbes and Sperlakis, 1979) of skeletal muscle. However, no identity is warranted between morphologically identifiable RR binding sites and sites where RR exerts pharmacological effects (Howell, 1974). RR is by no means a specific tool, and its validity and usefulness must be carefully checked by the investigator.

Our results show that RR slightly shifted the isometric force/pCa curve (Fig.

7), and this suggests an interaction between the acidic protein TnC (Hartshorne and Dreizen, 1973) and RR. Moreover, a partially purified TnC preparation bound, at equilibrium, up to 1.8 mol RR/mol (Salviati, G., unpublished). Nonetheless, the main action of RR in skinned fibers appeared to be on Ca^{2+} efflux from the SR. Two additional lines of evidence support this conclusion: (a) 5 μM RR inhibited the rate of tension rise by 93% (Fig. 6A); (b) force evoked by 5 mM caffeine was 98% of P_o ($n = 4$; not shown), and this implies (cf. Fig 7) that myoplasmic pCa after caffeine-induced Ca^{2+} release should be ~ 5.3 . At these pCa values, the effect of RR on force development is almost negligible (inset of Fig. 7).

It has been reported that, in intact frog skeletal muscle fibers, RR, by interacting with membrane-bound sialic acid, decreased surface negative charges and shifted the contraction threshold to more positive membrane potentials (Dörrscheidt-Käfer, 1979). Suzuki et al. (1980) also reported that, in frog skeletal muscle, RR decreased twitch and K contracture without affecting caffeine contracture, and postulated inhibition of trans-sarcolemmal Ca^{2+} influx. However, these effects, which might reasonably take place, are not comparable to ours, because skinning procedures render surface membranes leaky and destroy the transverse tubule-SR junction (Eastwood et al., 1979). The observation that the caffeine contracture was not affected by RR simply means that RR did not permeate across intact sarcolemma.

RR is also known to modify Ca^{2+} fluxes in mitochondria (Bernardi et al., 1984). None of the results reported here could be due to mitochondria, which are removed during the skinning (Eastwood et al., 1979).

Ca^{2+} Is Involved in Transverse Tubule-SR Coupling

The present results support the model in which Ca^{2+} plays a critical role in excitation-contraction coupling and/or Ca^{2+} release from the SR of skeletal muscle (see Stephenson, 1981a; Fabiato, 1983), and show that Ca^{2+} -gated Ca^{2+} channels, which are selectively localized in junctional TC (see Meissner, 1984), open when intermyofibrillar free Ca^{2+} is in the micromolar range ($\cong 3 \mu\text{M}$). Endo et al. (1970) have pioneered the concept of Ca^{2+} -induced Ca^{2+} release. However, the physiological relevance of their experiments has been obscured by the high levels of free Ca^{2+} needed to elicit Ca^{2+} release (Endo, 1981). On the other hand, Fabiato (1983, 1985) has recently shown that Ca^{2+} -induced Ca^{2+} release from the SR of mechanically skinned skeletal muscle fibers occurs, at physiological free Mg^{2+} , when bath free Ca^{2+} is changed very rapidly ($\cong 1 \text{ ms}$) from resting values to $\leq 1 \mu\text{M}$. At 3 μM free Ca^{2+} , Ca^{2+} release rates of 33.3 mmol/kg wet wt · s are obtained (Fabiato, A., personal communication), which are compatible with those in vivo. We did not measure Ca^{2+} efflux rates directly, but our calculated rates (see Discussion above) are not inconsistent with those in vivo. Thus, our results, as well as Fabiato's (1982, 1983, 1985), suggest that TC Ca^{2+} -gated Ca^{2+} channels may be involved in physiological Ca^{2+} release by enabling the massive Ca^{2+} release that activates contraction.

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