Force–Interval Relations of Twitches and Cold Contractures in Rat Cardiac Trabeculae

Effect of Ryanodine

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The twitch force (Ft)–interval relation of cardiac muscle reflects recovery of calcium release from the sarcoplasmic reticulum (SR). The calcium content of the SR is thought to be reflected by force developed during a contracture (Fc), induced by rapid cooling to near 0°C. In right ventricular trabeculae of rat, under control conditions, the Ft–interval relation consisted of recovery of Ft to steady state (early recovery), followed by a secondary increase of Ft up to a maximum at an interval of ~100 seconds (rest potentiation) and a decline of Ft at intervals >100 seconds (rest depression). The mechanisms that may underlie recovery of force after the last twitch at short intervals are 1) time-dependent transport of Ca2+ from the uptake compartment of the SR to the release compartment, 2) recovery of slow inward Ca2+ current during the action potential, and 3) recovery of the Ca2+ release channels in the SR. The Ft–interval relation was similar to the Ft–interval relation in that both a rest potentiation and a rest depression phase were present. However, at short interstimulus intervals (<1 second), Ft was independent of time, suggesting that the mechanism underlying early recovery was bypassed. Ryanodine (0.1–10 nM) reduced rest potentiation in a dose-dependent manner and accelerated rest depression of both Ft and Fc. At high ryanodine concentration, a significant Ft could only be induced after short intervals. Significant acceleration of rest depression was observed at low ryanodine concentrations, when Ft at intervals of 5 seconds was kept constant by increasing the stimulus frequency of [Ca2+]i, suggesting that the ryanodine effect was enhanced by increased [Ca2+]i. Ryanodine also increased the rate of decay of postextrasystolic potentiation in a dose-dependent manner. A significant effect was observed in 10 nM ryanodine. The twitch was not prolonged by ryanodine at these concentrations. These results suggest that the small magnitude of the twitch at short intervals is due to the finite time required by SR Ca2+ release channels to fully recover after a twitch. Furthermore, the results offer support for the hypothesis that ryanodine (in the nanomolar range) promotes Ca2+ leak from the SR in a dose-dependent manner and thereby limits Ca2+ accumulation during the interstimulus interval. Therefore, it may be expected that the negative inotropic effect of ryanodine is due to the SR Ca2+ depletion, and it is not necessary to postulate that ryanodine “blocks” the Ca2+ release channels in the SR. (Circulation Research 1991;69:937–948)

Studies by Fabiato and Fabiato1 on skinned cardiac cells have shown that a transient rise in the intracellular Ca2+ concentration ([Ca2+]i) induces Ca2+ release from an internal Ca2+ store, the sarcoplasmic reticulum (SR), thus causing contraction. For an intact cell (or intact preparation), the transient rise in [Ca2+]i, is brought about by Ca2+ entering the cell through sarcolemmal Ca2+ channels during an action potential. Relaxation is caused by Ca2+ returning to the SR and extrusion from the cell.2 Based on the above observation, on which the current model of calcium-induced calcium release is founded, four determinants of the magnitude of Ca2+ release from the SR may be suggested: 1) SR Ca2+ content, 2) magnitude and rate of rise of [Ca2+]i, which triggers Ca2+ release from the SR, 3) kinetics of SR Ca2+ release channels, and 4) availability of Ca2+ for release by the SR. In an intact preparation, the second determinant is dependent on the kinetics of sarcolemmal Ca2+ channels mentioned above.
Since the magnitude of an electrically stimulated twitch is predicted by the SR Ca$^{2+}$ release, and hence these determinants, the twitch force (Ft)–interval relation has been used to demonstrate the time course of recovery of Ca$^{2+}$ release from the SR. In rat cardiac trabeculae, the Ft–interval relation is characterized by three phases: an increase of Ft to steady state (early recovery), followed by a secondary slow increase of Ft above steady state up to an interval of ~100 seconds (rest potentiation), and a subsequent decrease in Ft at intervals >100 seconds (rest depression). Three possible mechanisms may be suggested to explain early recovery of force to the steady state: 1) the presence of a delay in transport of Ca$^{2+}$ ions between two hypothetical SR compartments, which have been proposed previously,3,4 2) recovery of the sarcoplasmic reticulum channels mediating the slow inward current carried by Ca$^{2+}$ (Ica),5,6 which in a dose-dependent manner triggers Ca$^{2+}$ release from the SR,7 3) recovery of the SR Ca$^{2+}$ release channels after the twitch, which also has been previously postulated.8

Ryanodine, a neutral plant alkaloid that binds specifically to the SR Ca$^{2+}$ release channel of terminal cisternae in cardiac and skeletal muscle,9 has been used in various studies11-14 to estimate the magnitude of SR Ca$^{2+}$ release and its contribution to the force in various species. In mammalian cardiac preparations, there is a decrease in Ft with exposure to ryanodine. It has been proposed that this negative inotropic effect may be due to an inhibition of the Ca$^{2+}$ release capability of the SR by blockage of Ca$^{2+}$ channels with ryanodine.11,13,17 Support for this suggestion is based on the observation that Ca$^{2+}$ content in SR vesicles is increased in the presence of micromolar concentrations of ryanodine.18 Calcium-45 efflux measurements as well as extracellular calcium concentration ([Ca$^{2+}$]ex) measurements by dyes and extracellular Ca$^{2+}$ microelectrodes19-23 have, on the other hand, suggested that ryanodine promotes Ca$^{2+}$ leak from the SR into the cytoplasm and subsequently to the interstitial space without activating the myofilaments.22 Furthermore, results of studies by Fleischer et al9 and Lattanzio et al24 on SR preparations of heart and skeletal muscle have shown that low concentrations of ryanodine lock the SR Ca$^{2+}$ release channels in an "open state," thereby causing a Ca$^{2+}$ leak. The latter observations suggest that the negative inotropic effect of ryanodine may be explained by induction of a Ca$^{2+}$ leak from the SR during the interstimulus interval, resulting in a smaller Ca$^{2+}$ release with each action potential because of a reduced Ca$^{2+}$ content of the SR. This hypothesis could be tested by measuring force of contracture (Fc) induced by rapidly cooling intact cardiac trabeculae to near 0°C, which has been shown to be proportional to the Ca$^{2+}$ content of the SR.25,26

The purpose of this study was twofold. By examining the effect of varied interstimulus intervals on Ft and Fc, we examined the possible underlying mechanism for the early recovery of force at short intervals. As well, we studied both Ft– and Fc–interval relations to evaluate whether the effects of ryanodine (in the nanomolar range) are consistent with inhibition of SR Ca$^{2+}$ release by a block of channels or whether the effect of ryanodine can be more readily explained by Ca$^{2+}$ loss from the SR.

Materials and Methods

Dissection and Perfuson Solutions

Sprague-Dawley rats of either sex were anesthetized with ether, and the hearts were quickly excised and perfused through the aorta. Free-running right ventricular trabeculae, attached between the atrioventricular ring and the free ventricular wall, were dissected. First the valvular end was separated from the atrial and ventricular tissue, and then the ventricular end was dissected. Muscles were mounted horizontally in a glass-covered chamber with a volume of 0.35 ml in which superfusion solution recirculated with a flow rate of 10 ml/min. The muscles were stimulated at 0.2 Hz (unless otherwise stated) with two parallel platinum electrodes. The stimuli were 5 msec in duration and 50% above threshold.

The dissection and perfusion solutions were similar except for the concentration of potassium, which was 20 mM in the dissection solution to arrest the heart and thus reduce possible damage to the muscle during dissection. The Krebs-Henseleit solution contained (mM) Na$^{+}$ 142.2, K$^{+}$ 5.0, Cl$^{-}$ 127.7, Mg$^{2+}$ 1.2, SO$_{4}^{2-}$ 1.2, H$_{2}$PO$_{4}^{-}$ 2.0, HCO$_{3}^{-}$ 19, Ca$^{2+}$ 0.7, and glucose 10. The solutions were equilibrated with 95% O$_{2}$–5% CO$_{2}$ to obtain a pH of 7.4 at 37°C. Under these conditions, the muscles were stable for the duration of the experiments (as long as 6–7 hours). As described previously,25 the use of 0.2 Hz, 0.7 mM Ca$^{2+}$, and a temperature of 25–26°C gives a Ft–interval relation with clearly distinguishable early recovery as well as rest potentiation and rest depression phases.

Force Measurements

Force was measured by means of a silicon semiconductor strain gauge (model # AE 801, SenSonor, Horten, Norway) with a short carbon extension arm. A stainless-steel wire was bent in a U shape (1.5 mm wide), and the free ends were glued onto the carbon arm, such that the U-shaped loop was positioned in the horizontal plane. The closed end of the loop was bent 90° upward and backward to form a basket.28 Muscles were positioned in the basket in the following manner: 1) The ventricular end was held, and the valvular end was carefully directed through the basket and then gently pulled through the loop without stretching the muscle. 2) The remnant of the ventricular end was gently clamped between the top and bottom wires of the basket, and the valvular end was attached to the stainless-steel hook of a servomotor (Cambridge Technology, Cambridge, Mass.). Muscles were measured in such a way that examination under microscope showed neither twisting in the
horizontal plane of the muscle nor any nonuniform shortening along the whole muscle. Muscles contracted while the length was kept constant.

Measurements of Sarcomere Length

Sarcomere length was measured using the optical diffraction technique described in detail previously.29 The principle is based on the diffraction pattern that is formed once monochromatic light passes through a diffraction grating. Because of the structural arrangements of the contractile proteins, the trabecula acts as a grating. Hence, illuminating a region (300–400 μm along the muscle length) of a thin trabecula by a He-Ne laser (wavelength of 632.8 nm, Spectra-Phys,ics, Eugene, Ore.) produces a diffraction pattern of a zero order and higher order band pairs. The spacing between the first-order bands is related to sarcomere length. The intensity distribution of the first-order diffraction pattern was scanned by using a 512-element linear photodiode array (Reticon, Sunnyvale, Calif.), and the sarcomere length was computed electronically after the correction for the scattered light from the zero-order diffraction. During the entire experiment, the same area (a nontranslating zone) of the muscle was illuminated and monitored. The video image of each preparation, obtained through the microscope, was continuously monitored.

Contract at Near 0°C

To study the Fc–interval relation, it was necessary to rapidly reduce the temperature of the superfusion solution from 26°C to near 0°C. To accomplish this, a double inflow system with flow rates equal to 10 ml/min (warm) and 14 ml/min (cold) was designed. The cold perfusion lines were jacketed while connected to a cooling system, and by using two cold exchangers, the temperature in the line was maintained at −2°C. The warm solution was switched to cold by means of miniature valves (The Lee Co., Westbrook, Conn.) in each line, positioned close to the inlets of the bath, thus minimizing the dead space. Because these were three-way valves, the solution passing through the valve in the off position could be recirculated to its reservoir, and the valve in the on position directed the solution to the bath. This arrangement prevented the stagnation of the cold solution in the valve that would have otherwise warmed up while the muscle was exposed to 26°C. A 6-V pulse of a duration determined by a Digitimer D4030 (UK) was used to operate the valves through mos-fet switches. The valve in the line of warm solution was kept on, and thus open to the bath, by one mos-fet until a pulse was generated by the timer. Then a pulse-driven switch stopped the flow of warm solution, and the valve in the cold line was simultaneously turned on to cool the muscle and hence cause contracture. With this arrangement, the bath temperature and the muscle surface were cooled to <5°C in ~350 msec and to near 0°C in ~500 msec, as measured by a thermocouple (Cole Parmer Instrument Co., Chicago, Ill.) with a time constant of ~1 msec. The time course of cooling was similar regardless of the interval preceding the contracture, and no substantial change was observed in pH with rapid cooling (<0.1 pH unit).

Addition of Ryanodine

Ryanodine (Calbiochem Corp., La Jolla, Calif.) was added to the perfusion solution, giving final concentrations of 0.1, 0.3, 1.0, 3.0, and 10 nM. On addition of ryanodine, the stimulus frequency was increased to 1.0 Hz to accelerate the action of ryanodine, as has been suggested by previous studies.30 After 20–25 minutes of equilibration, the stimulus rate was returned to 0.2 Hz.

Experimental Protocol

In one group of experiments after equilibration of at least 1 hour at 0.2 Hz, the Ft–interval relation, before and after ryanodine application, was examined at inter-stimulus intervals of 0.5–250 seconds. In a second group of experiments, the negative inotropic effect of ryanodine on Ft at intervals of 5 seconds was counteracted, and Ft was increased to control level by raising either the frequency of stimulation or [Ca2+], when the effect of each ryanodine concentration had reached a stable level. Thereafter, the Ft–interval relations were studied. In a third series, muscles were exposed to rapid change in temperature, and the absolute magnitude of Fc after varied intervals was studied in the presence and absence of ryanodine.

Statistical Analysis

The effects of ryanodine on Ft and Fc at varied intervals were examined using the rank sum test and one-way analysis of variance. Both these tests indicated the presence of significant depression of Ft and Fc with ryanodine at progressively longer intervals (p<0.05). The rate of decay of postextrasystolic potentiation was also examined before and after ryanodine; a significant (p<0.05) increase was observed in 10 nM ryanodine.

Results

Ft–Interval Relation and Ryanodine

Figure 1 shows the time course of Ft when a stimulus was inserted after the last contraction of a steady series (0.2 Hz) at a varied stimulus interval. The results are consistent with the Ft–interval relation, which has been previously documented in detail by Schouten et al.3 At short intervals, Ft was small but recovered rapidly. Ft, then, increased more slowly to a maximum at ~100 seconds. This phenomenon has been denoted rest potentiation. A decline in Ft, denoted as rest depression, at intervals >100 seconds was evident. With these long intervals (>100 seconds), we found neither signs of spontaneous sarcomere activity by microscopic observation nor evidence of spontaneous activity in the form of fluctuations of the intensity distribution of the laser diffraction pattern of the muscles; hence, the decline
of Ft could not have been caused by spontaneous Ca\(^{2+}\) release before stimulation. The fluid flow, however, usually caused some perturbations in the diffracted light and caused the slight noise seen in the sarcomere signal of Figure 1. It is also evident from Figure 1 that, with ryanodine, Ft after the last twitch was significantly depressed at long intervals and maximal rest potentiation occurred at a shorter test interval.

Figure 1 shows that, in muscles under control conditions, the postextrasystole was potentiated at all intervals at which the extrasystole was elicited. A striking observation was that, in the presence of ryanodine, the postextrasystole was potentiated after a twitch elicited at a short (<5 sec) interval (i.e., an extrasystole) following the last steady beat. However, when the extrasystole was elicited at long intervals (>10 seconds) the postextrasystole was depressed, while the amplitude of the subsequent contractions gradually returned to steady-state level.

Figure 2A shows that ryanodine diminished Ft at all intervals in a dose-dependent manner. The rapid recovery was not complete, but the negative inotropic effects of ryanodine were more pronounced at longer intervals, as evident from the diminished rest potentiation and accelerated rest depression. For example, at 10 nM ryanodine, Ft decreased by 46.4\(\pm\)3.2\%, 72.2\(\pm\)4.9\%, and 82.8\(\pm\)3.1\% (mean\(\pm\)SEM) at intervals of 5, 30, and 100 seconds, respectively, whereas rest potentiation was completely converted into rest depression (Figure 2A).

Because of the duration of these experiments (6–7 hours), it was important to examine if the pronounced changes in the characteristics of the Ft–interval relation were truly caused by ryanodine and not simply by time. Figure 2B confirms this. The addition of one concentration of ryanodine of 10 nM produced a curve similar to the curve obtained when 10 nM ryanodine was reached with cumulative doses (Figure 2A).

The Influence of Increased Stimulus Frequency and [Ca\(^{2+}\)]\(_{0}\) on the Early Recovery Phase of the Ft–Interval Relation After Ryanodine Exposure

To test if a muscle exposed to ryanodine could at all develop a force equal to steady-state Ft in the absence of ryanodine, we studied the Ft–interval relation at a frequency at which a 5-second interval produced Ft comparable to Ft at a 5-second interval before ryanodine attention. The inset of Figure 3 shows the recordings from such an experiment. Figures 3A and 3B show the complete Ft–interval relation after such intervention in one rat trabecula and in five trabeculae, respectively. It is clear that this intervention restored Ft during early recovery to control level despite the presence of ryanodine. In contrast to restoration of early recovery by raising the basal stimulus rate, rest depression was even more accelerated so that rest potentiation was abolished even at 1.0 nM ryanodine (Figures 3A and 3B). At this concentration, Ft decreased by 45.8\(\pm\)4.4\% and 73.8\(\pm\)5.6\% (mean\(\pm\)SEM) at intervals of 30 and 100 seconds, respectively; the difference with the effect of ryanodine at lower stimulus rate was significant (\(p<0.05\)).

The effect of ryanodine on early recovery could also be counteracted by raising [Ca\(^{2+}\)]\(_{0}\), as is shown by Figure 4. Again, whereas the influence of ryanodine on the early recovery phase of the Ft–interval relation could evidently be compensated for by increasing [Ca\(^{2+}\)]\(_{0}\), its influence on rest potentiation and rest
depression was enhanced. For example, 1.0 nM ryanodine at 0.73±0.05 Hz (Figure 3B) was sufficient to accelerate rest depression to a level similar to that obtained at 10 nM ryanodine and at a stimulus frequency of 0.2 Hz (Figure 2A). A similar effect of frequency has been noted previously. It is noticeable that, in the presence of higher [Ca\(^{2+}\)], 3 nM ryanodine exerts an influence similar to 10 nM ryanodine at a lower [Ca\(^{2+}\)] (Figure 4).

**Contractures at Near 0°C**

To examine the Ca\(^{2+}\) content of the SR available for contraction and to test whether ryanodine alters the net Ca\(^{2+}\) content, we examined Fc induced by rapid cooling of the muscle in the absence and presence of ryanodine. With rapid introduction of cold Krebs-Henseleit solution into the bath, the muscle developed a contracture as soon as the temperature reached ~0°C. To reduce the number of exposures to alternate cold and warm solutions and hence avoid possible diminished performance, only two concentrations of ryanodine were used.

Figure 5 shows the time course and magnitude of Fc after rapid introduction of cold Krebs-Henseleit solution at intervals of 5 and 100 seconds after the last steady-state twitch in control solution and in the presence of ryanodine. The Fc-interval relation of three muscles is summarized in Figure 6. The magnitude of Fc (before ryanodine) after intervals <1 second was strikingly constant. This contrasts with the increase of the amplitude of the twitch from a negligible force to steady-state Ft during early recovery (Figure 2A). Rest potentiation was also manifest in the contractures; that is, Fc was 20–30% larger at intervals of 100 seconds than Fc at 5-second intervals. This increase of Fc was only slightly smaller than the increase of Ft in the control solution (32–48%; compare with Figure 2A).

Fc decreased in the muscles exposed to ryanodine, but the time to maximal force of contraction induced by cooling was affected little (Figure 5). Ryanodine depressed Fc more at an interval of 100 seconds than at 5 seconds in a dose-dependent manner (compare Figures 5 and 6). Ryanodine (1.0 nM) caused acceleration of rest depression of Fc and, concomitantly, shortening of the time to maximal rest potentiation. Comparison of Figure 6 with Figure 2 shows that the magnitude of the effect of ryanodine on Fc was comparable to that on the Ft–interval relation. At 10 nM, ryanodine reduced both Fc and Ft at a poststimulus interval of 5 seconds by ~50%, eliminated rest potentiation, and reduced both Fc and Ft at an interval of 100 seconds by ~80%.

**The Effects of Ryanodine on Postextrasystolic Potentiation (Recirculated Fraction of Calcium)**

In muscles that contracted at a regular rate of 0.2 Hz, introduction of extrasystoles caused postextrasystolic potentiation of the next beats after resuming at 0.2 Hz. The level of postextrasystolic potentiation decayed to the steady state in an exponential manner (inset of Figure 7). It has been shown that the decay of postextrasystolic potentiation is accompanied by a decrease in the amplitude of intracellular calcium transients. The rate of decay was obtained in this study from the slope of the relation between force of subsequent potentiated beats. The linearity of the relation between the amplitude of postextrasystolic twitches and the force of each preceding potentiated contraction is striking and suggests that a constant fraction (B) of the calcium that elicits contraction reappears during the following beat. Therefore, we assumed that B can be used as a measure of the fraction of Ca\(^{2+}\) taken up by the SR after the
Figure 3. Frequency dependence of the effect of ryanodine. Inset: Recording showing that, in the presence of ryanodine, twitch force at an interval of 5 seconds has been kept constant by an increase in stimulus frequency. Increase in frequency was 0.6 Hz for 0.3 nM ryanodine. Panel A: Graph showing the twitch force–interval relation obtained from such experiments. A similar protocol as that in Figure 1 was followed except twitch force at an interval of 5 seconds after ryanodine was kept constant. The lines are polynomials that were fitted through the data in each case. \( F_{\text{test}} \) and \( F_{\text{control}} \) refer to twitch force at a test interval and twitch force at an interval of 5 seconds before ryanodine, respectively. Note the persistent accelerated rest depression at intervals >5 seconds with pronounced depression of twitch force. All twitch forces at intervals <5 seconds are restored to control level. Ryanodine (1.0 nM) suppressed rest potentiation significantly compared with 10 nM ryanodine needed in the absence of any frequency potentiation (see Figure 2A). Panel B: Graph showing average results of the twitch force–interval demonstrating the accelerated rest depression with ryanodine. Each point is mean ± SEM; n=5.

preceding release (Figure 7).\(^{32,33}\) If ryanodine depleted the SR of its \( \text{Ca}^{2+} \) content, one would predict that the recirculation fraction \( B_2 \) of \( \text{Ca}^{2+} \) via the SR would be reduced. To test this, we studied the rate of decay of postextrasystolic potentiation in the absence and presence of ryanodine. Figure 7 shows that ryanodine also increased the rate of decay of postextrasystolic potentiation in a dose-dependent manner, corresponding to a progressive decrease of \( B_2 \). A significant effect was observed in 10 nM ryanodine. Ryanodine, at these concentrations, did not alter the characteristics of the twitch. In particular, time to peak was unchanged (control, 120±3 msec; 10 nM ryanodine, 115±3 msec), and twitch was not prolonged. The time required for relaxation from maximal force to 50% of \( F_t \) was unchanged (105±0.7 msec in control solution versus 100±0.8 msec in muscles exposed to 10 nM ryanodine, respectively [mean±SEM]). The times to half relaxation were compared in contractions in which the absolute level of force development during the twitch was kept constant by adjusting [\( \text{Ca}^{2+} \)]. [\( \text{Ca}^{2+} \)] was 0.7 and 1.6 mM in the control solution and in the presence of 10 nM ryanodine, respectively.

Figure 4. Graph showing dependence of the ryanodine effect of [\( \text{Ca}^{2+} \)]. \( F_{\text{test}} \) is twitch force at a test interval; \( F_{\text{control}} \) is twitch force at an interval of 5 seconds before ryanodine. In the presence of ryanodine, raising [\( \text{Ca}^{2+} \)] only restored force to control level at short intervals. The depression of force at long interstimulus intervals by ryanodine was enhanced: 3 nM ryanodine was sufficient for a similar degree of rest depression as 10 nM ryanodine at a [\( \text{Ca}^{2+} \)] of 0.7 mM (compare with Figure 1).

Discussion

The results of this study confirm the existence of 1) early recovery during the \( F_t \)-interval relation, 2) rest potentiation, and 3) rest depression, as has been shown in rat cardiac muscle in detail.\(^{3,27}\) Rest potentiation and rest depression of force development are also prominent in the \( F_t \)-interval relation of contractions induced by rapid cooling. A novel finding is that shortly after a twitch (<1 second) the amplitude of a rapid cooling contracture is independent of time
observation was also that the rate of decay of postextrasystolic potentiation was increased by ryanodine in a concentration-dependent manner.

The effects of varying the interval between the last steady-state twitch on the amplitude of a contracture induced by rapid cooling, observed in our study, are consistent with previous studies and the proposed explanation of rest potentiation and rest depression. Moreover, the Fc-interval relation sheds further light on the process involved in the early rapid recovery of force development.

Recent studies have shown that rapid cooling of cardiac muscle to 0°C rapidly "freezes" the SR Ca2+ channels in an open state, which allows a large and possibly complete release of Ca2+ ions from the SR. Rapid cooling is neither accompanied by an action potential nor by a depolarization of sufficient magnitude to cause gated Ca2+ entry into the cell. The force of the contracture in response to cooling, therefore, is probably only determined by the amount of Ca2+ released by the SR during this procedure and by the sensitivity of the contractile filaments, which is lowered in the cold. The amount of Ca2+ released by the SR is probably determined by the Ca2+ content of the SR, as has been shown by the close correlation of the amplitude of the rapid cooling contracture and the SR Ca2+ content, as measured by atomic absorption spectrophotometry. Experiments by Dani et al40 on isolated myocytes showed that SR-related Ca2+ sequestration amounted to ~300 μmol/kg wet wt. Bridge25 has shown that nearly all of this Ca2+ is lost from the tissue during a rapid cooling contracture (i.e., ~260 μmol/kg wet wt). In the same study, a strong correlation between the magnitude of the rapid cooling contracture and the Ca2+ content of the tissue was documented. Although part of the Ca2+ released may have some other origin, the similarity between SR Ca2+ sequestration in isolated myocytes and the amount of Ca2+ that is lost with cooling suggests that cooling causes a near complete Ca2+ release from the SR. We have, therefore, used measurement of the amplitude of the contracture (Fc) induced by rapid cooling to obtain an estimate (albeit

**Figure 5.** Recordings showing contractures in a trabecula induced by quickly decreasing the superfusion solution temperature from 26°C to near 0°C. Left panels: Effect of 1.0 and 10 nM ryanodine on the magnitude of force of contracture (Fc) at interval of 5 seconds. SL indicates sarcomere length. Right panels: Same experimental conditions as in left panels but at interval of 100 seconds. Note significant Fc depression and slowing of relaxation after peak Fc. Two lower panels: Temperature (T) tracings. The beginning of the temperature tracing indicates the moment of solution change.

after the twitch, whereas the amplitude of an electrically triggered twitch would increase from zero to steady-state level as a function of time during the same interval. The effect of ryanodine consisted of a concentration-dependent decrease of force of both twitches and rapid cooling contractures; the decline of force due to ryanodine increased progressively with increasing interstimulus intervals. The ryanodine-dependent depression of force at short intervals could be eliminated by interventions that increase [Ca2+]; this effect was accompanied, however, by enhanced decline of force at longer intervals.

**Figure 6.** Graph showing the average force of contracture (Fc)–interval relation. SL indicates sarcomere length. Rest potentiation and rest depression are manifest in the control solution. Ryanodine accelerated rest depression in a manner similar to the reduction of rest depression of the twitch in Figure 2A. Note, however, that unlike the twitch force (Figure 2A), Fc is virtually constant at intervals <1 second, as is also illustrated in the inset, which shows a recording of a cold contracture induced after an interval of 640 msec. The first arrow indicates the introduction of cold solution, and the second arrow shows the rewarming. Data points are mean ± SEM, n=3.
not necessarily linear) of the Ca\(^{2+}\) content of the SR, as has been done in several previous studies.\(^{41-44}\)

**Early Recovery of Twitch Force**

The following three candidates may underlie the early rapid recovery of Ft: 1) a delay in transport of Ca\(^{2+}\) ions from a hypothetical uptake compartment (probably the longitudinal component of the SR) to a release compartment (probably the terminal cisternae) with a transport time constant of ~700 msec in rat at 25°C (see Reference 3), 2) recovery of Ca\(^{2+}\) current during action potential (I\(_{Ca}\)), and 3) recovery of SR Ca\(^{2+}\) channels after the last release.\(^8\)

The properties of the rapid cooling contracture as well as the recovery of rapid cooling contractures after the twitch do not suggest the presence of a process that transports Ca\(^{2+}\) from an uptake compartment to a release compartment in the SR (i.e., transport of Ca\(^{2+}\) longitudinally through the SR). Figure 5 shows clearly that the time to peak force of the contractures induced by rapid cooling is ~350 msec. If opening of SR Ca\(^{2+}\) release channels\(^{36}\) is the only effect of rapid cooling on the SR, it follows that the release of all Ca\(^{2+}\) present in the SR\(^{38}\) through these Ca\(^{2+}\) channels must have taken place at <350 msec. This is clearly shorter than the time constant of recovery of the twitch and inconsistent with a slow transport process between longitudinal SR and the terminal cisternae. It has been shown that the time course of Ca\(^{2+}\) transients during cooling contractures is even faster,\(^37\) and in fact, it is likely that the limiting factor for the rate of rise of force during a rapid cooling contracture is determined by the response of the contractile filaments to Ca\(^{2+}\) ions.\(^38\) The speed of the process of activation during rapid cooling is clearly inconsistent with the existence of a long delay (at 26°C) in the transport between the hypothetical uptake and release compartments within the SR, especially because one would expect that the rate of transport would slow down because of the cooling before the contracture starts. It is possible, but less likely, that rapid cooling opens Ca\(^{2+}\) pathways everywhere along the SR including the ryanodine receptors\(^{38}\) in the terminal cisternae.\(^35,46\) If this were the case, the relation between Fc and increasing intervals would not reveal a transport delay between the uptake and release sites in the SR. This possibility requires us to postulate ad hoc a more complicated effect of rapid cooling and awaits further research into the mechanism underlying Ca\(^{2+}\) release by the SR in response to cooling. The hypothesis that a separate uptake compartment would exist in the SR, insensitive to rapid cooling, is ruled out because of the evidence suggesting that the rapid cooling contracture assays all of the Ca\(^{2+}\) in the SR.\(^25,40\) For the same reason, one would expect that other Ca\(^{2+}\) sources (mitochondrial or extracellular) that potentially contribute to the recovery of the twitch should be revealed by the rapid cooling contracture.

For several reasons, we consider it unlikely that recovery of I\(_{Ca}\) underlies early recovery of Ft. Studies of calcium-induced release have shown that, provided that the SR is filled with Ca\(^{2+}\), I\(_{Ca}\) causes a graded and variable release of Ca\(^{2+}\) depending on rate of rise and magnitude of I\(_{Ca}\).\(^7,8\) If the SR is Ca\(^{2+}\)-depleted, the Ca\(^{2+}\) current will first load the SR and, hence, cannot induce a substantial contraction by itself.\(^2\) The importance of this loading has been shown in experiments where altering the duration of one action potential changed only the magnitude of subsequent contraction.\(^47\) Furthermore, measurement of Ca\(^{2+}\) in the extracellular clefs of cardiac muscle during contractions after rest have shown no link between Ca\(^{2+}\) entry and the magnitude of the corresponding contraction, especially at long rest intervals.\(^48\)

Since our experiments suggest that at short intervals the SR Ca\(^{2+}\) load is large, a large I\(_{Ca}\) should induce a significant release of SR Ca\(^{2+}\). It clearly does not. Hence the small magnitude of force at short intervals may reflect slow recovery of I\(_{Ca}\) or slow recovery of the SR Ca\(^{2+}\) channels.\(^8\) Studies on recovery of I\(_{Ca}\)\(^4,6\) however, suggest that this process is two to three times faster than recovery of Ft measured in our study. The time course of recovery of plateau duration of rat action potential\(^49\) is consistent with rapid recovery of I\(_{Ca}\) and has also been shown to be substantially faster than the recovery of Ft.\(^49\)

Therefore, by elimination of the other candidates, we favor the hypothesis that slow recovery of SR Ca\(^{2+}\) channels determines the rate of recovery of Ft.
Rest Potentiation and Rest Depression

Since there is an increase in the amplitude of Fc at intervals between 1 and 100 seconds, it appears that the SR accumulates Ca\(^{2+}\) during these intervals. This supports the earlier postulates\(^3,4\) that rest potentiation occurring in rat trabeculae is due to a net Ca\(^{2+}\) influx into the cell during rest and that this Ca\(^{2+}\) is then taken up by the SR. Rest potentiation has been shown in several species including man\(^34,50\) but is large in the rat, probably because of the relatively high Na\(^{+}\) level in the rat myocyte,\(^35\) which allows for a net influx of Ca\(^{2+}\) ions during the first 2 minutes after a twitch.

The decrease in Fc at intervals > 100 seconds (rest depression) can be interpreted as a reduction in the SR Ca\(^{2+}\) content at these intervals. Studies by Kitazawa\(^3\) have indeed demonstrated Ca\(^{2+}\) leak from the SR of skinned cardiac muscle with time. Furthermore, measurements by means of atomic absorption spectrophotometry have shown a reduced Ca\(^{2+}\) content in rabbit papillary muscles after long rest intervals.\(^2\) This also agrees with studies\(^20,22\) that have used Ca\(^{2+}\)-sensitive microelectrodes positioned in the extracellular clefts to reveal an increase of [Ca\(^{2+}\)]\(_{oc}\) during rest. Taken together, these studies suggest that Ca\(^{2+}\) leaks out of the SR during rest and leaves the cell by Ca\(^{2+}\) extrusion mechanisms.

The Effects of Ryanodine

Ryanodine affects SR function and depresses the force of contraction\(^15,16,52,53\) without influencing mechanisms such as myofilament Ca\(^{2+}\) sensitivity,\(^21\) Na\(^{+}\)-Ca\(^{2+}\) exchange\(^16,53\) and L\(_o\)\(^16,54,55\). Ryanodine binding sites have been identified only on the terminal cisternae of the SR of both cardiac and skeletal muscle.\(^45,46\) Although studies on SR vesicles of canine ventricular tissue and rabbit skeletal muscle have shown that high concentrations (\(\mu\)M) of ryanodine may block SR Ca\(^{2+}\) release and that lower concentrations (nM) may accelerate Ca\(^{2+}\) leak,\(^10,24\) the question of whether ryanodine exerts its effect on intact preparations by blocking the SR Ca\(^{2+}\) release channels or by depleting the SR of Ca\(^{2+}\) is unresolved. There is, however, agreement that the negative inotropic effect of ryanodine increases with the rate of stimulation, depolarization, or conditions in which Ca\(^{2+}\) release channels are partially open.\(^10,30,56\)

As evident from Figure 2A, ryanodine depresses rest potentiation and accelerates rest depression in a concentration-dependent manner. With 10 nM ryanodine, rest potentiation disappeared completely. At any interval, ryanodine caused a concentration-dependent negative inotropic effect. This effect became more pronounced at longer intervals. Since ryanodine does not affect I\(_o\) directly,\(^16,54,55\) it is more plausible to assume an effect on the Ca\(^{2+}\) release channels in the SR. The effects may have been 1) to block the channels\(^23\) or 2) to lock the SR Ca\(^{2+}\) channels in an open state.\(^10,24\) The former leads to an increase in SR Ca\(^{2+}\) content, and the latter leads to a time-dependent decrease of the SR Ca\(^{2+}\) content.

It is unlikely that the low concentrations of ryanodine that we have used in this study have caused the effect,\(^2\) that is, a block of the SR Ca\(^{2+}\) channels. If the twitch was reduced because ryanodine blocks Ca\(^{2+}\) channels, Ft should have been depressed, but the cooling contracture-interval relation in the presence of ryanodine should lie above that of the control. This is not what we observed (Figures 2 and 6). There is a progressive decrease of both Ft and Fc compared with the control as time elapses after the last steady-state twitch in the presence of ryanodine (Figure 6), suggesting a progressive loss of Ca\(^{2+}\) from the SR. Several other observations also support the hypothesis that ryanodine (in the nanomolar range) opens the SR Ca\(^{2+}\) channels and by this mechanism causes the relative decrease in force. For example, at 10 nM ryanodine, the postextrasystolic twitch subsequent to the extrasystole at an interval of 100 seconds was depressed. With resumption of regular stimulation at 0.2 Hz, the subsequent twitches were even smaller but gradually recovered, as one would expect on the basis of Ca\(^{2+}\) loading of the SR with repeated Ca\(^{2+}\) influx during the action potential.\(^2\) Also, an increase in the stimulation rate or an increase in [Ca\(^{2+}\)]\(_i\) did restore Ft to control level (Figures 3 and 4). Schouten et al\(^3\) have previously obtained evidence that increasing stimulation frequency or [Ca\(^{2+}\)]\(_i\) enhances Ft at all intervals, presumably because of an increase of [Ca\(^{2+}\)]\(_i\) and increase SR Ca\(^{2+}\) loading. In the presence of ryanodine, this was not observed: after restoration of force at short intervals by a higher frequency or higher [Ca\(^{2+}\)]\(_i\) the decline of Ft in the presence of ryanodine actually accelerated (Figures 3 and 4). These observations may be predicted if SR loses Ca\(^{2+}\) in the presence of ryanodine, because at short intervals the increase of Ca\(^{2+}\) in the SR with increased frequency or [Ca\(^{2+}\)]\(_i\) exceeds the loss. At long intervals, more and more Ca\(^{2+}\) is lost; thus, Ft will decline. Such restoration of force by means of increased frequency or [Ca\(^{2+}\)]\(_i\) would not be expected if ryanodine would block SR Ca\(^{2+}\) channels, because binding of ryanodine to the channel has been shown to be enhanced by elevated [Ca\(^{2+}\)].\(^57\)

We suggest that the loss of Ca\(^{2+}\) results from an increase in the background Ca\(^{2+}\) leak from the SR with ryanodine; evidently Ca\(^{2+}\) that has leaked out of the SR is expected to be extruded from the cell by the normal extrusion mechanisms.\(^44\) Such a postulated Ca\(^{2+}\) leak is in agreement with previous studies\(^20\) indicating that the appearance of Ca\(^{2+}\) in the extracellular space during the interstimulus interval is faster in the presence of ryanodine than in control conditions.

Of course, it is attractive to assume that ryanodine at nanomolar concentrations exerts only one effect, that is, to increase the probability that the Ca\(^{2+}\) channels in the SR occur in an open state with a low conductance.\(^10\) Such "partial" opening of SR Ca\(^{2+}\)
channels by ryanodine would lead to a progressive leak of Ca\textsuperscript{2+} from the SR as shown in Figure 6. On the other hand, ryanodine would be expected to exert little or no effect on the kinetics of Ca\textsuperscript{2+} release or recovery from Ca\textsuperscript{2+} release, as reflected by time to peak twitch force (control, 120±3 msec; treated, 115±3 msec) or kinetics of early recovery of FT development, as is indeed shown by our data (compare Figures 2A, 3, 4 and "Results"). The latter would be consistent with the proposed mechanism for early recovery of FT, which, as we have argued in the above, probably consists of recovery of SR Ca\textsuperscript{2+} channels after the preceding twitch.

**Ryanodine and the Rate of Decay of Postextrasystolic Potentiation**

Calcium in the cytosol is subject to extrusion from the cell as well as uptake by the SR. A change in the relative contribution of one mechanism (e.g., the extrusion mechanism\textsuperscript{58}) causes a change in the other.\textsuperscript{59} Hence, if ryanodine causes Ca\textsuperscript{2+} leakage from the SR and this Ca\textsuperscript{2+} is then extruded,\textsuperscript{44} the net amount of Ca\textsuperscript{2+} that is sequestered by the SR must be reduced. Hence, the amount of Ca\textsuperscript{2+} that recirculates from beat to beat between SR and the cytosol will be diminished. To examine this, we estimated the recirculating fraction of Ca\textsuperscript{2+} in the presence and absence of ryanodine, using a well-known experimental approach, the analysis of the rate of decay of postextrasystolic potentiation.\textsuperscript{32,33} If the interval between two excitations is short, less Ca\textsuperscript{2+} will be released from the SR, which will induce a small contraction as is shown in Figure 2. However, Ca\textsuperscript{2+} that has entered the myoplasm during the action potential will be taken up by the SR, adding to its Ca\textsuperscript{2+} load. If the next interval is long enough (equal to steady state), the release of this Ca\textsuperscript{2+} will cause a larger contraction. If the succeeding intervals are also sufficiently long, part of the released excess Ca\textsuperscript{2+} will be extruded through the sarcosome each time the muscle is stimulated, thus leaving the SR with progressively less Ca\textsuperscript{2+}. The resultant potentiation of the postextrasystolic twitches decays exponentially to the steady-state level, as is shown in the inset of Figure 7. The rate of decay of potentiation (obtained from the slope of the relation between the amplitude of successive beats) has been suggested to provide an estimate of Ca\textsuperscript{2+} taken up by SR (calcium recirculation through the SR [B\textsubscript{i}]) versus Ca\textsuperscript{2+} extruded through the sarcosome (1−B\textsubscript{i}).\textsuperscript{3} A faster decay indicates a smaller recirculation of Ca\textsuperscript{2+}. The results showed that indeed with ryanodine the decay rate increased, thus suggesting that the net reuptake of Ca\textsuperscript{2+} by the SR was reduced (Figure 7). A large effect was observed in the presence of 10 nM ryanodine.

Another consequence of a reduced net uptake of Ca\textsuperscript{2+} by the SR could be a reduced rate of relaxation. The observation that at concentrations of ryanodine used in this study (0.1–10 nM) relaxation of the twitch was not prolonged is probably due to the relatively small leak of Ca\textsuperscript{2+} over the time span (−200 msec) of relaxation of the twitch. When Ca\textsuperscript{2+} uptake by the SR is inhibited, there is a compensatory extrusion of Ca\textsuperscript{2+} through the Ca\textsuperscript{2+} pump and the Na\textsuperscript{+}−Ca\textsuperscript{2+} exchange in the sarcosome.\textsuperscript{3,58} Ryanodine indeed decreased the rate of relaxation of the longer-lasting rapid cooling contractures as shown in Figure 5. Cooling inhibits the mechanisms responsible for the removal of Ca\textsuperscript{2+} from the cytoplasm, leading to slowed relaxation of the contracture. In the presence of ryanodine, the rate of uptake of Ca\textsuperscript{2+} by the SR will be further reduced and cannot be compensated for by transsarcolemmal transport.

**Conclusion**

The major implications of this study can be summarized as follows: 1) The presence of a delay in the transport of Ca\textsuperscript{2+} from a hypothetical uptake compartment to a release compartment explains neither the short time to peak force of the rapid cooling contractures nor their magnitude after short intervals. The difference between the early phase of FT−interval relation and that of the Fc−interval relation can probably better be explained by recovery of SR Ca\textsuperscript{2+} release channels. 2) Ryanodine does not appear to inhibit the release of Ca\textsuperscript{2+} from the SR by blocking the Ca\textsuperscript{2+} release channels but, rather, enhances a Ca\textsuperscript{2+} leak from the SR. An enhanced leak of Ca\textsuperscript{2+} from the SR is sufficient to explain enhanced rest depression, the negative inotropic effect, curtailment of rest potentiation, and accelerated decay of postextrasystolic potentiation.

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