Inotropic Response to Hypothermia and the Temperature-Dependence of Ryanodine Action in Isolated Rabbit and Rat Ventricular Muscle: Implications for Excitation-Contraction Coupling

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We have used the sarcoplasmic reticulum (SR) inhibitor ryanodine to assess the contribution of the SR to the increase in twitch tension seen on cooling the mammalian myocardium. To select a suitable concentration of ryanodine, i.e., one that will exert a maximal effect at all temperatures studied, concentration-response curves for ryanodine action were constructed at 37°, 29°, and 23° C in ventricular muscle from rabbit and rat. Using a concentration of ryanodine (1 μM) that exerted a maximal effect at all temperatures studied, the ability of ryanodine to inhibit SR function at 37°, 29°, and 23° C was then confirmed by using rapid cooling contractures (RCCs) to provide an indirect assessment of the SR calcium content. To estimate the rest decay of the SR calcium content in the absence and presence of ryanodine (1 μM), RCCs were initiated after a range of rest intervals (0.3–300 seconds) in rabbit muscles maintained at 37°, 29°, or 23° C. In the absence of ryanodine, low temperatures elevated RCCs at all rest intervals studied. In the presence of ryanodine, RCCs were only seen at rest intervals shorter than 2.0 seconds, even at 23° C, the lowest temperature studied. Thus, even at 23° C, ryanodine appears to be effective at inhibiting SR calcium release in muscles stimulated at 0.5 Hz (i.e., after 2 seconds rest). Therefore, using this concentration of ryanodine (1 μM) and a stimulation rate of 0.5 Hz, we have investigated the contribution of the SR to the positive inotropic response to hypothermia. Under these conditions, the positive inotropic response to cooling in rabbit ventricle was almost unaffected by the inhibition of the SR with ryanodine. In rat ventricle, a tissue in which SR calcium release may dominate excitation-contraction (EC) coupling, the inotropic response to hypothermia was still observed, although developed tension was strongly depressed at all temperatures. These results suggest that a change in SR function is not the principal mediator of the large (400–500%) increase in force associated with cooling mammalian ventricular muscle from 37° to 25° C. The ryanodine-sensitive fraction of tension development was greatest at 37° C, suggesting that the relative contribution of the SR to tension development in rabbit ventricle is reduced at temperatures below 37° C. We investigated the influence of hypothermia on ryanodine-induced changes in action potential in both rabbit and rat ventricle, and the decline in the efficacy of ryanodine at low temperatures cannot be directly attributed to differential electrophysiologic effects at the different temperatures. (Circulation Research 1987;61:761–771)

Cooling from 37° to 22° C has long been known to result in a large inotropic response in the mammalian heart, but there is surprisingly little definitive information in the literature concerning the fundamental mechanisms underlying this phenomenon. The inotropic response to cooling has been attributed to a number of different mechanisms. These mechanisms include an increase in calcium influx during the action potential, an increase in the subsequent release of calcium triggered by the action potential, and a change in the sensitivity of the myofilaments to activator calcium. Action potential duration (APD) is known to be markedly prolonged by hypothermia, and this may have profound effects on trans-sarcolemmal calcium influx mediated by both the calcium inward current and the Na-Ca exchange mechanism. Goto et al. have reported an increase in peak calcium current (i_{Ca}) in response to cooling in bullfrog atrial muscle, and they have related this to the increase in tension development at low temperatures. An increase in i_{Ca}, however, does not appear to underlie the inotropic response to cooling seen in the mammalian myocardium since hypothermia appears to reduce the peak calcium inward current in rat and guinea pig ventricular muscle. It is possible that the Na-Ca exchange mechanism may play an important role in the positive inotropic response to hypothermia. The activity of the Na pump is likely to be reduced at low temperatures, resulting in a hypothermia-induced rise in the intracellular sodium ion concentration ([Na]). This elevation of [Na], may promote a
calcium influx during the plateau of the action potential or oppose a calcium efflux during diastole via the Na-Ca exchange mechanism.\textsuperscript{12,13} Although the activity of the Na-Ca exchange mechanism is itself temperature-dependent,\textsuperscript{14} its relatively low Q\textsubscript{10} means that this mechanism may contribute to the positive inotropic response to hypothermia either by loading the SR with calcium, raising diastolic free calcium, increasing the influx of activator calcium, or by any combination of the above mechanisms.

The situation is further complicated when the influence of temperature on the calcium sensitivity of cardiac myofilaments is considered. Brandt and Hibberd\textsuperscript{15} have demonstrated a decrease in the sensitivity of cat ventricular muscle to calcium on cooling from 29° to 20° C. Conversely, Fabiato\textsuperscript{4} has observed an increase in the calcium sensitivity of dog Purkinje fibers cooled from 22° to 15° C.

Hypothermia has been shown to increase myocardial calcium content,\textsuperscript{16} and this may be expected to load the SR with calcium and hence increase the contribution of SR calcium release to tension development. However, the uptake of calcium into isolated SR vesicles is reduced at low temperatures,\textsuperscript{17,18} and it is, therefore, unclear how the contribution of the SR to EC coupling is altered as the myocardial temperature is lowered below 37° C. To assess the contribution of the SR to the hypothermia-induced inotropy, it would be valuable to selectively inhibit the SR of intact muscle fibers and examine the influence of hypothermia. Ryanodine may be an agent that will allow this dissociation of the SR contribution. While ryanodine seems to be highly selective for SR inhibition,\textsuperscript{19,20} its exact mechanism of action appears to be complex,\textsuperscript{21,22} and its temperature-dependence has not been carefully characterized in mammalian cardiac muscle. Hypothermia is likely to influence not only EC coupling per se but also the potency of drugs, such as ryanodine, that act on the various steps within EC coupling.

The object of this study was to use ryanodine as a tool to investigate the role of the SR in the inotropic response to hypothermia. It was clear, however, that before such a study could be undertaken, the influence of temperature on the ability of ryanodine to inhibit the SR must be characterized. The first part of this paper, therefore, describes the influence of temperature on the action of ryanodine, while the latter portion describes the influence of a maximally effective concentration of ryanodine (as established in the preceding section) on the inotropic response to hypothermia. These studies have been conducted on ventricular muscle from both rabbit and rat, thus providing a comparison between a tissue in which EC coupling is relatively dependent on calcium influx (rabbit) and one in which SR calcium release predominates (rat).\textsuperscript{21,22} The results have been discussed in terms of the implications for the contribution of the SR to EC coupling at different temperatures and the possible mechanisms underlying the inotropic response to hypothermia.

### General

Superperfused right ventricular rabbit or rat papillary muscles or trabeculae were used in all studies. Male New Zealand white rabbits of approximately 1–2 kg body weight and male Sprague-Dawley rats of approximately 250 g body weight were killed by an overdose of sodium pentobarbital (plus 1,000 IU heparin) administered intravenously and intraperitoneally, respectively. Hearts were rapidly excised, perfused via the aorta to remove blood, and then small (0.1–0.5 mm diameter) right ventricular papillary muscles or trabeculae were selected and tied with fine suture. Muscles were then horizontally mounted in the superfusion chamber (volume 0.15 ml) with one end of the muscle tied to a static hook and the other to an isometric force transducer (Gould-Statham BG-10, Gould, Inc., Cleveland, Ohio). Muscles were field-stimulated with a 2-msec square wave of 1.5 times threshold at 0.5 Hz, were gradually stretched to the length at which developed force was maximal, and were then allowed to stabilize for 1 hour at 29° C.

### Materials and Methods

#### Solutions

The normal Tyrode’s solution contained (in mM) NaCl 140, KCl 6, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1, glucose 10, HEPES (N\textsubscript{2}-hydroxyethylpiperazine-N\textsuperscript{\textprime} 2-ethanesulphonic acid) 5. The pH of all solutions was adjusted to 7.40 at 23° C. The temperature-dependence of the pK\textsubscript{a} for HEPES will result in solutions becoming more acidic as the temperature is raised above this value. Thus, at 37° C, the pH of these solutions was 7.23. The change in pH associated with cooling from 37° to 23° C will therefore result in an alkalosis-induced inotropy. However, less than 20% of the positive inotropic effect of the lower temperature solutions can be attributed to the change in pH.\textsuperscript{24,25} The fraction of the hypothermia-induced inotropy that is caused by the alkalosis is, therefore, small with respect to the 400 to 500% increase in tension observed on cooling.\textsuperscript{1} To reduce the contractile activity of the heart and hence facilitate dissection, the CaCl\textsubscript{2} concentration was reduced to 0.1 mM during the dissection procedure. Ryanodine (Pen-nick Corp., Lyndhurst, N.J.) was dissolved in distilled water to produce a stock solution that was diluted immediately prior to use. All solutions were equilibrated with 100% O\textsubscript{2}.

#### Rapid Cooling Contractures

Rapid cooling contractures (RCCs), as described by Kurihara and Sakai\textsuperscript{26} and Bridge,\textsuperscript{27} were used to obtain a qualitative estimate of the releasable SR calcium content at 37°, 29°, and 23° C. It is important to note that RCCs are contractures induced by an acute rapid temperature jump to below 4° C and are used simply as an index of SR calcium availability. These RCCs should not be confused with the increase in stimulated twitch tension that is brought about by less dramatic changes in steady-state temperature (37° to 23° C).

RCCs were initiated by switching between parallel
water-jacketed superfuse inflow lines that converged at a solenoid controlled valve situated close to the muscle bath. One superfuse inflow line was maintained at either 37°C, 29°C, or 23°C, while the other was maintained at less than 0°C. Using this arrangement and a fast superfuse flow rate of 13 ml/min, the bath temperature could be reduced to less than 4°C approximately 300 msec after triggering the valve. In rabbit ventricle, the RCC, like the first postrest contraction, declines with increasing duration of quiescence preceding the cooling or the twitch. The progressive reduction in the amplitude of the RCC and the twitch following increasing periods of rest has been suggested to reflect the rest decay of SR calcium content.21-23 The influence of maintaining the preparation at either 37°C, 29°C, or 23°C on the rest decay of RCCs in the presence or absence of ryanodine was investigated.

The duration of the rest interval was measured from the time of the final stimulation pulse to the solution switch. Rest intervals of 5 seconds or less were generated (± 10 msec) using an electronic timer, which was triggered by the final stimulus and controlled the solenoid valve. Longer rest intervals were timed manually.

Action Potential Recordings

Action potentials were recorded via conventional 3 M KCl-filled microelectrodes of approximately 10–20 MΩ impedance. The upstroke of the action potential was recorded electronically using a differentiator that showed a linear response to 1,000 V/sec. Action potentials, dV/dt, and contraction profiles were displayed on a Tektronix 5100 oscilloscope and photographed. Action potential duration (to 95% repolarization) was measured both from the photographs and electronically using the circuit described by Kentish and Boyett.28

Results

Influence of Temperature on Ryanodine Action: Cumulative Ryanodine Concentration-Response Curves

Figure 1A shows cumulative concentration-response curves for the ability of ryanodine to reduce steady-state tension in rabbit ventricular muscle at 37°C, 29°C, or 23°C. It can be seen from Figure 1A that temperature exerts two distinct effects on the ryanodine concentration-response curve. First, cooling below 37°C decreased the efficacy of any single ryanodine concentration such that the concentration required to half-maximally inhibit tension (K1/2) was increased as the temperature was lowered. Second, the effect of cooling was to reduce the fraction of tension that was inhibitable by a maximal concentration of ryanodine. These two distinct effects are summarized in Table 1.

In the above experiments, the influence of the various ryanodine concentrations on the first postrest (30 seconds) beat (B1) was investigated at 37°C and 23°C. The first postrest beat has been suggested to be a sensitive index of SR function and is dramatically reduced by ryanodine.21 At 37°C the K1/2 for the in-

![FIGURE 1. Relation between the cumulative ryanodine concentration and peak developed tension at 37°, 29°, or 23°C in rabbit (A) and rat (B) ventricular muscle. Peak developed tension was expressed as a percentage of pre-ryanodine control value at each temperature. Muscles were stimulated at 0.5 Hz and stabilized for 1 hour at 29°C. Muscles were either maintained at 29°C or the temperature was changed to 37°C or 23°C and the muscles were allowed to re-equilibrate. The ryanodine concentration was then increased in a step-wise manner from 0.1 nM to 1 μM, and the muscles were allowed to equilibrate at each ryanodine concentration. Steady-state twitch tension was recorded at each concentration. In several experiments, the ryanodine concentration was increased to 10 μM, and no further decrease in force was observed. Data are expressed as mean ± SEM; n = 5 for each group.](http://circres.ahajournals.org/content/76/4/763)

Table 1. Influence of Temperature on the Ability of Ryanodine to Inhibit Tension Development of Rabbit and Rat Ventricular Muscle Assessed by the Concentration of Ryanodine Required to Half-Maximally Inhibit Tension (K1/2) and the Maximal Reduction in Tension Induced by Ryanodine

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Rabbit</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1/2 (nM)</td>
<td>Maximal tension reduction (%)</td>
</tr>
<tr>
<td>37°C</td>
<td>0.5</td>
<td>77 ± 6 (5)</td>
</tr>
<tr>
<td>29°C</td>
<td>5</td>
<td>35 ± 7 (5)</td>
</tr>
<tr>
<td>23°C</td>
<td>30</td>
<td>44 ± 3 (5)</td>
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Data taken from the experiments shown in Figure 1.
dine concentration (1 μM), B1 was reduced by 81% at 37°C and by 48% at 23°C.

The experiments described above for the rabbit ventricle were then repeated using rat ventricular muscle, and the concentration-response curves obtained are shown in Figure 1B. From Figure 1B it can be seen that the major difference between the dose-response curves obtained in the rabbit and rat is one of extent. That is, the rat myocardium, like the rabbit, shows decreasing sensitivity to ryanodine at lower temperatures, but the absolute reduction in tension at high ryanodine concentrations was greater in the rat than in the rabbit (see Table 1). The ability to markedly depress tension in the rat myocardium has been detailed previously, and this has been attributed to the relative importance of SR calcium release to EC coupling in that species. It is interesting to note that the estimates of K1/2 (see Table 1) are almost identical in both species.

In rat ventricular muscle, the temperature-dependence of the influence of ryanodine on the first postrest beat showed similar changes in K1/2 to those seen in rabbit ventricle. Compared with the rabbit, the maximal reduction of B1 at high ryanodine concentrations was, however, greater in the rat with B1 reduced by 99% at 37°C and by 91% at 23°C.

In a number of experiments in both rabbit and rat ventricular muscle, the ryanodine concentration was elevated to 10 μM, and no further decline in tension was observed at any of the temperatures studied. It is, therefore, clear that 1 μM ryanodine exerts a maximal effect on tension development at 37°, 29°, or 23°C. In these experiments, steady state was considered to have been established when developed tension changed by less than 10% within a 30-minute period. It is, however, conceivable that steady state was not truly established at low temperatures since the onset of ryanodine action is slow.

It was clearly important to establish that the hypothermia-induced decline in the ryanodine-sensitive fraction of tension reflects a real decline in the contribution of the SR to EC coupling at low temperatures and not simply a loss of ryanodine efficacy. To investigate this possibility, it would clearly be useful to be able to measure directly the size of the releasable pool of SR calcium under the various experimental conditions. However, this is not currently feasible, and we have, therefore, used RCCs to obtain a qualitative and indirect estimate of the SR calcium content at 37°, 29°, and 23°C in the absence and presence of ryanodine.

Use of Rapid Cooling Contractures to Assess the Influence of Temperature on Releasable SR Calcium in Absence and Presence of Ryanodine

The rapid cooling of cardiac muscle, as described by Kurihara and Sakai26 and Bridge,27 is thought to result in the release of SR calcium to the myofilaments and a consequent contracture that is indicative of the quantity of calcium released. RCCs are abolished in the presence of caffeine and are thought to be independent of trans-sarcolemmal calcium entry.27 Calcium entry via the Na-Ca exchange mechanism, similar to that underlying the low sodium contracture, is unlikely to contribute as RCCs are unaffected by reducing the sodium concentration of the cooling solution.27 In addition, calcium entry via the calcium channel does not appear to contribute to the RCC in normally polarized muscles because RCCs, recorded in sodium-free solutions, are unaffected by calcium channel blockade with nifedipine.22 RCCs are both reproducible and readily reversible and, therefore, provide a useful, though indirect, means of assessing the releasable calcium content of the SR. It should be noted that the RCC provides only a relative estimate of the SR calcium that can be released by rapid cooling. It is, therefore, important to emphasize that the calcium released by an action potential may not be the same as the calcium released in response to an RCC. However, it seems reasonable to assume that an experimental intervention that alters the SR calcium content releasable by cooling also alters the fraction of SR calcium that can be released by an action potential.

The aims of the experiments in this section were, therefore, twofold. First, to use the RCC amplitude as an index of SR calcium content at different temperatures, and second, to determine whether ryanodine, at a concentration that can induce a maximal effect at each temperature, completely inhibits SR calcium release during 0.5 Hz stimulation. Figure 2A shows the rest decay of RCCs at 37°, 29°, and 23°C. The data are expressed as a percentage of the previous 29°C steady-state twitch tension. The size of the RCC initiated at the beginning of the rest interval is assumed to reflect the maximum amount of SR calcium that can be released by rapid cooling (under these conditions) prior to a rest-dependent loss of SR calcium. The decline in the size of the RCC with increasing periods of rest reflects a decline in this pool of SR calcium and has been previously described in detail.22,27 From Figure 2A, it is apparent that, at the beginning of the rest period, the releasable pool of SR calcium was greater when the muscles were maintained at 23°C (RCC, 77 ± 8%) than when the muscles were maintained at 37°C (RCC, 47 ± 9%). The mean value obtained at 29°C was intermediate between these two points (RCC, 64 ± 6%) but was not significantly different from the 37°C value. The relative increase in releasable SR calcium content at the hypothermic temperatures was maintained throughout the rest period, and after a 300-second rest, both the 29° and 23°C RCCs were significantly different from the 37°C value. The rate of loss of releasable calcium from the SR, as reflected by the rest decay of the RCC, appears to be similar at all three temperatures although it is slightly slower at the lower temperatures. Assuming that the SR calcium will eventually fall to near zero after prolonged rest periods, the half-time for this decay to zero can be estimated to be approximately 5 minutes at 23°C and 2 minutes at 29° or 37°C.

The influence of ryanodine (1 μM) on the rest decay of the RCC assessed at 37°, 29°, and 23°C is shown
Muscles were then warmed to 37° C and ryanodine (1 μM) added. Ryanodine was added at 37° C to ensure a maximal effect, and muscles were then cooled to 29° C. The rest decay of the RCCs was then evaluated at 29°, 37°, and 23° C as before, only using rest intervals of 0.3, 0.5, 0.8, 1, 2, 3, 4, and 5 seconds. The magnitude of the RCCs recorded was expressed as a percent of the previous steady-state tension recorded in the absence of ryanodine to allow direct comparison between the control and ryanodine data. Data are expressed as mean ± SEM; n = 7.

In Figure 2B (note the sixtyfold change in the scale of the abscissa). At 37° C, RCCs were virtually absent even at the shortest rest intervals. This implies that at this temperature and in the presence of ryanodine, the SR was either depleted of releasable calcium under steady-state conditions or that this pool of releasable calcium was very rapidly lost from the SR within the first 300 msec following the stimulus. At 29° C, the rest decay of the RCC was slower than at 37° C and had a half-time of approximately 500 msec. At this temperature, RCCs were not seen at rest intervals longer than 1 second. A further slowing of the rest decay was seen in muscles maintained at 23° C. At 23° C, the half-time of decay was approximately 1 second and RCCs recorded after 2 seconds (an interval equivalent to the beat-to-beat interval in the steady-state observations) were not significantly elevated with respect to the 29° or 37° C values which were close to zero.

A minor complication of the interpretation of these results is, however, in the presence of ryanodine, at the lower temperatures and at the shorter rest intervals, there was incomplete relaxation of the final contraction before the rapid cooling contracture was initiated. It seems likely that the RCC reflects the sum of cytoplasmic and SR calcium. The initiation of the RCC at a time in the contraction cycle when the cytoplasmic calcium concentration is still elevated (i.e., after short rest intervals at 23° C) may invalidate the assumption that the RCC amplitude, recorded under these conditions, reflects the relative SR calcium release. The important observation from these studies is that after 2 seconds (the steady-state interval at a stimulation rate of 0.5 Hz) RCCs were virtually absent even at the lower temperatures. This observation is unaffected by the above limitation since relaxation was complete at all temperatures within 2 seconds after the stimulus. The conclusion from these experiments is that at a maximally effective ryanodine concentration and 0.5 Hz, SR calcium release as assessed by rapid cooling contractures is effectively eliminated at all temperatures studied. By using a maximally effective concentration of ryanodine, therefore, the contribution of the SR to the hypothermia-induced inotropy can now be assessed.

**Influence of Ryanodine on the Positive Inotropic Response to Hypothermia**

**Rabbit.** Figure 3A shows the positive inotropic response of rabbit ventricular muscle elicited by cooling from 37° C to either 33°, 29°, or 25° C in the absence or presence of ryanodine. Muscles were maintained at 37° C and then sequentially cooled to either 33°, 29°, or 25° C in the absence of ryanodine. Muscles were rewarmed to the 37° C control baseline between each period of hypothermia. To ensure that ryanodine exerted its maximal effect, muscles were then exposed to 1 μM ryanodine at 37° C and steady state reestablished. The cooling steps were then repeated in the presence of ryanodine. The increase in tension in response to cooling in the absence and presence of ryanodine was then expressed as a percentage of the pre-ryanodine 37° C control value.

The positive inotropic response of the mammalian myocardium to cooling below 37° C has been extensively documented, and it is evident from Figure 3A that in these experiments, developed tension was increased approximately fivefold by cooling from 37° to 25° C. At 37° C, 1 μM ryanodine reduced tension by 30 ± 8% with respect to the pre-ryanodine control value. From Figure 3A, it is clear that the inotropic response to cooling was little affected by the presence of ryanodine (0.1 μM). Cooling from 37° to 25° C resulted in a sevenfold increase in tension such that at 33°, 29°, or 25° C there was no significant difference in the inotropic response recorded in the absence or presence of ryanodine.
It should be noted that the single concentration of 1
μM ryanodine at 37°C reduced tension by 30% in
Figure 3A, while in the concentration-response study
shown in Figure 1A (and Table 1), the cumulative
administration of 1 μM ryanodine reduced tension by
77%. At 29°C, ryanodine reduced peak developed
tension by only 14% of control in Figure 3A compared
with 35% in Figure 1A. The single dose values from
Figure 3A may be more reliable for the maximal extent
of tension depression induced by ryanodine.* It is,
therefore, clear that in rabbit ventricular muscle the
increase in tension seen in response to cooling is
virtually unaffected by the inhibition of the SR with a
maximal concentration of ryanodine. This implies that
in this tissue the SR may not play a major role in the
hypothermia-induced inotropy.

Rat. Figure 3B shows the positive inotropic response
of rat ventricular muscle in response to cooling from
37°C to either 33°C, 29°C, or 25°C in the absence or
presence of ryanodine. In the absence of ryanodine,
developed tension increased approximately sixfold on
cooling from 37°C to 25°C, an equivalent inotropy to that
seen in rabbit ventricular muscle. In the presence of
ryanodine (1 μM), tension was reduced to 6 ± 5% at 37°C.
Cooling to 25°C resulted in an approximate thirteenfold
increase in tension to 82 ± 19% of the pre-ryanodine control value. This can be seen more clearly in the inset to Figure 3B in which the tension axis (0–100% control) has been expanded to
demonstrate the inotropic response. In the presence of
ryanodine, therefore, the inotropic response to cooling
was still evident. However, developed tension was
substantially reduced at all temperatures.

If the SR is not primarily responsible for the increase
in tension development at low temperatures in rabbit
ventricle (and to a lesser extent in rat ventricle), then
mechanisms other than those involving the SR must be
considered in the explanation of the inotropic response
to hypothermia. Ryanodine is known to influence the

*The cumulative concentration-response results (Figure 1 and
Table 1) were obtained from long experiments. These were
particularly long with respect to the initial control reference point
(up to 6 hours). Additionally, under control conditions, the decline
in steady-state force in long experiments was more apparent at 37°C
than at 29° or 23°C. In single concentration experiments (Figure
3), muscles were maintained at 37°C for only brief intervals, and
the control period preceded the test values by ≤1 hour. The
combination of these effects would lead to an overestimation of the
potency of ryanodine in the cumulative concentration-response
experiments, especially at 37°C. For example, from the single
concentration experiments in Figure 3A, ryanodine decreased twitch
tension by 30, 14, and 12% of control at 37°, 29°, and 25°C,
respectively. While this may be a more quantitative estimate, the
qualitative conclusion is the same, i.e., at temperatures below 37°C,
there is a decrease in the fraction of tension development that is
sensitive to ryanodine.

FIGURE 3. The inotropic response of rabbit (A) and rat (B) ventricular muscle
to cooling between 37° and 25° C in the absence and presence of 1 μM ryan-
odine. Inset shows the inotropic response of rat ventricular muscle to cooling
(shown in Panel B) on an expanded scale. Muscles were stabilized for 1 hour
at 37° C and stimulated at 0.5 Hz.

Muscles were then sequentially cooled to 33°, 29°, and 25° C and steady-state
tension recorded. Between each period of cooling, a period of re-equilibration
at 37° C was interposed. In this way, the inotropic response to cooling could
be related to the immediately previous pe-
riod of 37° C superfusion. To ensure a
maximal effect, ryanodine (1 μM) was
added at 37°C. Steady-state tension was
then re-established in the presence of
ryanodine. Steady-state tension was
then recorded at the three hypothermic
temperatures and was expressed as a
percentage of the steady-state tension
recorded at 37°C immediately prior to
the addition of ryanodine. The order in
which individual muscles were exposed
to the three hypothermic temperatures
was randomized in both the control and
ryanodine-free protocols. Data are ex-
pressed as mean ± SEM, n=5 for each
group in Panel A and n = 4  for each
group in Panel B.
shape of the action potential, and it is possible that differential effects of ryanodine on the action potential may maintain tension at low temperatures even when the SR is inhibited; that is, it is possible that ryanodine may preferentially prolong the action potential at low temperatures and thus indirectly limit the negative inotropic effects of ryanodine by maintaining tension development during the period of prolonged depolarization. It is, therefore, important to consider the influence of temperature on the ryanodine-induced changes in the action potential.

**Influence of Temperature on Ryanodine-Induced Changes in the Action Potential**

*Rabbit.* Figure 4A–C shows action potentials recorded at 37°C in the absence of ryanodine (A) and after 5 minutes (B) and 60 minutes (C) of ryanodine (100 nM) superfusion. As has been previously described, ryanodine was shown to induce a progressive lengthening of the action potential and a decline in developed tension. These changes were effectively complete within 30–40 minutes and Figure 4C shows steady-state action potential and tension profiles recorded after 60 minutes of ryanodine exposure at 37°C. Over this time course, ryanodine did not significantly alter the rate of rise of the action potential.

In these experiments, the administration of a single concentration of ryanodine (100 nM) at 37°C reduced tension by 36 ± 13% of the pre-ryanodine control value. This was a significantly smaller reduction in tension than was observed when the ryanodine concentration was increased to 100 nM in the cumulative dose-response study shown in Figure 1A (77 ± 6%) but was similar to the single concentration effect shown in Figure 3A (30 ± 8%) (see footnote page 766). Figure 4D–F shows a comparable series of action potential and tension profiles recorded from rabbit ventricular muscle maintained at 23°C. In the absence of ryanodine (Figure 4D), muscles maintained at 23°C show the prolongation and the slowing of the rate of rise of the action potential (note changes in scale) that are characteristic of hypothermia. The influence of ryanodine at 23°C can be seen to be qualitatively similar to that seen at 37°C with prolongation, and enhancement of the plateau region, of the action potential. In the example shown in Figure 4A–C, the rate of rise of the action potential was slightly increased, but this was not seen in the majority of muscles studied.

The changes in APD induced by ryanodine at the three temperatures studied are summarized in Table 2. From these data, it is apparent that the absolute increase in APD was 64 ± 24(5) msec at 23°C, 76 ± 17(5) at 29°C, and 83 ± 13(5) msec at 37°C. To consider the relative contribution of these changes in APD to EC coupling at the different temperatures, these data have been expressed as a percent change with respect to the

![Figure 4](http://circres.ahajournals.org/)

**Figure 4. Influence of temperature on action potentials recorded from rabbit ventricle muscle in the absence (A,D) and presence (B,C,E,F) of ryanodine (100 nM).** The upper trace of each panel shows dV/dt (offset with respect to the action potential), while the middle and lower traces of each panel show, respectively, action potential and tension records. Muscles were equilibrated at 0.5 Hz stimulation rate and were maintained at either 37° or 23°C. Panels A and D show control recordings made at 37°C and 23°C, respectively. Panels B (37°C) and E (23°C) show recordings made after 5 minutes of exposure to ryanodine (100 nM) and panels C (37°C) and F (23°C) after 60 minutes of ryanodine exposure. Qualitatively similar results were obtained when these experiments were done at 29°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Control (msec)</th>
<th>Ryanodine (10^-7M) (60 min) (msec)</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>222 ± 12 (5)</td>
<td>305 ± 24 (5)</td>
<td>137 ± 6 (5)</td>
</tr>
<tr>
<td>29°C</td>
<td>256 ± 29 (5)</td>
<td>332 ± 25 (5)</td>
<td>131 ± 9 (5)</td>
</tr>
<tr>
<td>23°C</td>
<td>372 ± 14 (5)</td>
<td>436 ± 32 (5)</td>
<td>117 ± 7 (5)</td>
</tr>
</tbody>
</table>

Muscles were stimulated at 0.5 Hz and action potentials were recorded under control conditions and throughout 60 minutes of ryanodine exposure. The 60-minute data are shown above. Data are expressed as mean ± SEM (n).
pre-ryanodine control value. The time-course of these changes in APD at 37°C, 29°C, and 23°C is shown in Figure 5. The rate of onset of the effects of ryanodine on APD can be seen to be temperature-dependent with the changes being most rapid at 37°C and slowest at 23°C. The time course of tension decline induced by ryanodine demonstrated a similar temperature dependence (not shown). The temporal association between the electrophysiologic changes and the tension changes may support the suggestion\(^\text{51,9,23}\) that these effects of ryanodine are mediated by a single intracellular action.

**Rat.** Figure 6A–C shows action potentials recorded from rat ventricular muscle maintained at 37°C in the absence of ryanodine (A) and after 5 minutes (B) and 60 minutes (C) of ryanodine exposure. After 5 minutes of superfusion at 37°C with 100 nM ryanodine, the late plateau phase of the rat action potential was reduced, as has been described previously by Mitchell et al.\(^\text{5}\) During this first 5 minutes of ryanodine exposure, the developed tension was seen to fall by 50%. A further reduction in the late plateau phase of the rat action potential and peak developed tension was observed and these changes equilibrated after approximately 45 minutes. In Figure 6C, at 37°C, tension was reduced to 1–2% of control (undetectable at the oscilloscope gain used in Figure 6C), and the late plateau phase of the action potential was considerably reduced with respect to the control action potential shown in Figure 6A. It is apparent from Figure 6A–C that ryanodine did not affect the rate of rise of the rat action potential at 37°C. The influence of hypothermia (23°C) on this process is shown in Figure 6D–F. From Figure 6D, it is apparent that hypothermia itself enhanced the late plateau phase of the rat action potential and reduced its rate of rise. After 5 minutes of ryanodine exposure (Figure 6E), the late plateau phase can be seen to be reduced but with little concomitant reduction in tension development. After 60 minutes of ryanodine exposure, the late plateau phase was further reduced and tension had equilibrated in this example at 25% of control. From Figure 6, it is apparent that the effects of ryanodine on the rat action potential and tension development are antagonized by hypothermia. Under steady-state conditions, 100 nM ryanodine abolished the late plateau phase of the rat action potential at 37°C, but at 23°C, this effect was considerably reduced. Similarly, at 37°C, ryanodine virtually eliminated tension development, while at 23°C, a considerable fraction of control tension remained in the presence of

![Figure 5. Time course for the ryanodine-induced changes in the duration of the action potential recorded from rabbit ventricular muscles maintained at either 37°, 29°, or 23° C. The experimental protocol was as described in Figure 4, and data are expressed as a percent of the initial pre-ryanodine control value at each temperature (mean ± SEM; n = 5 for each group).](image)

![Figure 6. Influence of temperature on action potentials recorded from rat ventricular muscle in the absence of (A,D) and presence (B,C,E,F) of ryanodine (100 nM). Upper trace of each panel shows dV/dt (offset with respect to the action potential), while the middle and lower traces of each panel show, respectively, action potential and tension records. Muscles were equilibrated at 0.5 Hz stimulation rate and were maintained at either 37° or 23° C. Panels A and D show control recording made at 37° and 23° C, respectively. Panels B (37° C) and C (23° C) show recordings made after 5 minutes of exposure to ryanodine and panels C (37° C) and F (23° C) after 60 minutes of ryanodine exposure.](image)
100 nM ryanodine, which is a submaximal concentration at that temperature (see Figure 1B).

**Discussion**

The basic observation of this study was that the positive inotropic response to hypothermia can be elicited even when the SR is inhibited with ryanodine. While it appears that at least a major fraction of the cooling-induced inotropy can occur in the absence of a contribution from the SR, this does not suggest that the SR does not normally contribute to this process. At low temperatures, the kinetics of SR calcium release may be slowed, and the RCC experiments indicate that there may be an increase in the SR calcium content. Whether this increase in SR calcium content results in an increased calcium release with each beat or whether the fractional release of SR calcium changes is unclear. If the fractional SR calcium release is unchanged at low temperatures, it seems unlikely that this increase in SR calcium content (which increased RCC magnitude by 62%) underlies the 400-500% increase in tension seen at low temperatures. It is clear that even if the SR calcium release is unchanged or even slightly enhanced, the relative contribution of the SR to tension development may be reduced at temperatures below 37°C. Further evidence for a decline in the role of the SR at low temperatures can be seen when the temperature-dependence of the ryanodine concentration-response curves (Figure 1 and Table 1) is considered. Hypothermia reduced the ability of ryanodine to inhibit tension, and this was manifest in two ways. First, the concentration of ryanodine necessary to produce a maximal effect, the percent reduction in tension observed was less in muscles maintained at temperatures below 37°C. Further, ryanodine-induced loss of releasable calcium from the SR, after 2 seconds of rest at temperatures between 23°C and 37°C, there is no release of calcium from the SR in the presence of caffeine. Therefore, we conclude that, at the 0.5-Hz stimulation rate used in the concentration-response studies, 1 μM ryanodine completely inhibits SR function at these temperatures. The reduction in the ryanodine-sensitive fraction of tension at temperatures lower than 37°C cannot be ascribed to a loss of ryanodine potency. In other words, once the ryanodine concentration is sufficient to exert its maximal effect at any given temperature, the SR calcium release at 0.5 Hz is effectively blocked. Since the maximal concentration of ryanodine did not markedly change the hypothermia-induced inotropy in rabbit ventricle (Figure 3A), most of the inotropy in this tissue is not due to an increase in SR calcium release.

If the SR is not primarily responsible for the increase in tension development at low temperatures in rabbit ventricular muscle, then mechanisms, other than those involving the SR, must be considered in the explanation of the inotropic response to hypothermia. Hypothermia, however, will affect every step in EC coupling, making it unlikely that a single mechanism underlies the inotropic response to cooling. At low temperatures, the activity of the systems responsible for regulating cytoplasmic calcium may be reduced, resulting in an elevation of the cytoplasmic calcium concentration. A rise in the resting free calcium concentration may in itself exert an inotropic effect. The subsequent release of SR calcium and the influx of calcium through the cell membrane upon excitation may result in a larger increment of tension if the resting free calcium concentration is elevated toward the steeper region of the pCa-tension curve. One factor that may contribute to the increase in cellular calcium content at low temperatures may be the elevation of [Na], that has been recently described on cooling ventricular muscle. The relation between [Na] and tension has been shown to be very steep around normal intracellular values. That is, a small change in [Na], is associated with a large change in tension presumably by favoring a calcium influx via the Na-Ca exchange mechanism. The reduction in the sodium gradient by a hypothermia-induced rise in [Na], would raise the intracellular calcium concentration under steady-state conditions. Such a rise in [Na], would also tend to enhance calcium entry and depress calcium efflux during the action potential via the Na-Ca exchange mechanism. The Na-Ca exchanger is only indirectly dependent on metabolic energy, via the establishment of the sodium gradient and hence has a relatively low Q10 between 35° and 25°C of 1.35 as compared to the Na-pump current, which has a Q10 of approximately 3.0 over the same temperature range (extrapolated from the data of Eisner and Lederer). The activity of the Na-Ca exchange mechanism is therefore likely to be less affected by the reduction of temperature than is the Na-pump. The Na-Ca exchange may therefore play a role in cellular calcium loading at low temperatures due to the hypothermia-induced decline in the sodium gradient.

It is interesting to note that calcium entry during individual contractions has been shown to be enhanced by reducing the sodium gradient with the cardioactive steroid acetylstrophanthidin. This acetylstrophanthidin-induced inotropy, like the hypothermia-induced inotropy, has been shown to occur in the presence of caffeine or ryanodine. In other experiments (not shown), caffeine also failed to inhibit the
hypothermia-induced inotropy. Thus, a functional SR is not required for increases in tension produced either by acetylstrophanthidin or by hypothermia. It is, therefore, possible that at low temperatures, the contribution of Na-Ca exchange to trans-sarcolemmal calcium influx, during an individual contraction, may be enhanced. In this regard, it is interesting to note that a hypothermia-induced increase in tension of a single twitch has been observed even when the temperature was only reduced for the duration of that single contraction.40 An enhanced calcium influx via the Na-Ca exchange mechanism would also be favored at low temperatures by the prolongation of the action potential. If the Na-Ca exchange is indeed involved in the elevation of the intracellular calcium concentration, either during a single beat or under steady-state conditions, then the APD and the configuration of the AP may be important determinants of tension development.

Ryanodine is known to prolong the action potential,21,22 and it is possible that if this prolongation is particularly marked at low temperatures, the negative inotropic effect of ryanodine could be reduced by the maintenance of tension during the longer plateau of the action potential. This, however, does not appear to be the case because the results described here indicate that the prolongation of the action potential was less at 23°C (both in absolute and relative terms) than at 29°C or 37°C. The decline in the ability of ryanodine to reduce tension at low temperatures, therefore, does not appear to be attributable to a disproportionate increase in APD at these temperatures.

It is interesting to compare rabbit and rat ventricular muscle in terms of their responses to ryanodine at different temperatures. At each temperature studied, the K1/2 for ryanodine action was almost identical in rat and rabbit (see Table 1). The difference between the two species, however, was the extent to which tension could be reduced with a maximal concentration of ryanodine. In rat ventricle, a maximally effective ryanodine concentration can almost abolish contractile force at all temperatures. This decline in the relative importance of the SR to tension development at lower temperatures suggests that other cellular mechanisms must be considered in any attempt to explain the inotropic response to hypothermia.

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References


Key Words • temperature • excitation-contraction coupling • ryanodine • myocardium • action potential
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