Ca²⁺ Release Induced by Rapid Cooling and Caffeine in Ferret Ventricular Muscles

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Abstract: The Ca²⁺ release induced by rapid cooling (RC) and caffeine in ferret ventricular muscles was investigated. For this purpose, the author measured the intracellular Ca²⁺ concentration ([Ca²⁺]_i) using aequorin. Rapid lowering of the temperature of the bathing solution from 30°C to low temperatures after the cessation of electrical stimulation transiently increased [Ca²⁺]_i. The peak of [Ca²⁺]_i was altered depending upon the temperature in RC. [Ca²⁺]_i reached 1.59 μM when the temperature was lowered from 30 to 4°C. Caffeine (15 mM) applied during cooling after the decline of RC-induced intracellular Ca²⁺ signal caused an increase in [Ca²⁺]_i. The author assumed that the total Ca²⁺ content in the SR was the sum of the peaks of [Ca²⁺]_i increased by RC (Ca_{RC}) and caffeine application (Ca_{caf}). The fractional Ca²⁺ release induced by RC [Ca_{RC}/“assumed” total released Ca²⁺ from the SR(%)] was 63.7% at 4°C. The peak of Ca²⁺

increased by RC was proportional to the “assumed” total released Ca²⁺ from the SR. No significant correlation was observed between RC-induced [Ca²⁺]_i change and the [Ca²⁺]_i before RC. However, in Na⁺-deficient solutions (Li⁺ or TMA⁺ replacement), the fractional Ca²⁺ release induced by RC was decreased; this inhibition shows a dependence upon extracellular Na⁺ concentration. In contrast, the fractional Ca²⁺ release by caffeine application after RC was enhanced. These results suggest that: 1) RC releases a fraction of the Ca²⁺ accumulated in the SR and 2) the mechanism of RC-induced Ca²⁺ release in mammalian cardiac muscle is different from that of RC-induced Ca²⁺ release in frog skeletal muscles, which requires an increase in [Ca²⁺]_i before RC. Extracellular Na⁺ might modify RC-induced Ca²⁺ release through a change in [Na⁺]_i. [Japanese Journal of Physiology, 47, 263–272, 1997]

Key words: intracellular calcium, low temperature, aequorin, sarcoplasmic reticulum.

When the temperature of the bathing solution is rapidly lowered, contracture is induced in skeletal, cardiac, and smooth muscles (rapid cooling contracture, RCC) [1–6]. In skeletal muscle fibers, treatment of the preparation with a low concentration of caffeine before rapid cooling (RC) is required for the initiation of RCC [1, 2, 7]; indicating that RCC is induced by the synergistic effects of caffeine and low temperature, which increase the intracellular Ca²⁺ concentration ([Ca²⁺]_i) before RC. On the other hand, pretreatment of the preparation with caffeine is not required for the initiation of RCC in mammalian cardiac and smooth muscles [4, 5, 8]. Therefore, the mechanism of increase in [Ca²⁺]_i induced by RC might vary

among different tissues.

RC-induced Ca²⁺ release from sarcoplasmic reticulum (SR) in skeletal muscle fibers is directly shown by measuring [Ca²⁺]_i change using aequorin [9]. Similarly, in mammalian cardiac muscles, RC is suggested to release the Ca²⁺ accumulated in the SR [5], and Bers *et al.* measured the [Ca²⁺]_i change in RCC in guinea-pig ventricular myocytes using a fluorescent Ca²⁺ indicator, Indo-1 [8]. Recently, Sitsapesan *et al.* [10] reported that the open probability of the Ca²⁺ release channels in sheep cardiac muscle, which are incorporated into an artificial lipid bilayer, increases at 5°C compared to that at 23°C. This result suggests that RC enhances the opening of Ca²⁺ release chan-

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nels in the SR and the resultant Ca^{2+} increase induces RCC. However, the details of RC-induced Ca^{2+} release in mammalian cardiac muscles have not been fully investigated, particularly in relation to the RC of skeletal muscles and caffeine-induced Ca^{2+} release, which are considered to be induced through the CICR mechanism [11].

In order to investigate the characteristics of RC-induced Ca^{2+} release in mammalian cardiac muscles, aequorin was injected into the superficial cells of ferret papillary muscles, and $[\text{Ca}^{2+}]_i$ changes induced by RC were measured under various conditions and discussed.

The preliminary results have already been presented in abstract form [12–14].

METHODS

Preparations. Ferrets (body weight 600–1,200 g) were anesthetized by intraperitoneal injection of pentobarbitone ($150 \text{ mg} \cdot \text{kg}^{-1}$), and the hearts were quickly removed. The blood in the heart was washed out by retrograde perfusion of normal Tyrode's solution at 30°C through the aorta. Then, the right ventricle wall was quickly opened and the papillary muscles were dissected out in normal Tyrode's solution at 30°C . The diameter of the preparation was $0.3\text{--}1.5 \text{ mm}$ ($0.8 \pm 0.03 \text{ mm}$, mean $\pm$ standard error of the mean, $n=34$) and the length was $2.5\text{--}5.5 \text{ mm}$ ($4.0 \pm 0.1 \text{ mm}$, $n=34$). The preparation was horizontally mounted in a muscle chamber with a pair of platinum black wires for electrical stimulation. One end of the preparation was connected to a fixed hook and the other end was connected to the arm of a tension transducer (Kulite, BG10, NJ, USA). The preparation was stimulated by a square pulse with a 5 ms duration at $1.5 \times$ threshold unless otherwise mentioned. Before starting the experiment, the preparation was slowly stretched to develop maximal tension.

Solutions. The normal Tyrode's solution used for dissection and aequorin injection was as follows (mM): Na^+ , 135; K^+ , 5; Ca^{2+} , 2; Mg^{2+} , 1; Cl^- , 102; HCO_3^- , 20; HPO_4^{2-} , 1; SO_4^{2-} , 1; acetate, 20; glucose, 10; insulin, 5 units $\cdot \text{l}^{-1}$; pH, 7.3–7.4 at 30°C when equilibrated with 5% CO_2 +95% O_2 . Tyrode's solution buffered with N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), which was used in the experiments (HEPES-Tyrode's solution), had the following composition (mM): Na^+ , 128; K^+ , 5; Ca^{2+} , 2; Mg^{2+} , 1; Cl^- , 117; SO_4^{2-} , 1; acetate, 20; glucose, 10; HEPES, 5; and insulin, 5 units $\cdot \text{l}^{-1}$. The pH was adjusted to 7.3–7.4 with NaOH at 30°C and equilibrated with 100% O_2 . A high K^+ solution (150 mM

K^+), which was also used for the accumulation of Ca^{2+} in the SR, was made by adding 145 mM KCl to the Tyrode's solution without compensation for osmolality. When the extracellular Na^+ concentration ($[\text{Na}^+]_o$) was changed, NaCl and sodium acetate were replaced by LiCl and lithium acetate or by tetramethyl ammonium chloride (TMACl) and TMA acetate with compensation for osmotic pressure. The $[\text{Na}^+]_o$ used was 0, 32, 64, 96, and 128 mM. K^+ -free solution was also used to increase the intracellular Na^+ concentration ($[\text{Na}^+]_i$) [15].

Aequorin injection, light detection, and conversion to $[\text{Ca}^{2+}]_i$. Aequorin (purchased from Dr. J. R. Blinks) was dissolved in the solution containing 200–300 mM KCl and 10 mM HEPES at pH 7.0 with a final concentration of 100–150 μM . For aequorin injection, glass micro-pipettes with a resistance of 30–50 M Ω were prepared and filled with aequorin. Aequorin was injected into 100–200 superficial cells of each preparation by applying high-pressure ($4\text{--}7 \text{ kg} \cdot \text{cm}^{-2}$) nitrogen gas. Aequorin light signals were detected with a photomultiplier placed just above the preparation and were simultaneously recorded with tension. Light signals during RC and with caffeine application were recorded through 1 and 10 Hz low-pass filters, respectively. The light signals induced by RC and by caffeine were converted to $[\text{Ca}^{2+}]_i$ using the following equation [16];

$$\frac{L}{L_{\max}} = \left(\frac{1 + K_R [\text{Ca}^{2+}]}{1 + K_{\text{TR}} + K_R [\text{Ca}^{2+}]} \right)^n,$$

where n , K_R , and K_{TR} are constants calculated using the relation between aequorin luminescence and Ca^{2+} concentration ($[\text{Ca}^{2+}]$). $L/L_{\max}$ is the normalized aequorin light in which peak light at different $[\text{Ca}^{2+}]$ was normalized to the maximal light ($L_{\max}$) at saturated $[\text{Ca}^{2+}]$. $L_{\max}$, obtained by discharging all aequorin in the HEPES-Tyrode's solution at the end of each experiment, was calculated using the rate constant of the decay of aequorin light at saturating $[\text{Ca}^{2+}]$ [17]. As aequorin luminescence is markedly influenced by temperature, we made *in vitro* calibration curves at each temperature (Fig. 1A). The method for measuring the aequorin calibration curve is reported elsewhere [16, 17]. At each temperature, the pH of the solutions with different $[\text{Ca}^{2+}]$ was adjusted to 7.0, and solutions with low $[\text{Ca}^{2+}]$ were made using ethyleneglycol-bis- $[\beta\text{-aminoethylether}]\text{-N,N,N',N'}$ -tetraacetic acid (EGTA). The constants used for converting the light signal to $[\text{Ca}^{2+}]$ are presented in Table 1. Since the relation between $\log L_{\max}$ and temperature was linear (Fig. 1B), $L_{\max}$ was calculated at

Fig. 1. Effects of temperature on the relation between aequorin luminescence and $[Ca^{2+}]$. **A:** *In vitro* calibration curves measured at different temperatures (see METHODS for details). The curves were normalized to L_{max} at each temperature. **B:** The relation between temperature and $\log L_{max}$ were measured at different temperatures.

Table 1. Constants for conversion of aequorin light to $[Ca^{2+}]_i$; n , K_R , and K_{TR} at different temperatures.

Temperature (°C)	n^*	K_R	K_{TR}
30	3.51	3.17×10^6	67.5
24	3.97	3.59×10^6	51.5
18	3.18	6.76×10^6	147.9
12	3.72	5.05×10^6	76.5
7	3.42	7.53×10^6	119.1
4	3.03	15.94×10^6	255.3

* For the details of n , K_R , and K_{TR} , see text.

each temperature using the relation and used for the conversion of the light signal of the preparation to $[Ca^{2+}]_i$. Caffeine (20 mM) did not seriously alter the relation between fractional luminescence ($\log L/L_{max}$) and $[Ca^{2+}]_i$.

Measurement of the membrane potential.

The membrane potential of the superficial cells was measured using a conventional glass microelectrode filled with a 3 M KCl solution. The floating method was employed to keep the microelectrode in the cell

before and during contraction. The membrane potential change induced by RC was measured in HEPES-Tyrod's solution.

Protocols. For loading Ca^{2+} in the SR at 30°C, repetitive electrical stimulation (2 Hz for 5 min) was applied to the preparation [5] or the preparation was treated with 150-mM K^+ solution for 10 min. After Ca^{2+} loading, the temperature of the bathing solution was rapidly (<1 s) lowered from 30°C to various temperatures (4, 7, 12, 18, and 24°C).

1. *Investigation of the origin of Ca^{2+} in RC.* The preparation was treated with ryanodine (5 μ M) for 60 min to lock Ca^{2+} release channels in the SR in the open state. After treatment with 150-mM K^+ solution to load Ca^{2+} in the SR, Ca^{2+} was released by RC.

2. *The ratio of Ca^{2+} released by RC to the "assumed" total released Ca^{2+} from SR.* $[Ca^{2+}]_i$ increased by RC returned to near the resting level before RC within less than 20 s after the commencement of RC. Caffeine (15 mM) was rapidly applied at 30 s after the commencement of RC to release all of the remaining Ca^{2+} in the SR. As the effects of 15 and 30 mM caffeine on Ca^{2+} release were not significantly different, 15 mM caffeine was used for this purpose. At low temperatures, particularly below 4°C, the rate of Ca^{2+} uptake by the SR is decreased, and only a small fraction of RC-induced intracellular Ca^{2+} is slowly removed by SR. Almost all Ca^{2+} released by RC is transported by mitochondria because ruthenium red and NaN_3 , inhibitors of the mitochondrial Ca^{2+} transport, significantly prolong the decay of RC-induced Ca^{2+} [18]. Therefore, the increase in $[Ca^{2+}]_i$ induced by caffeine after RC reflects the apparent remaining Ca^{2+} in the SR (the Ca^{2+} not released by RC and the small amount of Ca^{2+} transported by the SR during RC). I estimated the fraction of Ca^{2+} released by RC from the SR. For the calculation of the fraction of Ca^{2+} released by RC, I used the "assumed" total released Ca^{2+} from the SR (not true total released Ca^{2+}), which was defined as the sum of Ca^{2+} released by RC (Ca_{RC}) and Ca^{2+} released by caffeine (Ca_{caf}). Since a major fraction of the released Ca^{2+} is bound to troponin C, the true total released Ca^{2+} is the sum of $[Ca^{2+}]_i$ and the amount of Ca^{2+} bound to troponin C during RC and caffeine application. However, I assumed that Ca^{2+} binding to troponin C altered in proportion to $[Ca^{2+}]_i$ and that the affinity of troponin C for Ca^{2+} did not significantly alter in each trial of RC and caffeine application. Thus, the fraction of Ca^{2+} released by RC can be expressed as $Ca_{RC}/(Ca_{RC} + Ca_{caf})$ (%).

3. *Effect of intracellular ion concentration on the amount of Ca^{2+} released by RC.* For the investiga-

tion of the relation between the pre-cooling $[Ca^{2+}]_i$ and $Ca_{RC}/(Ca_{RC} + Ca_{caf})$ (%), the mean value of $[Ca^{2+}]_i$ during the 10 s period before RC was used for the pre-cooling $[Ca^{2+}]_i$. For changing $[Ca^{2+}]_i$ before RC, I used various $[K^+]_o$ (0, 5, 150 mM) and $[Na^+]_o$ (0, 32, 64, 96, 128 mM) solutions.

To observe the effect of $[Na^+]_i$ on RC, Na^+ in the HEPES-Tyrode's solution was replaced with equimolar Li^+ or TMA^+ , as described above, because it is reported that $[Na^+]_i$ proportionally decreases as $[Na^+]_o$ is decreased [19–21]. The preparation was continuously stimulated at 0.2 Hz in the Na^+ -deficient solutions for 60–120 min at 30°C, which was considered to be long enough to decrease $[Na^+]_i$. Then, the stimulation frequency was increased to 2 Hz and stimulated for 5 min before RC for loading Ca^{2+} in the SR. Thus, the relation between $[Na^+]_o$ and the fractional amount of Ca^{2+} released by RC, $Ca_{RC}/(Ca_{RC} + Ca_{caf})$ (%), was measured.

Chemicals. The following chemicals were used: caffeine (Nacalai Tesque Inc., Kyoto, Japan) and ryanodine (Agri-Systems, Inc., PA, USA). All chemicals used were of analytical grade.

Statistics. Measured values are shown as mean $\pm$ standard error of the mean (SEM) in the text and in the figures. Student's *t*-test (two-tailed for paired samples) was used to test for significant differences of the measured values. A significant difference was verified at $p < 0.05$. A coefficient analysis was also used to test the independence of two factors.

RESULTS

Effects of RC on $[Ca^{2+}]_i$, tension, and membrane potential

When the temperature of the solution was rapidly lowered from 30 to 4°C, 20 s after the cessation of repetitive stimulation (2 Hz for 5 min), $[Ca^{2+}]_i$ transiently increased and the peak of $[Ca^{2+}]_i$ reached $1.59 \pm 0.06 \mu M$ ($n=17$) (Fig. 2). Tension was initiated following the change in $[Ca^{2+}]_i$, and the peak of tension reached $27.5 \pm 3.1 \text{ mN} \cdot \text{mm}^{-2}$ ($n=15$) (Fig. 2). In a separate experiment, the membrane potential and tension were simultaneously measured during RC. The resting membrane potential before RC was $-74 \pm 1 \text{ mV}$ ($n=3$). The membrane was slightly depolarized to $-67 \pm 2 \text{ mV}$ ($n=3$) immediately after RC, and then it further depolarized to $-60 \pm 1 \text{ mV}$ ($n=3$) 30 s after the beginning of RC (Fig. 2). The action potential was not triggered by RC. However, some preparations showed a twitch-like contraction in RC. These preparations were discarded. The results of the membrane potential were consistent with those re-

Fig. 2. Effects of rapid cooling on $[Ca^{2+}]_i$, tension, and membrane potential. Rapid cooling (RC) from 30 to 4°C after repetitive stimulation (2 Hz for 5 min) induced a change in $[Ca^{2+}]_i$ and tension. The noisy record with a faster change is $[Ca^{2+}]_i$, which is followed by a change in tension. Membrane potential change during RC is shown in the lower trace. The microelectrode was kept in the cell before and during RC. Slight depolarization is induced by RC and the depolarization continues during cooling.

ported in guinea-pig papillary muscles [5]. Therefore, the RC-induced Ca^{2+} release in ferret ventricular muscles is not due to significant membrane depolarization.

Effects of ryanodine on $[Ca^{2+}]_i$ change induced by RC

Ryanodine ($5 \mu M$) significantly suppressed the increase in $[Ca^{2+}]_i$ induced by RC after treatment with a 150-mM K^+ solution to accumulate Ca^{2+} in the SR (data not shown). Therefore, $[Ca^{2+}]_i$ increased by RC in ferret ventricular muscle is considered to be due to Ca^{2+} released from the SR as in other specimens.

The ratio of the amount of Ca^{2+} released by RC to the "assumed" total released Ca^{2+} from the SR

After the preparation was stimulated at 2 Hz for 5 min, the peak of $[Ca^{2+}]_i$ induced by RC from 30 to 4°C (Ca_{RC}) was $1.57 \pm 0.04 \mu M$ ($n=9$) and the peak of $[Ca^{2+}]_i$ induced by caffeine application 30 s after RC (Ca_{caf}) was $0.90 \pm 0.05 \mu M$ ($n=9$) in HEPES-Tyrode's solution (Fig. 3A). Ca_{RC} was in proportion to the "assumed" total released Ca^{2+} , and the ratio of Ca_{RC} /"assumed" total released Ca^{2+} of the SR was $63.7 \pm 0.9\%$ (Fig. 3B and C). Therefore, RC might release approximately 63.7% of the Ca^{2+} stored in the SR even though the Ca^{2+} content of the SR varied. In this cal-

Fig. 3. The ratio of $[Ca^{2+}]_i$ induced by RC to the "assumed" total released Ca^{2+} during cooling. $[Ca^{2+}]_i$ was transiently increased by RC from 30 to 4°C after repetitive stimulation (2 Hz for 5 min) and then, during cooling, it gradually returned to the level before RC. Then, caffeine (15 mM) was rapidly applied during cooling and the remaining Ca^{2+} in the SR was released (A). The ratio $Ca_{RC} / (Ca_{RC} + Ca_{Cal})$ (%) was $63.7 \pm 0.9\%$ (in HEPES-Tyrod's solution, $n=9$). The amount of Ca^{2+} released by RC ranged from 1.10 to $2.73 \mu M$ (in the solution with various $[K^+]_o$ and normal $[Na^+]_o$, $n=23$) and increased in proportion to the increase in "assumed" total Ca^{2+} . Total released Ca^{2+} in the abscissa is "assumed" total released Ca^{2+} (see METHODS section for the details of "assumed" total released Ca^{2+}) (B). However, this ratio was relatively constant (from 50.3 to 68.4%) ($n=23$) and was not significantly influenced by the change in "assumed" total released Ca^{2+} (C).

ulation, some fraction of Ca^{2+} released by RC is transported to the SR during cooling, but this fraction is considered to be fairly small (less than 5%) compared to the Ca^{2+} transported to the mitochondria [18].

$[Ca^{2+}]_i$ change induced by RC from 30°C to various temperatures

In frog skeletal muscle fibers treated with a low concentration of caffeine, Ca^{2+} is released when the solution temperature is decreased to lower than 4°C (critical temperature) [2]. In order to determine the critical temperature in ferret ventricular muscles, I measured $[Ca^{2+}]_i$ when the temperature was lowered from 30°C to various temperatures. The peak of $[Ca^{2+}]_i$ induced by RC increased depending on the change in temperature of RC, and the relation between the temperature in RC and the peak of $[Ca^{2+}]_i$ was fit-

Fig. 4. $[Ca^{2+}]_i$ change induced by RC from 30°C to various temperatures. After repetitive stimulation (2 Hz for 5 min) in HEPES-Tyrod's solution, the temperature of the bathing solution was rapidly lowered from 30°C to various temperatures (24, 18, 12, 7, and 4°C) and $[Ca^{2+}]_i$ was measured (A). B shows the relation between the temperature in RC and the peak of $[Ca^{2+}]_i$ change induced by RC ($n=2-17$, in each temperature). This relation was fitted by two lines. r means coefficients of regression lines.

ted by two linear lines (Fig. 4A and B). Therefore, a clear critical temperature was not observed but the RC-induced Ca^{2+} release reached a nearly maximal level below 4°C. When the temperature was lowered to 24, 18, 12, 7, and 4°C, the peak of $[Ca^{2+}]_i$ was 0.51 , 0.68 ± 0.07 , 1.44 ± 0.10 , 1.57 ± 0.09 , and $1.59 \pm 0.06 \mu M$, respectively.

Influence of pre-cooling $[Ca^{2+}]_i$ on the RC-induced Ca^{2+} release

Since RC-induced Ca^{2+} release from the SR is dependent upon pre-cooling $[Ca^{2+}]_i$ in frog skeletal muscle fibers treated with caffeine [7, 9], I tested whether or not the RC-induced $[Ca^{2+}]_i$ change in ferret cardiac muscles is influenced by $[Ca^{2+}]_i$ before RC. The relation between the amount of Ca^{2+} released by RC and $[Ca^{2+}]_i$ prior to RC was observed under various condi-

tions which are thought to alter $[Ca^{2+}]_i$. Then, the fractional Ca^{2+} released by RC, $Ca_{RC}/(Ca_{RC}+Ca_{caf})$ (%) (see METHODS for the definition), was calculated. This indicator was not influenced by the change in the "assumed" total Ca^{2+} content in the SR (see above) which is expected to be altered under some experimental conditions used. The $[Ca^{2+}]_i$ before cooling varied under various conditions as shown in Fig. 5 ($n=110$, 17 experiments). The fractional Ca^{2+} released by RC was 18.1–75.2% ($n=110$, 17 experiments). As shown in Fig. 5, the peak of $[Ca^{2+}]_i$ increased by RC did not show a dependence upon the pre-cooling $[Ca^{2+}]_i$. Therefore, in contrast to the RC-induced Ca^{2+} release in skeletal muscle fibers [7, 9], an increase in $[Ca^{2+}]_i$ before RC is not necessary for RC-induced Ca^{2+} release in ferret cardiac muscles.

The relation between $[Na^+]_o$ and $[Ca^{2+}]_i$ increased by RC

The peak of $[Ca^{2+}]_i$ increased by RC and by caffeine applied after RC was measured at various $[Na^+]_o$. Ca_{RC} and Ca_{caf} were measured and the percentage of Ca_{RC} to the "assumed" total released Ca^{2+} was plotted against $[Na^+]_o$. As shown in Fig. 6A and B, the RC-induced Ca^{2+} (Ca_{RC}) was decreased at low

Fig. 5. Dependence of the RC-induced Ca^{2+} release on pre-cooling $[Ca^{2+}]_i$. Change in $[Ca^{2+}]_i$ induced by RC (Ca_{RC}) and by caffeine application (Ca_{caf}) during cooling and the average $[Ca^{2+}]_i$ during the 10 s before RC (pre-cooling $[Ca^{2+}]_i$) was measured. The bottom line is the baseline which was measured by closing a photomultiplier shutter. The graph shows the relation between pre-cooling average $[Ca^{2+}]_i$ and the fractional Ca^{2+} released by RC. Fractional Ca^{2+} release by RC was calculated using the equation $Ca_{RC}/(Ca_{RC}+Ca_{caf})$ (%) (see METHODS for details).

$[Na^+]_o$, which was observed in both Li^+ and TMA^+ replacements. The pooled data is shown in Fig. 6C. On the other hand, the fractional Ca^{2+} release by RC was not increased in K^+ -free solution, which can elevate $[Na^+]_i$ (Fig. 7A and B). In the K^+ -free solution, the resting $[Ca^{2+}]_i$ was already increased before RC, suggesting an increase in $[Na^+]_i$. However, the fractional Ca^{2+} release by RC in the K^+ -free solution was $64.7 \pm 0.3\%$, which did not significantly differ from that in the HEPES-Tyrod's solution with 5-mM K^+ ($63.7 \pm 0.9\%$). Therefore, the effect of $[Na^+]_i$ on the RC-induced Ca^{2+} release is saturated at $[Na^+]_i$ in HEPES-Tyrod's solution with 5-mM K^+ .

DISCUSSION

The origin of Ca^{2+} and mechanism of Ca^{2+} release in RC. RCC can be induced in ferret ventricular muscles as in other preparations [1, 5, 6]. This study directly proved that the RCC in ferret ventricular muscles was induced by an increase in $[Ca^{2+}]_i$. Therefore, RCC in ferret ventricular muscles is not due to the change in the Ca^{2+} sensitivity of the contractile elements.

As in skeletal muscle fibers and other mammalian cardiac muscles, the origin of Ca^{2+} increased by RC of ferret ventricular muscle is the Ca^{2+} stored in the SR, because RC-induced $[Ca^{2+}]_i$ change was inhibited by ryanodine (data not shown).

This study demonstrates that the amount of Ca^{2+} released by RC increased as the temperature in RC was lowered (Fig. 4). It is reported that low temperatures increase the open probability of Ca^{2+} release channels in the SR, although single-channel conductance is linearly reduced by lowering the temperature [10]. Thus, Ca^{2+} is released through the Ca^{2+} release channels of the SR as a net effect of cooling.

The amount of Ca^{2+} and the fraction of Ca^{2+} released by RC. It is reported that in guinea-pig ventricular myocytes, the peak of $[Ca^{2+}]_i$ during RC reaches $10 \mu M$ (measured with Indo-1) and that almost all of the Ca^{2+} accumulated in the SR could be released by RC [8]. In ferret papillary muscles, the peak of $[Ca^{2+}]_i$ during RC, measured with aequorin, was $1.59 \pm 0.06 \mu M$ (Fig. 2). The quantitative difference of the measured values of $[Ca^{2+}]_i$ increased by RC in both studies might be due to the differences in experimental conditions; indicators employed (aequorin and Indo-1), species used (ferret and guinea-pig), preparations used (papillary muscles and single cardiac myocytes), and temperatures during experiments (from 30 to 4°C and from room temperature to 0–1°C).

Fig. 6. Effects of [Na⁺]_o on the Ca²⁺ release induced by RC and caffeine application. Na⁺ in the HEPES-Tyrod's solution was replaced with Li⁺ (A) or TMA⁺ (B). In each panel, caffeine (15 mM) was applied 30 s after RC at a low temperature. The time of the application of caffeine for all records is indicated in the top panel. [Na⁺]_o is shown at the left side. For TMA⁺ replacement, the top panel shows the record in 0-mM [Na⁺]_o, the middle panel in 64-mM [Na⁺]_o, and the bottom panel in 128-mM [Na⁺]_o solution. The bottom line in each panel represents the baseline when the photomultiplier shutter was closed. C gives the pooled data showing the relation between [Na⁺]_o in the solution in which Na⁺ was replaced with Li⁺ or TMA⁺ and the fractional Ca²⁺ release induced by RC ($n=2-9$ in each [Na⁺]_o).

This study shows that, in ferret papillary muscles, caffeine was applied during cooling after the transient RC-induced [Ca²⁺]_i change increased [Ca²⁺]_i, suggesting that some of the remaining Ca²⁺ in the SR can be further released by caffeine. The decrease in [Ca²⁺]_i during cooling can be explained as follows [18]. RC releases Ca²⁺ by opening Ca²⁺ release channels of the SR. The fact that caffeine could release Ca²⁺ after RC suggests that all Ca²⁺ could not be released by RC or that some of the Ca²⁺ released by RC is transported to the SR. However, almost all of the Ca²⁺ released by RC is transported by mitochondria because ruthenium red, an inhibitor of Ca²⁺ uniport of mitochondria, significantly prolongs the decay time of the Ca²⁺ signal during RC [22]. NaN₃ also shows similar effects on the decay of [Ca²⁺]_i during cooling. Since 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (TBQ) and thapsigargin (inhibitors for the Ca²⁺ pump in the

SR) are ineffective for the decay time of RC-induced Ca²⁺ signal, the SR cannot substantially remove Ca²⁺ from the myoplasm during cooling [18]. At low temperatures below 4°C, the Na⁺-Ca²⁺ exchanger with high Q_{10} is also inhibited [18]. Therefore, the Ca²⁺ released by caffeine after RC mainly reflects the remaining Ca²⁺ in the SR which was not released by RC. The decay of Ca²⁺ signal in RC and caffeine application is limited by the regenerative Ca²⁺ released from the SR. The longer decay time during caffeine application as compared to that during RC suggests that caffeine releases Ca²⁺ more effectively from the SR. Thus, all Ca²⁺ stored in the SR can be released by caffeine but RC probably does not release all Ca²⁺ in the SR.

Ions influencing the Ca²⁺ release in RC.

1. *Effects of [Ca²⁺]_i on RC-induced Ca²⁺ release.* In frog skeletal muscle fibers, an increase in

Fig. 7. The effect of $[Na^+]_i$ on the Ca^{2+} release from the SR induced by RC. **A:** The fractional Ca^{2+} release induced by RC and caffeine application during cooling in solutions with different $[Na^+]_o$ and $[K^+]_o$. In the 0-mM $[Na^+]_o$ and 5-mM $[K^+]_o$ solution, $[Na^+]_i$ was expected to be lower than that in 128-mM $[Na^+]_o$ and 5-mM $[K^+]_o$ solution. Higher $[Na^+]_i$ was expected in the solution with 128-mM $[Na^+]_o$ and 0-mM $[K^+]_o$ due to inhibition of the sarcolemmal Na^+-K^+ pump. The bottom line in each panel is the baseline to measure $[Ca^{2+}]_i$. **B:** In the pooled data of the fractional Ca^{2+} released by RC in the three different solutions, no significant difference was observed between the fractional Ca^{2+} release by RC in normal $[Na^+]_i$ and that in higher $[Na^+]_i$. However, a significant difference was observed when $[Na^+]_i$ was decreased.

$[Ca^{2+}]_i$ before RC is required for a large increase in Ca^{2+} during RC; when $[Ca^{2+}]_i$ reaches a critical level, a large amount of Ca^{2+} (higher than $16 \mu M$) can be released [9]. To increase $[Ca^{2+}]_i$ before RC, a low concentration of caffeine is employed because RC itself cannot sufficiently increase $[Ca^{2+}]_i$ for triggering large Ca^{2+} release in skeletal muscles. Therefore, RC-induced Ca^{2+} release in skeletal muscle fibers is assumed to be due to the enhancement of the CICR mechanism [7]. This hypothesis is further supported by the fact that procaine, an effective inhibitor for

CICR [23], can inhibit RCC in skeletal muscles [2, 9]. In contrast to RCC in skeletal muscle fibers, the amount of Ca^{2+} released by RC in ferret papillary muscles was not influenced by $[Ca^{2+}]_i$ before RC, which was varied using various experimental conditions, and no critical Ca^{2+} concentration, which caused a large release of Ca^{2+} , was observed (Figs. 4 and 5). In addition, the amount of Ca^{2+} released by RC in ferret papillary muscles (about $2 \mu M$) was significantly lower than that released by frog skeletal muscles (about $16 \mu M$). Therefore, the Ca^{2+} release mechanism during RC of ferret papillary muscle is not the same as that in skeletal muscle from the quantitative and qualitative aspects.

There are two possible explanations regarding the mechanism of RC-induced Ca^{2+} release in ferret papillary muscles. One possibility is that RC of ferret cardiac muscles releases Ca^{2+} from the SR through the CICR mechanism as in the case of skeletal muscle RCC, but stimulation of the CICR mechanism by RC of the cardiac muscle is weak. Therefore, $[Ca^{2+}]_i$ increased by RC of the cardiac muscle is lower than that in skeletal muscles, although enhancement of the CICR mechanism by caffeine application prior to RC is required for frog skeletal muscles. The fact that caffeine (an activator of CICR) [11] releases more Ca^{2+} in ferret cardiac muscles as compared to RC supports this hypothesis. However, ruthenium red, a potent inhibitor for CICR [24], could not inhibit RC-induced Ca^{2+} release even when the Ca^{2+} transients in twitch, which is considered to be due to CICR, were inhibited [22]. This contradicts the hypothesis that RC of the cardiac muscle releases Ca^{2+} through the CICR mechanism. The other possibility is that RC directly opens the Ca^{2+} release channels through a different mode from CICR, which is suggested by Sitsapasan *et al.* [10].

2. Influence of $[Na^+]_o$ on RC-induced $[Ca^{2+}]_i$ change. This study demonstrates that the fractional Ca^{2+} release induced by RC was decreased in Na^+ -deficient solutions. In the Na^+ -deficient solution in which Li^+ was used as a substitute, $[Na^+]_i$ decreased [20, 21] and the intracellular Li^+ concentration ($[Li^+]_i$) increased [25]. Consequently, both factors, a decrease in $[Na^+]_i$ and an increase in $[Li^+]_i$, should be considered to influence the RC-induced Ca^{2+} release. However, a similar result was observed when Na^+ was replaced by TMA⁺. Therefore, the increase in $[Li^+]_i$ is not a factor in the inhibition of Ca^{2+} release in RC in Na^+ -deficient solutions. In the TMA⁺ replacement solution, the intracellular pH (pH_i) was reported to be altered [20]. However, Li^+ replacement does not alter the pH_i [19]. Therefore, pH_i is not a factor responsible

for the inhibition of RC-induced Ca²⁺ release in Na⁺-deficient solutions. Thus, a change in [Na⁺]_i might be a possible factor to explain the effect of low [Na⁺]_o on RC-induced Ca²⁺ release.

If [Na⁺]_i is altered, the Na⁺-Ca²⁺ exchanger in mitochondria is also influenced. In low [Na⁺]_i, the Na⁺-Ca²⁺ exchanger in mitochondrial membrane is inhibited and mitochondria should contain more Ca²⁺, and thus, the Ca²⁺ content in the SR is secondarily diminished. If this is the case, both RC-induced and caffeine-induced Ca²⁺ release should be decreased. However, my results contradict this hypothesis because caffeine-induced Ca²⁺ release was rather increased. Therefore, the effect of [Na⁺]_i on the mitochondrial transport is not a factor which explains the results. For RC-induced Ca²⁺ release, intracellular Na⁺ might play a role, although this is speculative.

The dependence of RC-induced Ca²⁺ release on [Na⁺]_i is almost saturated under the control condition in HEPES-Tyrod's solution because RC-induced Ca²⁺ release was not altered in K⁺-free solution, which increases [Na⁺]_i (Fig. 7).

The different sensitivities of caffeine-induced Ca²⁺ release and RC-induced Ca²⁺ release to low [Na⁺]_o ([Na⁺]_i) (Fig. 7) also suggest two possibilities: 1) RC releases Ca²⁺ in a different mode from CICR, which operates in caffeine-induced Ca²⁺ release; and 2) RC does not strongly stimulate the CICR mechanism as in the case of caffeine as discussed above.

Finally, the RCC in skeletal muscle fiber is reproducible even in a high K⁺-solution with low [Na⁺]_o [2], which is different from what we observed in the RCC in ferret cardiac muscles. Therefore, RC-induced Ca²⁺ release in ferret papillary muscles qualitatively and quantitatively differs from that in skeletal muscles, which is considered to be due to enhancement of the CICR mechanism [7]. Further studies are required to clarify the molecular mechanism regarding RC-induced Ca²⁺ release in cardiac muscles.

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