

# Role of $\text{Ca}^{2+}$ in the rapid cooling-induced $\text{Ca}^{2+}$ release from sarcoplasmic reticulum in ferret cardiac muscles

Etsuko Tanaka · Masato Konishi · Satoshi Kurihara

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**Abstract** Rapid lowering of the solution temperature (rapid cooling, RC) from 24 to 3°C within 3 s releases considerable amounts of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) in mammalian cardiac muscles. In this study, we investigated the intracellular mechanism of RC-induced  $\text{Ca}^{2+}$  release, especially the role of  $\text{Ca}^{2+}$ , in ferret ventricular muscle. Saponin-treated skinned trabeculae were placed in a glass capillary, and the amount of  $\text{Ca}^{2+}$  released from the SR by RC and caffeine (50 mM) was measured with fluo-3. It was estimated that in the presence of ATP about 45% of the  $\text{Ca}^{2+}$  content in the SR was released by RC. The amount of SR  $\text{Ca}^{2+}$  released by RC was unchanged by the replacement of ATP by AMP-PCP (a non-hydrolysable ATP analogue and agonist for the ryanodine receptor but not for the  $\text{Ca}^{2+}$  pump of SR), suggesting that the suppression of the  $\text{Ca}^{2+}$  pump of SR at low temperature might not be a major mechanism in RC-induced  $\text{Ca}^{2+}$  release. The free  $\text{Ca}^{2+}$  concentration of the solution used for triggering RC-induced  $\text{Ca}^{2+}$  release was estimated to be only about 20 nM with fluo-3 or aequorin. When this solution was applied to the preparation at 3°C, only a small amount of  $\text{Ca}^{2+}$  was released from SR presumably by the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) mechanism. Thus, in mammalian cardiac muscles, RC releases a part of the (<50%) stored  $\text{Ca}^{2+}$  contained in the SR, and the mechanism of RC-induced  $\text{Ca}^{2+}$  release may

differ from that of CICR, which is thought to play a role in frog skeletal muscle fibres that express ryanodine receptors of different types.

**Keywords** Calcium · Sarcoplasmic reticulum · Rapid cooling ·  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release

## Introduction

Rapid lowering the solution temperature is known to induce contracture in skeletal, cardiac and smooth muscles (rapid cooling contracture, RCC) [1–3]. Among these muscles, treatment of the preparation with low concentrations of caffeine is required for RCC in frog skeletal muscle [4]. In RCC of frog skeletal muscle, caffeine releases a small amount of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) by the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) mechanism, and low temperature inhibits  $\text{Ca}^{2+}$  uptake by SR. Thus, it was supposed that both caffeine and low temperature synergistically increase  $\text{Ca}^{2+}$  concentration around the SR and work to facilitate the CICR mechanism, leading to a massive  $\text{Ca}^{2+}$  release from SR [5].

However, in mammalian cardiac muscles, pre-treatment with caffeine is not required for RCC [3], and the intracellular  $\text{Ca}^{2+}$  concentrations before rapid cooling (RC) measured in intact ferret papillary muscles do not correlate with the amount of  $\text{Ca}^{2+}$  released by RC [6], suggesting that the mechanism of RC-induced  $\text{Ca}^{2+}$  release differs from that of CICR. Thus, the  $\text{Ca}^{2+}$  release mechanism by RC in cardiac muscle may differ from that in skeletal muscle.

In this study, we investigated the intracellular mechanism of RC-induced  $\text{Ca}^{2+}$  release in ferret ventricular muscle. We focused on whether the RC-induced  $\text{Ca}^{2+}$

E. Tanaka (✉) · S. Kurihara  
Department of Cell Physiology, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan  
e-mail: etsukotamanawa@kfz.biglobe.ne.jp

M. Konishi  
Department of Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan

release in cardiac muscle could be explained by CICR. For this purpose, quantitative measurement of  $\text{Ca}^{2+}$  concentrations around SR before RC is essential. Therefore, we used the previously established methodology with saponin-treated skinned preparations in which the SR functions are known to be well preserved [7, 8]. The saponin-skinned trabeculae were placed in a glass capillary, and various solutions with different compositions were directly applied to the SR to estimate the amount of released  $\text{Ca}^{2+}$  from the SR and  $\text{Ca}^{2+}$  content in SR.

A part of the results was presented in abstract form [9].

## Materials and methods

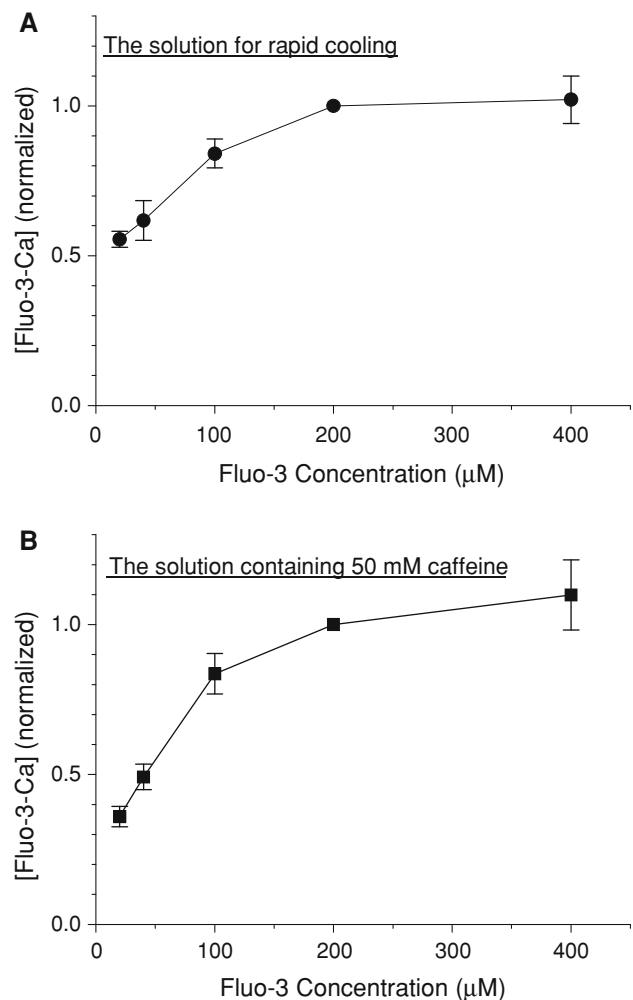
All experiments were performed in accordance with the Guidelines on Animal Experimentation of The Jikei University School of Medicine.

### Preparations

Male ferrets weighing 600–1,200 g were anaesthetised by intraperitoneal injection of pentobarbitone ( $150 \text{ mg kg}^{-1}$ ), and the hearts were quickly removed. The blood in the heart was washed out by retrograde perfusion of normal Tyrode's solution at  $30^\circ\text{C}$  through the aorta. Then, the right ventricle wall was quickly opened, and papillary muscles or trabeculae were dissected out in normal Tyrode's solution at  $30^\circ\text{C}$ . The papillary muscles or trabeculae were cut along the longitudinal axis in the relaxing solution with 10 mM EGTA and 4.6 mM ATP at  $4^\circ\text{C}$ . The diameter of the preparation was 180–305  $\mu\text{m}$  ( $241 \pm 5 \mu\text{m}$ , mean  $\pm$  standard error of the mean,  $n = 38$ ) and the length was 2.1–6.0 mm ( $3.8 \pm 0.2 \text{ mm}$ ,  $n = 38$ ). The preparation was tied at both ends to a tungsten wire (diameter 50  $\mu\text{m}$ , length 12 mm) with silk thread and treated with saponin (10  $\mu\text{g/ml}$ ) in the relaxing solution for 30–40 min. The saponin-treated preparation was then inserted into a glass capillary tube (internal diameter 500–600  $\mu\text{m}$ ) and fixed inside the capillary by bending the ends of the tungsten wire. The fluorescence signal was measured in the central portion (1 mm long) of the preparation.

### Experimental apparatus

The experimental apparatus used was slightly modified from the original apparatus shown in Fig. 1 of Kawai and Konishi [7]. The glass capillary containing the preparation was placed on an inverted microscope (IMT2-F4, Olympus, Tokyo, Japan). One end of the glass capillary was connected to a silicon tube as a solution inlet. The tube was passed through either one of two heat exchangers set at 24 and  $3^\circ\text{C}$ , and was connected to a step-motor-controlled



**Fig. 1** The relation between fluo-3 concentration and  $[\text{Ca} \cdot \text{Fluo-3}]$  in the presence or absence of 50 mM caffeine. After  $\text{Ca}^{2+}$  loading,  $\text{Ca}^{2+}$  was released from the SR into a glass capillary lumen by rapid cooling (a) or 50 mM caffeine (b). GOM1.5F and GORMOCaff solutions (see Table 1) were used for a and b, respectively, at  $3^\circ\text{C}$ . The fluorescence signal was measured with five different concentrations of fluo-3 and was calibrated in terms of  $\Delta[\text{Fluo-3-Ca}]$  (see Eq. 1 in text). Each symbol represents mean  $\pm$  SEM ( $n = 5$ )

multiposition (16 channels) valve (EMT-O-CSD16UWP-HC, Valco Instruments Co. Inc., Houston, TX, USA) for the selection of perfusing solution. A multiposition valve actuator for the rotation of ports was carried out using a personal computer (PC-9801NS/A, NEC, Tokyo, Japan). The other end of the capillary (a solution outlet) was linked to a peristaltic pump (Perista Bio-minipump, ATTO Co., Tokyo, Japan) via a silicon tube. The rate of solution flow was controlled by changing the rotor speed of the pump.

Excitation light from a xenon lamp (UXL-75XB, Ushio Inc., Tokyo, Japan) was passed through a 480-nm filter (excitation filter, half bandwidth 15 nm, RDF480, Omega Optical Inc., Brattleboro, VT, USA), and the fluorescence emitted from the capillary of a wavelength longer than

530 nm was detected through a barrier filter (emission filter, OG530, Melles Griot, Carlsbad, CA, USA) with a photomultiplier (R03-RB12M, Hamamatsu Photonics). The output of the photomultiplier was fed to a photometer system (P101/P102, Nikon Co., Tokyo, Japan). Storage and analysis of the data were carried out on a personal computer (PC-9801DX, NEC, Japan). The A/D converter, pump, pinch valve and shutter for the excitation light (EC-601, Copal, Tokyo, Japan) were controlled by the stimulator pulses of a preset sequence (DPS-1300D, Dia Medical System Co., Ltd., Tokyo, Japan).

Solutions and chemicals

The normal Tyrode’s solution used for dissection was as follows (mM): Na<sup>+</sup>, 135; K<sup>+</sup>, 5; Ca<sup>2+</sup>, 2; Mg<sup>2+</sup>, 1; Cl<sup>-</sup>, 102; HCO<sub>3</sub><sup>-</sup>, 20; HPO<sub>4</sub><sup>2-</sup>, 1; acetate, 20; glucose, 10; insulin, 5 units l<sup>-1</sup>; pH, 7.3–7.4 at 30°C when equilibrated with 5% CO<sub>2</sub> + 95% O<sub>2</sub>.

The composition of the relaxing solution used for cutting out the preparations and for treating the preparations with saponin was as follows (mM): K<sub>2</sub>ATP (adenosine 5'-triphosphate, dipotassium salt), 5.2; EGTA (ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid), 10.0; PIPES (piperazine-N-N'-bis (2-ethane sulfonic acid)), 20.0; Mg<sup>2+</sup>, 1.5; MgMs<sub>2</sub>, 5.6; KMs, 90.8; NaMs, 10.0; Leupeptin, 10 µg/ml; pH, 7.0 adjusted by KOH at 4°C.

The composition of the solutions used for measuring fluorescence is shown in Table 1. Ionic constituents were computed by solving multi-equilibrium equations using binding constants compiled by Martell and Smith [10]. All solutions contained 20 mM PIPES, 5 µM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and 10 µM DCB (2',4'-dichlorobenzamil hydrochloride). Ionic strength was

0.2 M. Loading and assay solutions contained 10–20 µg/ml Leupeptin. pH was adjusted to 7.0 by KOH at 3 or 24°C. K<sub>2</sub>ATP, AMP (adenosine 5'-monophosphate), K<sub>2</sub>AMP-PCP (β,γ-methyleneadenosine-5'-triphosphate), saponin and CCCP were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DCB and fluo-3 (pentapotassium salt) were purchased from Invitrogen (OR, USA). PIPES and caffeine were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), EGTA from Fluka Chemie AG of Sigma-Aldrich Co. and Leupeptin from Peptide Institute, Inc. (Osaka, Japan). All chemicals used were of analytical grade. Aequorin was purchased from Dr. J.R. Blinks.

Estimation of total Ca<sup>2+</sup> released from the SR

After RC, Ca<sup>2+</sup> was released from the SR to the capillary lumen filled with the “assay” solution containing 20 µM fluo-3 (G0M1.5F, Table 1). The fluorescence intensity of fluo-3 in the assay solution was low in the absence of Ca<sup>2+</sup> release from the SR (*F*<sub>0</sub>), but it rose to higher levels (*F*) after the onset of Ca<sup>2+</sup> release from the SR. To quantify the amount of released Ca<sup>2+</sup>, we used the method previously established in our laboratory [7]. At the end of each experiment, the assay solution plus 1 mM Ca<sup>2+</sup> was introduced into the capillary that contained the muscle preparation to estimate the fluorescence of Ca<sup>2+</sup>-bound fluo-3 (*F*<sub>max</sub>). The fluorescence of Ca<sup>2+</sup>-free fluo-3 (*F*<sub>min</sub>) was measured by introducing the assay solution plus 1 mM EGTA. *F*<sub>min</sub> was slightly lower than *F*<sub>0</sub> because of slight contamination of Ca<sup>2+</sup> in the assay solution (1.3 µM estimated with fluo-3). The change in fluo-3 fluorescence after Ca<sup>2+</sup> release (*F* – *F*<sub>0</sub>), could be calibrated in terms of the change in concentration of Ca<sup>2+</sup>-bound fluo-3 (Δ[Fluo-3 · Ca]) by the following equation:

**Table 1** Composition of solutions

Solution	K <sub>2</sub> ATP (mM)	AMP (mM)	EGTA (mM)	CaMs <sub>2</sub> (mM)	Mg <sup>2+</sup> (mM)	MgMs <sub>2</sub> (mM)	KMs (mM)	NaMs (mM)	Caffeine (mM)	Fluo-3 (µM)
Load: 24°C										
CaG1M1.5	4.1	0	1	0.4	1.5	5.5	117.5	10	0	0
Wash: 24°C										
G1R	0	0	1	0	1.5	1.6	143.9	10	0	0
G1OR	0	0	10	0	1.5	2.1	116.5	10	0	0
Pre-assay: 24°C										
G0RM1.5F	0	0	0	0	1.5	1.5	147	10	0	20
G0RM0F	0	0	0	0	0	0	151.5	10	0	20
Assay: 3°C										
G0M1.5F	4.6	0	0	0	1.5	5.5	120.7	10	0	20
G0RM0CafF	0	25	0	0	0	0	95.1	10	50	20

All solutions contained 10 mM PIPES, 5 µM CCCP and 10 µM DCB. Ionic strength was 0.2 M. Load and assay solutions contained 10–20 µg/ml leupeptin. pH was adjusted to 7.0 by KOH at 3 or 24°C. The meanings of the abbreviations are as follows: G0, 1, 10; 0, 1, 10 mM EGTA, M0, 1.5; 0, 1.5 mM free Mg<sup>2+</sup>, R; no ATP, Ca; pCa 6.5, Caf; 50 mM caffeine, F; 20 µM fluo-3

$$\Delta[\text{Fluo-3} \cdot \text{Ca}] = [\text{Fluo-3}]_{\text{tot}}(F - F_0)/(F_{\text{max}} - F_{\text{min}}), \quad (1)$$

where  $[\text{Fluo-3}]_{\text{tot}}$  was the total fluo-3 concentration in the assay solution and was 20  $\mu\text{M}$  in the present study.

The amount of  $\text{Ca}^{2+}$  released from the SR, defined as the change in total  $\text{Ca}^{2+}$  concentration in the capillary lumen ( $\Delta[\text{Ca}]_{\text{tot}}$ ), could be expressed as the sum of changes in concentrations of free  $\text{Ca}^{2+}$  ( $\Delta[\text{Ca}^{2+}]$ ),  $\text{Ca}^{2+}$  bound to fluo-3 ( $\Delta[\text{Fluo-3} \cdot \text{Ca}]$ ) and  $\text{Ca}^{2+}$  bound to other buffers (e.g., intrinsic intracellular buffers and ATP in the solution) ( $\Delta[\text{B} \cdot \text{Ca}]$ ):

$$\Delta[\text{Ca}]_{\text{tot}} = \Delta[\text{Ca}^{2+}] + \Delta[\text{Fluo-3} \cdot \text{Ca}] + \Delta[\text{B} \cdot \text{Ca}]. \quad (2)$$

When the fluo-3 concentration is high enough to predominantly bind released  $\text{Ca}^{2+}$  (i.e.,  $\Delta[\text{Fluo-3} \cdot \text{Ca}] \gg \Delta[\text{Ca}^{2+}] + \Delta[\text{B} \cdot \text{Ca}]$ ),  $\Delta[\text{Ca}]_{\text{tot}}$  could be approximated with  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$ . Figure 1a shows the relation between the fluo-3 concentration in the assay solution (G0M1.5F, Table 1) and  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$ . After the SR was fully loaded with  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  release was induced by RC. The calibrated  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$  increased as the fluo-3 concentration was increased up to 200  $\mu\text{M}$ , but was saturated at higher fluo-3 concentrations (200–400  $\mu\text{M}$ ). The result suggested that the fluo-3 concentration of 200  $\mu\text{M}$  was high enough to predominantly bind released  $\text{Ca}^{2+}$  from the SR and that  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$  obtained at high fluo-3 concentrations ( $\geq 200 \mu\text{M}$ ) could be an index of the amount of  $\text{Ca}^{2+}$  released from the SR (i.e.,  $\Delta[\text{Ca}]_{\text{tot}}$  expressed in units of  $\mu\text{M}$  in the capillary space). With 20  $\mu\text{M}$  fluo-3 primarily used in the present study for economic reasons, on the other hand,  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$  had to be scaled to attain the value that would be obtained if the fluo-3 concentration was sufficiently high ( $\geq 200 \mu\text{M}$ ). The scaling factor of 1.80 estimated from Fig. 1a was used in the following sections.

We used the other assay solution that contained 50 mM caffeine and 25 mM AMP (G0RM0CafF, Table 1) to release all  $\text{Ca}^{2+}$  in the SR. High concentrations of caffeine and AMP should maximise  $\text{Ca}^{2+}$  release, and the  $\text{Ca}^{2+}$  pump was expected to be inactive in the absence of ATP. Because caffeine significantly influences fluo-3 fluorescence and affinities of intracellular buffer sites for  $\text{Ca}^{2+}$ , we carried out a separate set of calibrations of fluo-3 fluorescence signals; at the end of each experiment,  $F_{\text{max}}$  and  $F_{\text{min}}$  were estimated by the introduction of the G0RM0CafF assay solution plus 1 mM  $\text{Ca}^{2+}$  and the assay solution plus 1 mM EGTA, respectively. The relation between the fluo-3 concentration and  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$  was constructed in the G0RM0CafF assay solution (Fig. 1b). Then  $\Delta[\text{Fluo-3} \cdot \text{Ca}]$  was calculated by Eq. 1 and was scaled by a factor of 2.78 (estimated from Fig. 1b) to yield  $\Delta[\text{Ca}]_{\text{tot}}$ .

## Measurement of free $\text{Ca}^{2+}$ concentration in the solutions for rapid cooling

The free  $\text{Ca}^{2+}$  concentration of the solution for RC (G0M1.5F, Table 1) was measured with two different methods. The first method used fluo-3 fluorescence measured in the glass capillary that contained the muscle preparation without loading SR with  $\text{Ca}^{2+}$ , as described below in the next section (protocol D1, Fig. 3). We calculated  $[\text{Ca}^{2+}]$  with

$$[\text{Ca}^{2+}] = K_d [(F_0 - F_{\text{min}})/(F_{\text{max}} - F_0)], \quad (3)$$

where  $K_d$  denotes a dissociation constant of fluo-3 for  $\text{Ca}^{2+}$ . For the value of  $K_d$ , we used 0.476  $\mu\text{M}$  determined previously at 22°C [7]. Note that little temperature dependence of the  $K_d$  value has been reported for  $\text{Ca}^{2+}$ -fluo-3 bindings [11].

The second method used a  $\text{Ca}^{2+}$  photoprotein, aequorin; aequorin luminescence of the G0M1.5F solution before application to the preparation was measured in vitro at 3°C in the absence of the preparation. Aequorin was dissolved in the solution containing 150 mM KCl and 10 mM PIPES at pH 7.0 to a final concentration of 150  $\mu\text{M}$ . The luminescence was converted to  $[\text{Ca}^{2+}]$  using the following equation [12];

$$[\text{Ca}^{2+}] = (L/L_{\text{max}})^{1/n} + (L/L_{\text{max}})^{1/n} \times (K_{\text{TR}} - 1) / (K_{\text{R}}(1 - (L/L_{\text{max}})^{1/n})) \quad (4)$$

where  $L$  is the aequorin light obtained from the solutions.  $L_{\text{max}}$  is the maximal light at saturating  $\text{Ca}^{2+}$ . For constants  $n$ ,  $K_{\text{R}}$  and  $K_{\text{TR}}$ , we used, respectively, 3.11,  $3.62 \times 10^6$  and 85.62 obtained previously at 3°C (see Methods in [6]).

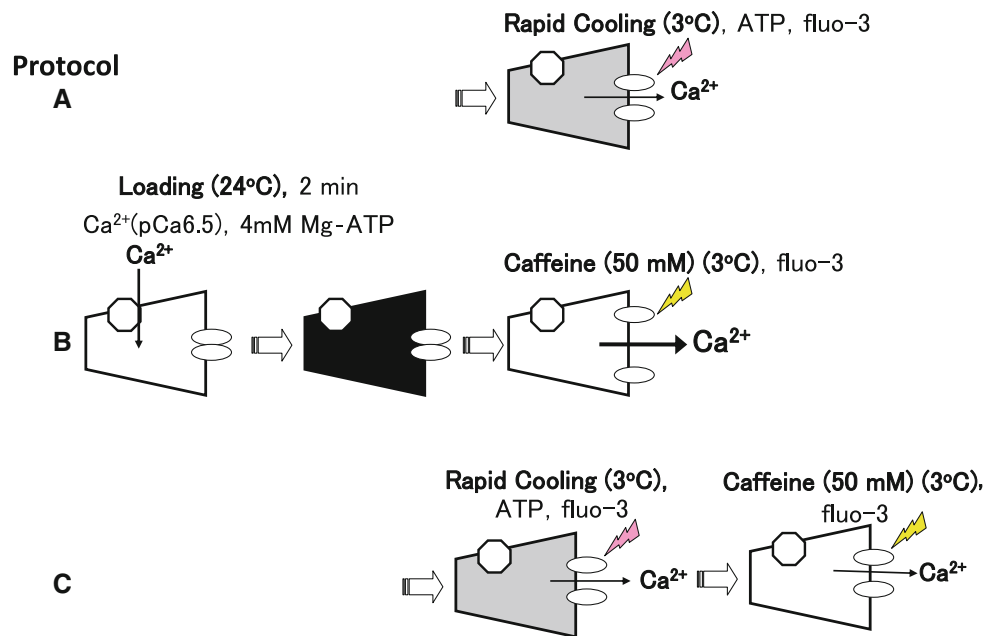
## Experimental protocol

SR was loaded with  $\text{Ca}^{2+}$  by incubation of the preparation at pCa 6.5 in the loading solution (CaG1M1.5, Table 1) for 2 min at 24°C, the condition optimised to achieve nearly full loading of the SR as in preliminary experiments (data not shown).

### 1. Measurements of the fractional $\text{Ca}^{2+}$ release by RC

*Protocol A* After loading SR with  $\text{Ca}^{2+}$  and washout of the  $\text{Ca}^{2+}$  in the preparation with the  $\text{Ca}^{2+}$ -free solution (G1R) at 24°C for 1 min, fluo-3 was introduced (G0RM1.5F), and the bathing solution temperature was lowered from 24 to 3°C within 3 s (G0M1.5F, Table 1).  $\text{Ca}^{2+}$  was released into a glass capillary (RC-induced  $\text{Ca}^{2+}$  release), and the fluorescent  $\text{Ca}^{2+}$  signal was measured (A in Figs. 2, 3).

*Protocol B* After  $\text{Ca}^{2+}$ -loading and washing the preparation in the same manner as in protocol A, fluo-3 was



**Fig. 2** Overview of protocols. After loading SR with  $\text{Ca}^{2+}$  at pCa 6.5 in the solution containing 4.1 mM ATP (CaG1M1.5, see Table 1) for 2 min at 24°C,  $\text{Ca}^{2+}$  release was triggered by rapid cooling (protocol A), 50 mM caffeine (protocol B), and rapid cooling followed by 50 mM caffeine (protocol C).  $\text{Ca}^{2+}$  released from the SR was bound to fluo-3 in a glass capillary lumen, and the resultant fluorescence signal was measured as described in the text. Protocol A Temperature of a solution was lowered from 24 to 3°C within 3 s with

simultaneous application of 4.6 mM ATP and 20  $\mu\text{M}$  fluo-3 (G0M1.5F, see Table 1). Protocol B A solution that contained 50 mM caffeine, 25 mM AMP and 20  $\mu\text{M}$  fluo-3 (G0RM0CafF, see Table 1) was applied at 3°C. Protocol C Rapid cooling (temperature of G0M1.5F lowered to 3°C) and 50 mM caffeine (G0RM0CafF) were sequentially applied. The meanings of *dark* and *shaded* trapezoids are full  $\text{Ca}^{2+}$  and decreased  $\text{Ca}^{2+}$  in SR, respectively

introduced (G0RM0F, Table 1), and the total  $\text{Ca}^{2+}$  content in SR was released by 50 mM caffeine at 3°C (B in Figs. 2, 3).

**Protocol C** When 50 mM caffeine was applied to the preparation following RC-induced  $\text{Ca}^{2+}$  release, residual  $\text{Ca}^{2+}$  in SR was measured (C in Figs. 2, 3). In protocol C, the preparation was washed for 1.5 min (by G10R for 1 min and by G1R for 30 s) between RC and the application of 50 mM caffeine.

Protocols D1 and D2 lack SR  $\text{Ca}^{2+}$  loading steps, but otherwise were very similar to protocols A and B, respectively (Fig. 3).

For estimation of the amount of  $\text{Ca}^{2+}$  released by RC or caffeine, the fluo-3 fluorescence signals obtained without SR  $\text{Ca}^{2+}$  loading (protocol D1 for RC, and protocol D2 for caffeine) were subtracted from the fluorescence signals obtained with SR  $\text{Ca}^{2+}$  loading (protocol A or C for RC; protocol B or C for caffeine). We counted an average of 5 s around the peak of the subtracted signal.

The fractional SR  $\text{Ca}^{2+}$  release by RC was calculated using the values obtained from protocol A and B.

## 2. Effect of adenine nucleotides on $\text{Ca}^{2+}$ release by RC

Protocol A was modified to study effects of ATP on RC-induced  $\text{Ca}^{2+}$  release. For removal of ATP, the assay solution of protocol A (G0M1.5, Table 1) was replaced by

G0RM1.5 that did not contain ATP. When AMP-PCP, a non-hydrolysable ATP analogue, was used, 4.6 mM  $\text{K}_2\text{ATP}$  in the assay solution (G0M1.5F) was simply substituted with 4.6 mM  $\text{K}_2\text{AMP-PCP}$ .

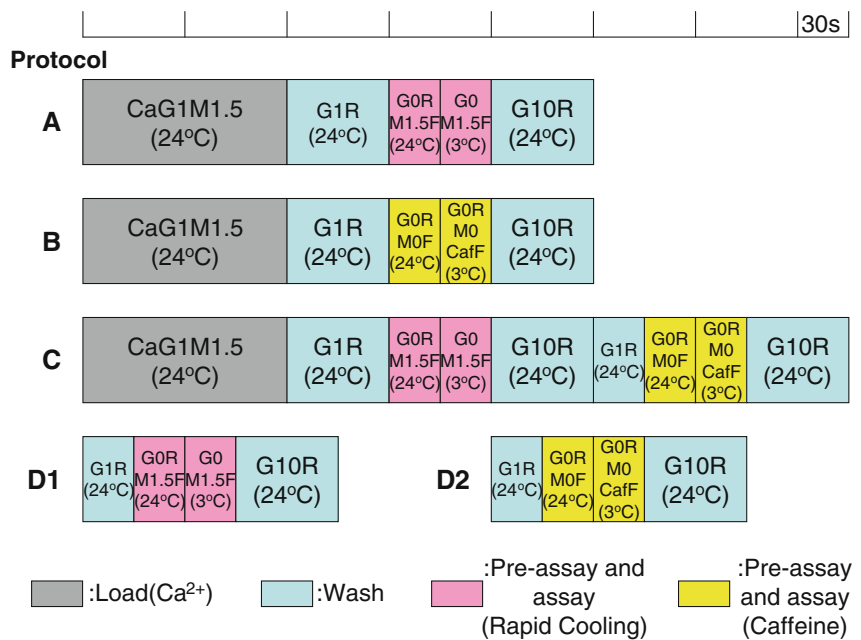
## 3. Measurement of the amount of $\text{Ca}^{2+}$ released by the CICR mechanism at 3°C

The protocol was identical to protocol A (above), except for modifications of the solution temperatures. Following  $\text{Ca}^{2+}$  loading in SR at 24°C for 2 min, the preparation in a capillary was washed with the  $\text{Ca}^{2+}$ -free solution (G1R, Table 1), which contains neither ATP nor  $\text{Ca}^{2+}$ , for 1 min. During this period, the temperature of the solution was slowly lowered from 24 to 3°C. After introduction of fluo-3 at 3°C (G0RM1.5F, Table 1), the solution for RC containing ATP and fluo-3 (G0M1.5F, Table 1) was applied at 3°C to the pre-cooled preparation. The change of fluo-3 fluorescence in a glass capillary was measured as described above for protocol A.

## Statistical analysis

We used two-tailed Student's *t* test, and a significant difference of  $p < 0.05$  was verified. Statistical values were given as mean  $\pm$  SEM.

**Fig. 3** Timetable for solution exchange. Protocol A–C correspond to, respectively, protocol A–C in Fig. 2. Protocol D1 and D2, which lack the Ca<sup>2+</sup> loading step, provide the “background” for the RC and caffeine assays, respectively



## Results

Free Ca<sup>2+</sup> concentration in the solution for rapid cooling

We measured the contaminated free Ca<sup>2+</sup> concentration in the solution used for RC containing 4.6 mM ATP and 20 μM fluo-3 with either fluo-3 or aequorin. The average free Ca<sup>2+</sup> concentration was estimated to be 19 ± 2 nM (*n* = 11) measured with fluo-3 (protocol D1 in Fig. 3) and 20 ± 2 nM (*n* = 3) with aequorin at 3°C. There was no significant difference between the two values measured with fluo-3 and that with aequorin. Thus, the free Ca<sup>2+</sup> concentration around SR was about 20 nM (pCa 7.7) before Ca<sup>2+</sup> release was induced by RC.

Fractional Ca<sup>2+</sup> release induced by rapid cooling

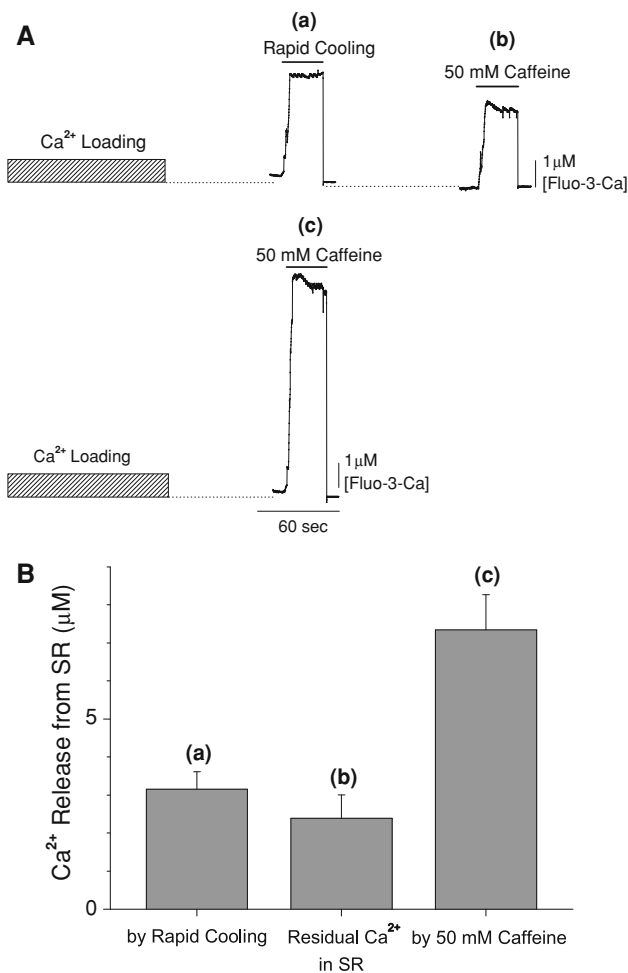
Figure 4a shows typical traces of the fluorescence measurements with protocols C (upper) and B (lower) (Figs. 2, 3) obtained from the same preparation. After Ca<sup>2+</sup> loading of the SR, RC induced a large increase in the [Fluo-3-Ca] in the capillary space (a). The preparation was briefly washed with Ca<sup>2+</sup>-free solutions (G10R and G1R, see protocol C in Fig. 3), and a subsequent application of 50 mM caffeine (plus 25 mM AMP) caused a substantial increase in [Fluo-3-Ca] (b), indicating that the SR still contained a significant amount of Ca<sup>2+</sup> after RC. When the SR was reloaded with Ca<sup>2+</sup> and the caffeine assay (50 mM caffeine plus 25 mM AMP) was repeated without RC in the same preparation, an increase in [Fluo-3-Ca] was greater by about a factor of two than that induced by RC (c).

From pooled data shown in Fig. 4b, the amount of SR Ca<sup>2+</sup> released by RC (a) was, on average, 3.16 ± 0.46 μM. On the other hand, the residual SR Ca<sup>2+</sup> after RC (b) was, on average, 2.39 ± 0.61 μM. This value was significantly smaller (*p* < 0.01) than the 4.19 ± 0.49 μM predicted by the total amount of releasable Ca<sup>2+</sup> (*c* = 7.34 ± 0.93 μM) minus the amount Ca<sup>2+</sup> released by RC (a). This difference is likely due to Ca<sup>2+</sup> leakage from the SR into solutions that contained EGTA during 1.5 min washing periods inserted between RC and caffeine applications (see Fig. 3, protocol C). In saponin-treated cardiac muscles of mice, Morimoto et al. [13] reported a substantial Ca<sup>2+</sup> leakage from the SR; about 50% of the SR Ca<sup>2+</sup> content was lost when the preparation was perfused with the solution that contained 1 mM EGTA for 1.5 min at 22°C.

Thus, the fraction of Ca<sup>2+</sup> released by RC was, on average, 44.7 ± 1.7% of the total SR Ca<sup>2+</sup> content in 16 preparations.

Rapid cooling with ATP opens the considerable SR Ca<sup>2+</sup> release channels

With the standard solution containing 4.6 mM ATP (G0M1.5F, Table 1), a considerable amount of Ca<sup>2+</sup> was released from the SR by RC (Fig. 5a), whereas in the absence of ATP (G0RM1.5F, Table 1) RC caused a much a smaller Δ[Fluo-3-Ca] signal (Fig. 5b), suggesting that Ca<sup>2+</sup> release channels of SR hardly opened. The amount of Ca<sup>2+</sup> released by RC in the presence and absence of ATP were 2.06 ± 0.27 and 0.41 ± 0.09 μM, respectively (Fig. 5c).



**Fig. 4** The fraction of SR  $\text{Ca}^{2+}$  released by rapid cooling. **A** Examples of fluorescence traces obtained with protocols C (*upper*) and B (*lower*). Fluo-3 fluorescence signals have been calibrated in terms of [Fluo-3-Ca]. With protocol C (*upper traces*), rapid cooling caused a rise of [Fluo-3-Ca] (*a*). After a brief wash with  $\text{Ca}^{2+}$ -free solutions, subsequent application of 50 mM caffeine also caused an increase of [Fluo-3-Ca] (*b*) that reflected the release of residual  $\text{Ca}^{2+}$  in SR. With protocol B (*lower trace*), a large increase in [Fluo-3-Ca] was observed with the application of the solution containing 50 mM caffeine (*c*). At the end of assay periods in *a-c* (indicated by horizontal bars), rapid decreases of [Fluo-3-Ca] were due to closing an optical shutter, which terminated the fluorescence recordings. **B** Columns *a-c* summarise the  $\text{Ca}^{2+}$  release from SR ( $\Delta[\text{Fluo-3-Ca}]$ ) obtained from the type of experiment shown in *a-c*, respectively, in **A**. Columns show mean  $\pm$  SEM of five preparations

#### Rapid cooling-induced $\text{Ca}^{2+}$ release in the presence of ATP or AMP-PCP

The SR  $\text{Ca}^{2+}$  pump still works (albeit slowly) in the presence of ATP at  $3^\circ\text{C}$  [14]. Therefore, SR could reuptake a considerable part of the  $\text{Ca}^{2+}$  released by RC and might decrease the free  $\text{Ca}^{2+}$  concentration in the glass capillary lumen. Inhibition of the SR  $\text{Ca}^{2+}$  pump could lead to an increase in the free  $\text{Ca}^{2+}$  concentration around SR and

might trigger the CICR. In order to examine a possible role of CICR in the RC-induced  $\text{Ca}^{2+}$  release, we compared the fraction of SR  $\text{Ca}^{2+}$  released by RC in the presence of ATP and AMP-PCP, a non-hydrolysable ATP analogue. The fraction of  $\text{Ca}^{2+}$  released by RC was  $44.7 \pm 1.7\%$  with 4.6 mM ATP (see above) and was  $45.6 \pm 8.2\%$  with 4.6 mM AMP-PCP (Fig. 6). There was no difference between these two values. The present results suggest that the RC-induced  $\text{Ca}^{2+}$  release is not influenced by hydrolysis of ATP, in other words, by the activity of SR  $\text{Ca}^{2+}$  pump.

#### The $\text{Ca}^{2+}$ -induced $\text{Ca}^{2+}$ release at $3^\circ\text{C}$

After  $\text{Ca}^{2+}$  loading in the SR, the temperature of the solution for the wash (G1R), which contains neither ATP nor  $\text{Ca}^{2+}$ , was slowly lowered from  $24$  to  $3^\circ\text{C}$  for 1 min, and then the solution for RC (containing 4.6 mM ATP and  $\sim 20$  nM free  $\text{Ca}^{2+}$ ) was applied at  $3^\circ\text{C}$  to the preparation (slow cooling). Then, we measured the change of  $\text{Ca}^{2+}$  concentration in a glass capillary. The typical data are shown in Fig. 7, and the mean value was  $0.23 \pm 0.10$   $\mu\text{M}$  ( $n = 5$ ).

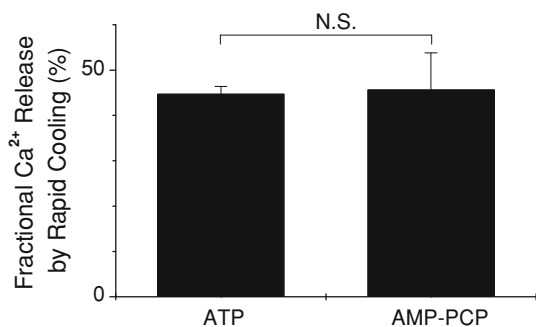
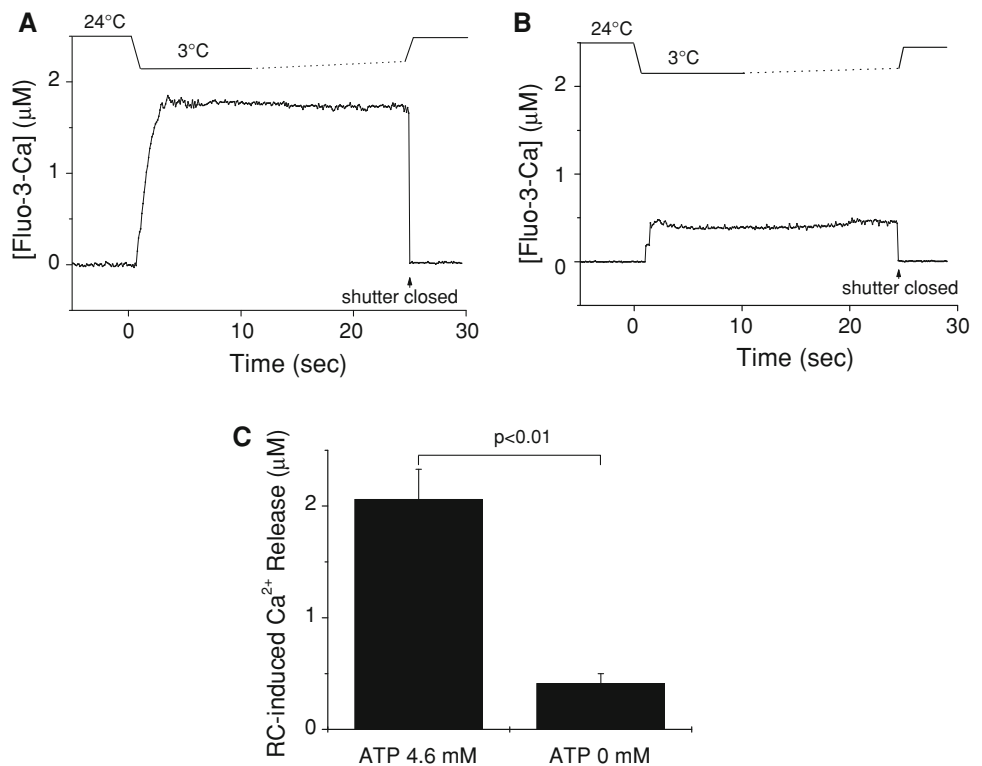
Because the temperature around the preparation was kept constant at approximately  $3^\circ\text{C}$ , the small  $\text{Ca}^{2+}$  release induced by the solution exchange was not due to cooling. Rather, it was thought that  $\text{Ca}^{2+}$  was released from the SR by changes in ATP and free  $\text{Ca}^{2+}$  concentrations (i.e., CICR). Aside from the initial rise, the [Fluo-3-Ca] signal also showed a gradual rise that started about 10 s after the solution exchange (Fig. 7). We might speculate that the CICR mechanism was gradually accelerated as the free  $\text{Ca}^{2+}$  concentration around the SR increased.

## Discussion

### Role of $\text{Ca}^{2+}$ in the rapid cooling-induced $\text{Ca}^{2+}$ release

In frog skeletal muscle, a slight increase in the intracellular  $\text{Ca}^{2+}$  concentration around the SR before RC was required for the RC-induced  $\text{Ca}^{2+}$  release. To increase the intracellular  $\text{Ca}^{2+}$  concentration before RC, a low concentration of caffeine was employed because the RC itself could not sufficiently increase the intracellular  $\text{Ca}^{2+}$  concentration for triggering a considerable  $\text{Ca}^{2+}$  release in skeletal muscles [4]. Therefore, the RC-induced  $\text{Ca}^{2+}$  release in skeletal muscles was assumed to be due to the enhancement of the CICR mechanism [5]. During RCC in frog skeletal muscle, the change in the aequorin light signal showing the intracellular  $\text{Ca}^{2+}$  concentration occurred in three phases. The first phase was a transient change of intracellular  $\text{Ca}^{2+}$  concentration accompanying slight

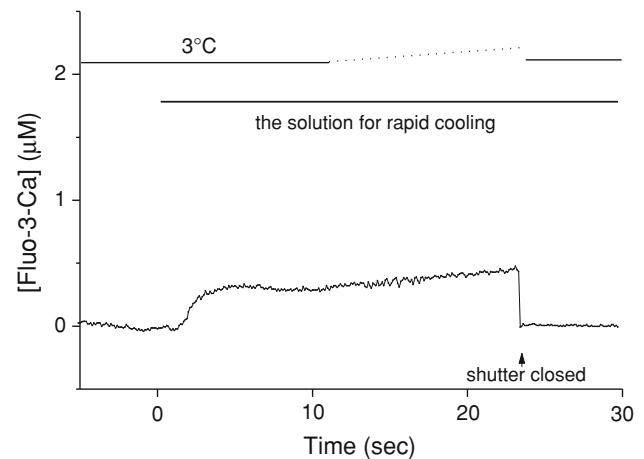
**Fig. 5** Rapid cooling-induced  $\text{Ca}^{2+}$  release in the presence or absence of ATP. **a, b** Traces show changes in solution temperature (*top*) and [Fluo-3-Ca] (*bottom*) in the presence (**a**) or absence (**b**) of ATP. The fluorescence recording was terminated by closing an optical shutter at the times indicated by *arrows*. **c** Summary of RC-induced  $\text{Ca}^{2+}$  release ( $\Delta[\text{Fluo-3-Ca}]$ ) estimated in the type of experiments shown in **a** and **b**. *Left and right columns* represent mean  $\pm$  SEM of data obtained, respectively, in the presence ( $n = 16$ ) and in the absence ( $n = 7$ ) of ATP



**Fig. 6** Fraction of SR  $\text{Ca}^{2+}$  released by rapid cooling in the presence of either ATP or AMP-PCP.  $\text{Ca}^{2+}$  release was induced by rapid cooling in the presence of 4.6 mM ATP (*left*,  $n = 16$ ) or in the presence of 4.6 mM AMP-PCP (*right*,  $n = 4$ ). The amount of  $\text{Ca}^{2+}$  released by rapid cooling was normalised to the total amount of releasable  $\text{Ca}^{2+}$  in the SR (*ordinate*). Each bar represents the mean  $\pm$  SEM

tension. During the second phase, the light signal slowly increased as cooling produced maximum tension development. The third phase was an additional large light signal induced after the second phase, even though the tension was saturated. The second and third phases were more sensitive to low concentrations of procaine, an effective inhibitor for CICR, than the first phase [4].

On the other hand, pretreatment of the preparation with caffeine is not required for the initiation of RCC in mammalian cardiac and smooth muscles [2, 3]. In contrast to RCC in skeletal muscles, the amount of  $\text{Ca}^{2+}$  released by



**Fig. 7**  $\text{Ca}^{2+}$  release from the SR induced by the solution for rapid cooling at 3°C. After  $\text{Ca}^{2+}$  loading of the SR, the temperature of the solution for the wash (G1R, Table 1) and the pre-assay solution (G0RM1.5F, Table 1) was gradually lowered from 24 to 3°C, and then the assay solution that contained ATP and fluo-3 (G0M1.5F) was applied at 3°C to the pre-cooled preparation as indicated at the top. Fluo-3 fluorescence signals were measured and were calibrated in terms of [Fluo-3-Ca]. An optical shutter was closed at a time indicated by an *arrow*

RC in ferret ventricular muscles was not influenced by the intracellular  $\text{Ca}^{2+}$  concentration before RC [6].

In the present study, in skinned cardiac muscles, the free  $\text{Ca}^{2+}$  concentration in the solution used for RC (G0M1.5F, see Table 1) was about 20 nM when estimated with either fluo-3 or aequorin, which seems lower than the level



required to activate the CICR. It has been reported that the CICR is hardly activated at  $\text{Ca}^{2+}$  concentrations below 100 nM (i.e.,  $\text{pCa} < 7$ ) [15–17]. Thus, the contribution of CICR may be minor, if it exists at all, for the initiation of  $\text{Ca}^{2+}$  release induced by RC. This is supported by two lines of evidence. (1) When the preparation was perfused with the solution containing 20 nM free- $\text{Ca}^{2+}$  and 4.6 mM ATP (G0M1.5F, Table 1) with the temperature set constant at 3°C (Fig. 6), the [Fluo-3-Ca] signal rose slowly with the delayed onset (~5 s after the solution exchange), and the [Fluo-3-Ca] signal was, on average,  $0.23 \pm 0.10 \mu\text{M}$ . This value was only about 10% of that achieved by RC ( $2.06 \pm 0.27 \mu\text{M}$ ). (2) The SR  $\text{Ca}^{2+}$  pump still works, albeit slowly, in the presence of ATP even at lower temperatures [14]. When the SR  $\text{Ca}^{2+}$  pump is completely inhibited (by replacement of ATP with AMP-PCP), the CICR may be enhanced because of the local increase in the free  $\text{Ca}^{2+}$  concentration around the SR. The fractional  $\text{Ca}^{2+}$  release induced by RC was unchanged even when ATP was replaced by AMP-PCP. Overall, the results suggest that CICR might not play an important role in the RC-induced  $\text{Ca}^{2+}$  release.

#### Mechanisms of opening the $\text{Ca}^{2+}$ release channels by rapid cooling

Global conformational changes upon binding ligands were observed with the gap junction protein [18] and with the nicotinic acetylcholine receptor [19]. Orlova et al. [20] demonstrated the three-dimensional structure of the rabbit skeletal muscle  $\text{Ca}^{2+}$  release channel in an open state using electron cryomicroscopy and angular reconstitution. In contrast to its closed state, in the open state reconstruction, a central cavity was revealed in the transmembrane region of the channel in the presence of  $\text{Ca}^{2+}$  and ryanodine. The opening of the channel is associated with a 4° rotation of its transmembrane region with respect to its cytoplasmic region and with significant mass translocations within the entire cytoplasmic region of the channel tetramer.

RC alone hardly opens the SR  $\text{Ca}^{2+}$  release channels. In the present study, RC could release considerable  $\text{Ca}^{2+}$  when ATP or AMP-PCP co-existed (Figs. 5, 6). Sitsapesan et al. [21] also confirmed that the SR  $\text{Ca}^{2+}$ -release channels in the artificial lipid bilayer were not activated at low temperatures in the absence of cytosolic  $\text{Ca}^{2+}$  or a cardiotoxic agent, sulmazole. Another study revealed that the presence of 10  $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  and 100  $\mu\text{M}$  cytosolic ATP increased the mean open probability from 0.052 to 0.284 in sheep cardiac ryanodine receptor channels incorporated into planar phospholipid bilayers [22].

At low temperature and in the presence of  $\text{Ca}^{2+}$  [21], ryanodine, caffeine and adenine compounds [22], the  $\text{Ca}^{2+}$  release channels seem to be stable in an open state

compared with that in closed state. In other words, low temperature is considered one of the so-called opening factors on the SR  $\text{Ca}^{2+}$  release channels. In cardiac, skeletal and smooth muscles, in the presence of one or more of those opening factors, the  $\text{Ca}^{2+}$  release channels might be stable in an open state. In addition, it was considered that the rapid lowering of the temperature may be important to synchronize the opening of a large population of  $\text{Ca}^{2+}$  release channels on SR membranes.

#### Fractional amount of $\text{Ca}^{2+}$ release by rapid cooling

Bers et al. measured intracellular  $\text{Ca}^{2+}$  transients during RCCs in guinea pig ventricular myocytes using the fluorescent  $\text{Ca}^{2+}$  indicator, Indo-1. They demonstrated that RC of myocytes from 22 to 0–1°C could release a large amount of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i > 10 \mu\text{M}$ ) from the SR and suggested the use of RCCs as a useful means of assessing the SR  $\text{Ca}^{2+}$  content in intact cardiac muscle cells [23].

In our previous study, we estimated the fraction of SR  $\text{Ca}^{2+}$  released by the RC in intact papillary muscles of ferrets [6]. The intracellular free  $\text{Ca}^{2+}$  concentration was measured with aequorin, and  $\text{Ca}^{2+}$  release was first induced by RC (from 30 to 4°C) and subsequently by 15 mM caffeine. This experimental protocol was somewhat similar to that employed in the present study (Fig. 4a, upper). Both RC and subsequent application of caffeine caused transient rises in  $[\text{Ca}^{2+}]_i$ , and the peak changes in  $[\text{Ca}^{2+}]_i$  induced by RC and caffeine averaged, respectively, 1.59 and 0.90  $\mu\text{M}$  [6]. From these results, the fraction of SR  $\text{Ca}^{2+}$  released by RC was calculated to be 64% [=1.59/(1.59 + 0.9) × 100], which was higher than that estimated in the present study (44.7%; see “Results”). The difference may be attributed to the experimental methods employed in the previous study, in which peaks of  $\Delta[\text{Ca}]_i$  measured with or without 15 mM caffeine were directly compared. The increase in apparent  $\text{Ca}^{2+}$  sensitivity was produced by caffeine in skinned cardiac and skeletal muscle fibres of the rat [24]. Furthermore, the complex of troponin (Tn) C with Tn I and Tn T resulted in an increase in the affinity of Tn C for  $\text{Ca}^{2+}$  in the presence of caffeine in the bovine heart [25]. Therefore, the amount of Ca bound to Tn C should be greater at any given  $[\text{Ca}^{2+}]_i$ . It follows that the use of  $\Delta[\text{Ca}]_i$  measured during caffeine application would erroneously underestimate the total amount of  $\text{Ca}^{2+}$  released from the SR ( $\Delta[\text{Ca}]_{\text{tot}}$ ), unless an appropriate correction was made. Consequently, a fraction of  $\Delta[\text{Ca}]_{\text{tot}}$  induced by RC would have been overestimated in the previous study. In the present study, on the other hand,  $\Delta[\text{Ca}]_{\text{tot}}$  was deduced by scaling  $\Delta[\text{Fluo-3} \cdot \text{Ca}]_{\text{tot}}$  with factors separately estimated in the presence (2.78) and in the absence (1.80) of 50 mM caffeine (Fig. 1). Thus, we consider that the revised value in the present study (44.7%) is more

relevant as an estimate of the fraction of SR  $\text{Ca}^{2+}$  released by RC.

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