Contribution of Mitochondria to the Removal of Intracellular Ca^{2+} Induced by Caffeine and Rapid Cooling at Low Temperatures in Ferret Ventricular Muscles

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Abstract: We investigated the role of mitochondria in the removal of intracellular Ca^{2+} which was increased by caffeine (15 mM, Caf), rapid lowering of the solution temperature from 30 to 4°C (rapid cooling, RC), and electrical stimulation (0.07 Hz, ES). For this purpose, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured using aequorin from the superficial cells of ferret papillary muscles. The three maneuvers induced transient changes in $[\text{Ca}^{2+}]_i$ with different time courses. The decay time of the aequorin light signal (DT) in the Caf-induced Ca^{2+} release was significantly prolonged by the inhibitors for Na^+ - Ca^{2+} exchanger (Ni^{2+} , Na^+ -free solution) at higher temperatures ($\geq 12^\circ\text{C}$). In the caffeine application at lower temperatures ($\leq 12^\circ\text{C}$), the inhibitors for mitochondria (ruthenium red, NaN_3)

significantly prolonged the DT but other inhibitors were ineffective. In the RC-induced Ca^{2+} release, DT was significantly prolonged by the mitochondrial inhibitors but other inhibitors were not effective. In the ES-induced Ca^{2+} release, each inhibitor for the sarcoplasmic reticulum (SR) (thapsigargin and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone) prolonged the DT at all temperatures. The inhibitors for Na^+ - Ca^{2+} exchanger slightly prolonged the DT only at higher temperatures, and the mitochondrial inhibitors did not alter the DT at any temperature. These results suggest that mitochondria substantially transport Ca^{2+} when the Ca^{2+} uptake by the SR and Na^+ - Ca^{2+} exchanger are inhibited and $[\text{Ca}^{2+}]_i$ is increased with a slower time course. [Japanese Journal of Physiology, 47, 251–262, 1997]

Key words: mitochondria, intracellular Ca^{2+} , aequorin, caffeine, rapid cooling.

In mammalian cardiac muscles, contraction is triggered by an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Some of the increased intracellular Ca^{2+} which initiate contractions are bound to Ca^{2+} binding proteins. At the same time, Ca^{2+} is removed by Ca^{2+} removal mechanisms (sarcoplasmic reticulum (SR), Na^+ - Ca^{2+} exchanger, and Ca^{2+} pump in the cell membrane), and then a decrease in $[\text{Ca}^{2+}]_i$ precedes relaxation [1, 2]. In the physiological contraction-relaxation cycle, the SR and Na^+ - Ca^{2+} exchanger are primarily responsible for the removal of intracellular Ca^{2+} and $[\text{Ca}^{2+}]_i$ is transiently altered (Ca^{2+} transient). The time course of the decay of the Ca^{2+} transients differs under various conditions which alter the

activity of each Ca^{2+} removal mechanism and the affinity of the Ca^{2+} binding proteins [3]. Mitochondria occupy approximately 30% of the cytosolic volume in mammalian cardiac muscle and have a large capacity to uptake intracellular Ca^{2+} [4]. Therefore, mitochondria have been considered to contribute to the removal of intracellular Ca^{2+} under pathophysiological conditions in which the Ca^{2+} removal mechanisms except mitochondria are apparently inhibited and $[\text{Ca}^{2+}]_i$ is significantly increased [5, 6]. A recent study suggests that mitochondria can accumulate Ca^{2+} even under physiological conditions [7]. However, the details of the interrelation of these intracellular Ca^{2+} removal mechanisms (SR, Na^+ - Ca^{2+} exchanger, and mito-

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chondria) are not fully understood, although the characteristics of the mitochondrial Ca^{2+} transport mechanism have been extensively studied [8–10].

The aim of this study is to clarify the conditions under which the mitochondria play a significant role in the removal of intracellular Ca^{2+} . For this purpose, we used aequorin as the Ca^{2+} indicator and measured the change in $[\text{Ca}^{2+}]_i$, which is increased with different maneuvers (caffeine (Caf), rapid cooling (RC), and electrical stimulation (ES)) at different temperatures; the former two maneuvers (Caf and RC) directly and continuously release Ca^{2+} from the SR [11–15] and the time courses of the decay of the Ca^{2+} signal during Caf and RC stimulation are slower compared to that in ES at the same temperature. This is due to the continuous Ca^{2+} release from the SR and the apparent inhibition of Ca^{2+} removal by the SR [12, 14, 15] (see DISCUSSION for details).

A portion of these results has already been presented in abstract form [16–18].

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 . The right ventricle wall was opened and papillary muscles were quickly dissected out at 30°C . The diameter of the preparation was 0.4–1.5 mm ($0.8 \pm 0.02 \text{ mm}$, mean $\pm$ standard error of the mean, $n=68$) and the length was 2.2–5.8 mm ($4.1 \pm 0.1 \text{ mm}$, $n=68$). The preparation was mounted horizontally 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 (BG10, Kulite, NJ, USA). The preparation was stimulated by a square pulse with a 5 ms duration at 1.5-fold threshold unless otherwise stated. Before starting the experiment, the preparation was slowly stretched to the length which produced the maximal tension of the preparation.

Solutions. The normal Tyrode's solution used for dissection and aequorin injection was composed of the following (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; and insulin, 5 units $\cdot \text{l}^{-1}$ with a pH of 7.3–7.4 at 30°C equilibrated with 5% CO_2 +95% O_2 . The Tyrode's solution buffered with N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), which was used in the experiments (HEPES-Tyrode's solu-

tion), 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 saturated with 100% O_2 . When the extracellular Na^+ concentration ($[\text{Na}^+]_o$) was zero, NaCl was isotonicity replaced by LiCl or tetramethyl ammonium chloride (TMACl).

Aequorin injection, light detection, and conversion to $[\text{Ca}^{2+}]_i$. For measuring $[\text{Ca}^{2+}]_i$, we used aequorin (purchased from Dr. J. R. Blinks), which 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. After completion of the injection, aequorin light signals were detected with a photomultiplier (EMI 9789A, Ruislip, U.K.) placed immediately above the preparation, and the signals were simultaneously recorded with tension. In this study, the tension record was not shown because Caf and RC cannot simultaneously activate all of the cells in the preparation, and tension did not reflect the contraction of the whole preparation. Therefore, although the absolute value of developed tension per cross sectional area was not measured, relative change of tension was compared. Both maneuvers sufficiently released Ca^{2+} in the superficial cells injected with aequorin, and the light signals of aequorin could be used as an indicator for the change in $[\text{Ca}^{2+}]_i$. Light signals induced by RC and Caf were recorded through 1 and 10 Hz low-pass filters, respectively. Light signals induced by ES at $4\text{--}7^\circ\text{C}$ were recorded through a 10 Hz low-pass filter. At other temperatures, light signals were not filtered.

In the figures, the changes in $[\text{Ca}^{2+}]_i$ are shown as the aequorin light signal, and the time courses of the light signals induced with different maneuvers were compared in the presence or absence of inhibitors for each Ca^{2+} removal mechanism. The change in the time course of the light signal was used to identify the main mechanism responsible for Ca^{2+} removal from the myoplasm. In some experiments, we converted the aequorin light signal to $[\text{Ca}^{2+}]_i$ using the following equation [19] for quantitative consideration;

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

where n , K_R , and K_{TR} are constants calculated using the relation between aequorin luminescence and the

Ca²⁺ concentration ($[Ca^{2+}]$). L/L_{max} is the normalized aequorin light in which the peak light at different $[Ca^{2+}]$ (L) was normalized to the maximal light (L_{max}) obtained at saturated $[Ca^{2+}]$. L_{max} was calculated using the rate constant for the decay of aequorin light at saturated $[Ca^{2+}]$ after discharging all of the aequorin in the cells in HEPES-Tyrod's solution at the end of each experiment [20]. As aequorin luminescence is markedly influenced by temperature, we measured the relation between aequorin luminescence and $[Ca^{2+}]$ in a cuvette at 30 and 4°C. The method for measuring the aequorin calibration curve has already been reported elsewhere [19, 20]. The constants used for the conversion of the light signal to $[Ca^{2+}]$ were calculated using the calibration curves: n , K_R , and K_{TR} are 3.51, 3.17×10^6 , 67.5 at 30°C and 3.03, 15.94×10^6 , 255.3 at 4°C, respectively. Caf (20 mM) did not significantly affect aequorin luminescence.

Protocol. The preparation was regularly stimulated at different frequencies depending on the temperature to accumulate Ca²⁺ in the SR, and then Caf (15 mM) was rapidly applied at different temperatures. For RC, we applied repetitive ES (2 Hz for 5 min) to the preparation at 30°C, which was for accumulation of Ca²⁺ in the SR. After loading Ca²⁺ in the SR, the temperature of the bathing solution was rapidly (<1 s) lowered from 30 to 4°C. This RC method is known to directly release Ca²⁺ from the SR [11, 13, 21]. ES (0.07 Hz) was applied to the preparation at different temperatures to compare the time courses of the decay of the Ca²⁺ signals induced by RC and Caf application.

Two inhibitors for each Ca²⁺ removal mechanism were used independently to identify and confirm the main mechanism which functions at different temperatures: for Na⁺-Ca²⁺ exchanger, NiCl₂ (10 mM, Ni) [22, 23] and Na⁺-free solution (Li⁺ or TMA⁺ replacement, Na⁺-free) [24]; for SR, thapsigargin (0.5 μM, Thap) [25] and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (10 μM, TBQ) [26]; for mitochondria, ruthenium red (50 μM, RR) [27] and NaN₃ (2 mM, NN) [28]. The preparations were treated with the inhibitors for 20 to 120 min by applying ES (0.2 Hz) regularly before recording, which equilibrated the preparation with the inhibitors.

The decay time of the aequorin light signal to decrease from the peak to 37% of the peak (63% decay time) was measured. The zero level of the light signal was measured when a shutter was closed. The decay times of the light signal measured in the presence or absence of the inhibitors were compared by normalizing the peaks of the two signals to identify the primary Ca²⁺ removal mechanism. In noisy light signals,

the peak was determined as the highest point after smoothing the signal. If two peaks were recognized in the light signal, the midpoint was employed for the peak. Furthermore, when 63% decay time lasted far beyond the end of the record, 63% decay time was the time measured from the peak to the end of the record (≤ 140 s for Caf, ≤ 30 s for RC).

Chemicals. The following chemicals were used: caffeine (Nacalai Tesque Inc., Kyoto, Japan), NiCl₂ (Nacalai Tesque Inc.), thapsigargin (Thap) (Calbiochem Corp., La Jolla, CA, USA), 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (TBQ) (Aldrich Chemical Company Inc., Milwaukee, WI, USA), and NaN₃ and ruthenium red (Nacalai Tesque Inc.). Thap and TBQ were dissolved in dimethylsulfoxide (DMSO) (Wako Pure Chemical Ind. Ltd., Tokyo, Japan) as the stock solution, and then diluted with the HEPES-Tyrod's solution before use.

Statistics. Measured values were expressed as the mean $\pm$ standard error of the mean (SEM) in the text and figures. Student's *t*-test (two-tailed for unpaired samples) was used to test the significant difference of the measured values. Significant difference was verified at $p < 0.05$.

RESULTS

The decay time of the aequorin light signals induced by Caf application, RC, and ES at a low temperature

The increased $[Ca^{2+}]_i$ induced by Caf application, RC, and ES spontaneously decayed even at 4°C. The decay time of the light signal induced with each Ca²⁺ releasing maneuver at 4°C was longer than that in Caf application and ES measured at 30°C. The decay time of the light signal induced by Caf application and RC at 4°C was 5.33 ± 0.36 s ($n=19$) and 3.50 ± 0.19 s ($n=38$), respectively. On the other hand, the decay time of the light signal induced by ES at 4°C was 1.71 ± 0.07 s ($n=28$), the shortest among the three maneuvers (Fig. 1). In addition to the decay time, the time to peak light in ES was faster than that in Caf application and RC.

Relation between the peak light of aequorin and the decay time

Since a change in $[Ca^{2+}]_i$ is suggested to influence the time course of the decay of the Ca²⁺ signal [29], we measured the relation between the peak Ca²⁺ concentration and the 63% decay time in the Caf-induced Ca²⁺ release. The peak $[Ca^{2+}]_i$ increased by Caf was altered by changing the Ca²⁺ content in the SR; by changing the stimulation period before Caf applica-

Fig. 1. Changes in the light signal of aequorin induced with caffeine (Caf) (A), rapid cooling (RC) (B), and electrical stimulation (ES) (C). Caf (15 mM) was rapidly applied at 4°C. In RC, the temperature of the solution was rapidly lowered from 30 to 4°C within 1 s. Before the Caf application, the preparation was regularly stimulated at 0.07 Hz for about 5 min at 4°C. In RC, the preparation was stimulated at 2 Hz for 5 min before cooling. ES at 0.07 Hz was applied at 4°C.

tion or by using K^+ -free solution. The peak $[Ca^{2+}]_i$ ranged from 0.63–2.56 μM and the 63% decay time varied from 3.64 to 8.72 s at 4°C. However, no significant correlation between these two parameters was observed ($n=20$, 11 experiments) (Fig. 2A). In RC, the peak $[Ca^{2+}]_i$ was between 1.10–2.86 μM , and the decay time of the light signal was 1.20–5.06 s at 4°C. As in the case of Caf, no significant correlation was observed ($n=23$, 12 experiments) (Fig. 2A). Therefore, we could compare the decay time of the light signal increased by Caf or RC in the presence or absence of the inhibitors without considering the influence of the change in peak $[Ca^{2+}]_i$. On the other hand, in ES, the decay time became shorter if peak $[Ca^{2+}]_i$ was higher at 30°C ($n=5$, $r=0.90$, $p<0.05$) (Fig. 2B), as previously reported in ferret ventricular muscles [3]. However, the slope of the relation was not so steep such that the dependence of the decay time on peak $[Ca^{2+}]_i$ was very small. Therefore, if a large difference in the decay time of the light signal was produced by an inhibitor, the main Ca^{2+} removal mechanism could be evaluated by comparing the time course of the decay time of the light signal even in ES.

Fig. 2. The relation between peak $[Ca^{2+}]_i$ and the decay time of the light signals induced by three different Ca^{2+} -releasing maneuvers. **A:** Ca^{2+} release with 15 mM Caf at 4°C (○) and RC from 30 to 4°C (●). No significant correlation between the peak $[Ca^{2+}]_i$ and the 63% decay time of the light signal was observed ($n=20$, 11 experiments in Caf, $n=23$, 12 experiments in RC). **B:** Ca^{2+} release by ES at 30°C. The 63% decay time of the light signal was inversely proportional to the peak $[Ca^{2+}]_i$ ($n=5$, one experiment, $r=0.90$, $p<0.05$).

Prolongation of the decay time of Caf-induced light signal in the presence of Ca^{2+} removal mechanism inhibitors at various temperatures

At 30°C, Caf induced a transient change in $[Ca^{2+}]_i$ and the decay time of the light signal was 0.60 ± 0.06 s ($n=18$) (Figs. 3A and 4). The decay time was not altered by TBQ as shown in Fig. 3A. When Na^+ - Ca^{2+} exchanger was inhibited by either Ni or Na^+ -free, the peak of the light signal increased and the decay time of the light signal was significantly prolonged to 2.53 ± 0.49 s ($n=7$, $p<0.01$) (Figs. 3A and 4). However, neither RR nor NN showed a significant effect on the decay time although the peaks of the light sig-

A. 30°C

B. 4°C

Fig. 3. Effects of the inhibitors for each Ca²⁺ removal mechanism on the decay time of the light signals induced by Caf at 30°C (A) and 4°C (B). The left panel (no-SR) shows the light signals in the presence or absence of the inhibitors for the sarcoplasmic reticulum (SR), Thap and TBQ at 30 and 4°C. The middle panels (no-EX) show the effects of Na⁺-Ca²⁺ exchanger inhibitors, Ni (upper panel) and Na⁺-free (lower panel) at 30 and 4°C. The right panels (no-MT) show the effect of mitochondrial inhibitors, RR and NN at 30 and 4°C. The insets are the normalized light signals in which the peaks of the light signals were normalized to match their peaks.

nal were significantly increased by RR. These results indicate that the Na⁺-Ca²⁺ exchanger is primarily responsible for the removal of Ca²⁺ released by Caf at 30°C.

At 4°C, the decay time of the Caf-induced light signal was markedly prolonged by each mitochondrial inhibitor (RR, NN) (Figs. 3B and 4). However, the inhibitors for SR and Na⁺-Ca²⁺ exchanger did not significantly alter the decay time (Figs. 3B and 4). The decay time in the absence of the mitochondrial inhibitors was 5.33 ± 0.36 s ($n=19$), and that in the presence of the inhibitors was $>86.82 \pm 18.44$ s ($n=8$, $p<0.01$). RR and NN showed similar effects on the decay time. Therefore, mitochondria significantly contribute to the removal of Ca²⁺ increased with Caf at 4°C.

Since the effects of the inhibitors for Na⁺-Ca²⁺ exchanger and mitochondria on the decay of the light

signal differed at 30 and 4°C, we analyzed the temperature-dependent changes in the effects of the inhibitors. As shown in Fig. 4A, the effect of Ni was lessened as the temperature of the solution was lowered. On the other hand, the effect of RR, which prolonged the decay time of the light signal, seemed to become more obvious at temperatures lower than 18°C. Each SR inhibitor, however, did not show a temperature dependence, due to the regenerative Ca²⁺ release from the SR. The temperature dependence of the effects of the inhibitors on the decay time of the light signal in Caf application was summarized and is shown in the graph of Fig. 4B. The graph indicates that each mitochondrial inhibitor significantly prolongs the decay time at temperatures below 12°C, and that each Na⁺-Ca²⁺ exchanger inhibitor is more effective at temperatures higher than 12°C.

From these results, we postulated that the Ca²⁺ re-

Fig. 4. Effects of the inhibitors for each Ca^{2+} removal mechanism on the decay time of the light signals induced by Caf at different temperatures. A: In each panel, the normalized light signals induced by Caf in the presence or absence of an inhibitor are superimposed. The effects of Ni and RR are shown in the upper and middle rows, respectively. The effects of the inhibitors for the SR are shown in the lower row. **B** shows the pooled data. Each point is the mean value of 2 to 19 measurements. To avoid confusion, SEM was omitted and only the mean values were plotted. Statistical significance against the control was tested when the number of measurements was greater than three. no-EX at 12 and 30°C were significantly different from the control, that at 4°C was not significantly different from the control, and other points were not tested due to the small number of experiments.

leased by Caf is primarily removed by the Na^+ - Ca^{2+} exchanger at higher temperatures and that the Ca^{2+} uptake by mitochondria contributes more greatly to Ca^{2+} removal when the Na^+ - Ca^{2+} exchanger is inhibited at lower temperatures.

Alterations in the decay time of RC-induced light signals induced by each Ca^{2+} removal mechanism inhibitor

In the presence of each inhibitor of the SR (Thap, TBQ), the RC-induced peak $[\text{Ca}^{2+}]_i$ was decreased, probably due to a decrease in the Ca^{2+} content in the SR, but the decay time of the light signal was not significantly altered (Fig. 5). Similarly, no significant change in the decay of the light signal was recognized when the Na^+ - Ca^{2+} exchanger was inhibited by either Ni or Na^+ -free. Therefore, both mechanisms (SR and

Na^+ - Ca^{2+} exchanger), which are apparently inhibited in RC (due to low temperature for Na^+ - Ca^{2+} exchanger and continuous Ca^{2+} release for SR), do not contribute to Ca^{2+} removal. On the other hand, each mitochondrial inhibitor (RR, NN) increased the peak $[\text{Ca}^{2+}]_i$ induced by RC and significantly prolonged the decay time of the light signal. The decay time was 3.50 ± 0.19 s ($n=38$) and $>18.12 \pm 3.19$ s ($n=11$, $p<0.01$) in the absence and presence of the mitochondrial inhibitors, respectively (Fig. 5A and B).

Therefore, as in the case of Caf application at low temperatures, mitochondria removes a sufficient amount of Ca^{2+} if Ca^{2+} is continuously released from the SR and the function of the Na^+ - Ca^{2+} exchanger is inhibited at low temperatures as in the case of Caf.

Prolongation of the decay time of ES-induced light signals induced by each Ca^{2+} removal mechanism inhibitor

The results of the effects of the inhibitors on the decay time of the light signals induced by ES differed from those induced by Caf and RC. At 30°C, each inhibitor for the SR (Thap, TBQ) and Na^+ - Ca^{2+} exchanger (Ni, Na^+ -free) significantly decreased the ES-induced peak of $[\text{Ca}^{2+}]_i$ and prolonged the decay time of the light signals (Figs. 6A and 7). The decay time of the light signals in the control was 0.05 ± 0.002 s ($n=34$). The SR inhibitors prolonged the decay time to 0.15 ± 0.02 s ($n=6$, $p<0.01$) (Fig. 7B). Each inhibitor for the Na^+ - Ca^{2+} exchanger (Ni, Na^+ -free)

Fig. 5. Changes in the light signals induced by the inhibitors for each Ca²⁺ removal mechanism in RC. A: Light signals increased by RC in the presence or absence of the inhibitors for each Ca²⁺ removal mechanism. The left panels (no-SR) show the effects of the inhibitors of the SR, Thap and TBQ. The middle panels (no-EX) show the effects of Na⁺-Ca²⁺ exchanger inhibitors (Ni and Na⁺-free). The right panels (no-MT) show the effects of mitochondrial inhibitors (RR and NN). In the insets, the peaks which were significantly different were normalized and superimposed. **B:** Graph showing the 63% decay time of the light signal in the presence or absence of the inhibitors for each Ca²⁺ removal mechanism. The decay time was measured in the presence of the inhibitors and the data were pooled. No-SR, no-EX, and no-MT correspond to treatment of the preparation with the inhibitors for the SR, Na⁺-Ca²⁺ exchanger, and mitochondria, respectively.

prolonged the decay time of the Ca²⁺ transient; however, the effect of Ni was more pronounced (Fig. 6A). The difference is probably due to prolongation of the action potential by Ni [23]. Therefore, only the decay time in Na⁺-free was measured and compared with that in the control. Na⁺-free slightly but significantly prolonged the decay time to 0.07 ± 0.01 s ($n=6$, $p<0.01$) (Fig. 7B). However, the mitochondrial inhibitors (RR, NN) did not significantly alter the decay time (Figs. 6A and 7).

At 4°C, the decay time of the light signal without the inhibitors was prolonged to 1.71 ± 0.07 s ($n=28$). In contrast to 30°C, at 4°C only the SR inhibitors significantly prolonged the decay time to 3.85 ± 0.42 s ($n=6$, $p<0.01$) (Fig. 7B). The inhibitor for the Na⁺-Ca²⁺ exchanger (Na⁺-free) did not significantly prolong the decay time. In addition, the mitochondrial inhibitors did not alter the decay time of the ES-induced light signals even at 4°C (Figs. 6B and 7).

As in the case of Caf, the temperature dependence of the effect of the inhibitors on the decay time of ES-induced light signals was measured and is summarized in Fig. 7. The results indicate that each inhibitor

of the SR significantly prolonged the decay time at high and low temperatures. Although the measured mean values at 30°C in the presence (closed square) and absence (open square) of inhibitors are very close due to the scaling, these are significantly different ($p<0.01$). The Na⁺-Ca²⁺ exchanger inhibitor (Na⁺-free) was effective only at higher temperatures. On the other hand, the mitochondrial inhibitors were ineffective at all temperatures tested.

DISCUSSION

Relevance of the measurement of the decay time of the aequorin light signal as an indicator of the Ca²⁺ removal mechanism. In this study, the [Ca²⁺]_i increase induced by various stimulation maneuvers was measured from the superficial cells of aequorin-injected ferret papillary muscles. Although the velocities of the spread of the activation by Caf and the temperature change throughout the whole preparation were much slower than that by action potential, superficial cells can be immediately and sufficiently activated with the rapid application of

A. 30°C

B. 4°C

Fig. 6. Effects of the inhibitors for each Ca^{2+} removal mechanism on the decay time of the light signals induced by ES at 30°C (A) and 4°C (B). The left panels (no-SR) show the light signals in the presence or absence of the inhibitors for the SR (Thap and TBQ). The middle panels show the effect of Na^+ - Ca^{2+} exchanger inhibitors (Ni and Na^+ -free). The right panels show the effect of mitochondrial inhibitors (RR and NN). Significantly different peaks were normalized to show the time course (insets). Upward small deflections indicate the application of stimulus.

Caf and RC. Therefore, the slower time course of the light signals in Caf application and RC are not due to the slow activation by Caf and temperature change.

Aequorin luminescence is influenced by temperature. However, the light signals of aequorin in tissue measured at different temperatures were not limited by the temperature-dependent kinetics of aequorin luminescence, because the kinetics of aequorin luminescence in a cuvette were much faster than the measured values in tissue even at a low temperature [30].

The decay of the Ca^{2+} signal in ES showed a slight dependence on peak $[\text{Ca}^{2+}]_i$ (Fig. 2B) [29]. On the other hand, during Caf application and RC, the decay time of the light signals was not influenced by the

change in peak $[\text{Ca}^{2+}]_i$ (Fig. 2A). The difference may be due to the different main Ca^{2+} removal mechanism (the SR in ES, mitochondria in Caf application and RC) and the longer duration of Ca^{2+} release from the SR during Caf application and RC. The change of peak $[\text{Ca}^{2+}]_i$ induced by ES in the presence of the SR or Na^+ - Ca^{2+} exchanger inhibitors was not as large as shown in the records of the light signals, because the change in the peak of the light signal is an exaggerated expression of $[\text{Ca}^{2+}]_i$ (the third power of $[\text{Ca}^{2+}]_i$) [19]. In addition, a 200% change in $[\text{Ca}^{2+}]_i$ during ES induces approximately a 15% change in decay time (Fig. 2B). However, the change in decay time which we observed and discussed (Figs. 6 and 7) was larger

Fig. 7. Temperature-dependent changes in the decay time of the light signals in ES and the effect of the inhibitors for Ca²⁺ removal mechanisms. **A:** In each panel, the normalized light signals induced by ES in the presence or absence of an inhibitor are superimposed. The effects of TBQ, Na⁺-free, and RR are shown in the upper, middle, and lower panels at 30, 12, and 4°C. The pooled data are shown in **B**. Concerning the effect of the inhibitors for the Na⁺-Ca²⁺ exchanger, Ni prolonged the decay time more than Na⁺-free because of the effect of Ni on the action potential. Therefore, only the decay time in the presence of Na⁺-free was plotted in **B**. Each point is the mean value of 2 to 35 measurements. To avoid confusion, SEM was omitted and only mean values were plotted. Statistical significance was tested when the number of measurements was greater than three. The points of no-SR and no-EX are overlapped, but these values were significantly different from the control in **B**.

than 4-fold the control. Therefore, the observed change is not limited by this factor (peak [Ca²⁺]_i-dependence of the decay time of the Ca²⁺ signal).

Some of the intracellularly increased Ca²⁺ are bound to troponin-C [3, 31], and the change in the affinity of troponin-C for Ca²⁺ is suggested to influence the decay time of the light signals in twitch: an increase in tension shortens the decay time of Ca²⁺ signal [3]. This is not the case in this study because tension was altered in parallel with the change in the [Ca²⁺]_i (tension was not shown in the figure).

Therefore, the measurement of [Ca²⁺]_i change using aequorin from the superficial cells can be used for the evaluation of the Ca²⁺ handling mechanism in the three maneuvers employed in this study.

Ca²⁺ release and removal in Caf application, RC, and ES. A higher concentration of Caf is known to open the Ca²⁺ release channels in the SR by enhancing the Ca²⁺-induced Ca²⁺ release mecha-

nism, and Ca²⁺ can be regeneratively released [12, 15]. The Ca²⁺ release continues until all Ca²⁺ in the SR is released because Ca²⁺ release dominates Ca²⁺ uptake by the SR. Therefore, the decay time of the Caf-induced light signal is longer than with the other two maneuvers (Figs. 1 and 8A). Since Ca²⁺ uptake by the SR is apparently inhibited by Caf, Ca²⁺ is removed by the Na⁺-Ca²⁺ exchanger at higher temperatures. Therefore, inhibition of the Na⁺-Ca²⁺ exchanger significantly prolonged the decay time and the SR inhibitors were apparently ineffective at 30°C. However, at a low temperature, the activity of the Na⁺-Ca²⁺ exchanger with a relatively high *Q*₁₀ [22] is inhibited. Therefore, at temperatures lower than about 12°C, the Na⁺-Ca²⁺ exchanger inhibitors did not show a significant effect (Fig. 4) because the mechanism is already inhibited at a low temperature. Thus, the increased Ca²⁺ by Caf at low temperatures should be transported to mitochondria with a low *Q*₁₀ [32, 33] and a large capacity. Therefore, the mitochondrial inhibitors showed a significant effect on the decay time of the light signals in Caf application at low temperatures.

Although Ca²⁺ removal from the myoplasm depends on the temperature of the solution, temperature also influences the Ca²⁺ release mechanism. It is now widely accepted that rapid lowering of the temperature of the solution directly releases Ca²⁺ from the SR [11, 13, 21] because low temperatures increase the open

Fig. 8. The contribution of Ca^{2+} release and Ca^{2+} removal mechanisms to Ca^{2+} signals in Caf application (A), RC (B), and ES (C) at 4°C . The inner dotted lines in A and B are the decay of Ca^{2+} signal in ES (C). The decay of Ca^{2+} signal in ES is assumed to be limited by the rate of Ca^{2+} uptake by the SR, which is decreased at low temperatures. Ca^{2+} release ceases faster than the Ca^{2+} uptake by the SR in ES. Noisy lines in A and B (next to the inner dotted line) show the measured Ca^{2+} signal in Caf application (A) and RC (B). The decay of the Ca^{2+} signal by Caf is longer than that by RC. The label, "continuous release," for Caf and RC means continuous and longer Ca^{2+} release as compared to the decay of Ca^{2+} by ES. The area in each figure indicates the relative contribution of the Ca^{2+} removal by mitochondria, SR, and others. The label, "others" includes the sarcolemmal Ca^{2+} pump and Na^+ - Ca^{2+} exchanger. Ca^{2+} uptake by the SR is involved in the Ca^{2+} removal by ES and RC. Mitochondrial Ca^{2+} transport contributes more to the Ca^{2+} removal in Caf application than in RC. Since, in RC, the decay of Ca^{2+} was not completely blocked by the inhibitors of mitochondria, some Ca^{2+} might be transported by other Ca^{2+} removal mechanisms including the SR, which we could not demonstrate using the SR inhibitors in RC. Therefore, the SR and others are incorporated in the scheme.

probability of the Ca^{2+} release channels of the SR [14]. Thus, Ca^{2+} is continuously released from the SR by RC, which is shown as a slower time course of the light signal as compared to that in ES at the same low temperature (Figs. 1 and 8B). Since Ca^{2+} is continuously released in RC, the apparent Ca^{2+} uptake by the SR is inhibited as in the case of Caf application. The

Na^+ - Ca^{2+} exchanger is also inhibited at a low temperature. Therefore, the inhibitors of the SR and Na^+ - Ca^{2+} exchanger were ineffective and the mitochondrial inhibitors were more significantly effective for the decay time of the light signal in RC (Fig. 5).

In contrast to Caf application and RC, ES releases Ca^{2+} from the SR at a faster rate, which is followed by faster inactivation of the Ca^{2+} release channels [34]. Thus, the decay time of the Ca^{2+} signal in ES is shorter compared to those in Caf application and RC at the same temperature (Fig. 1). In ES, in which Ca^{2+} release does not continue unlike the case of Caf application and RC (Fig. 8C), the released Ca^{2+} is primarily removed by the SR, which is the fastest Ca^{2+} transport mechanism among the possible Ca^{2+} removal mechanisms even at 4°C . Therefore, the SR could transport Ca^{2+} at a slower rate even at low temperatures. Thus, the SR inhibitors significantly prolonged the decay time of the light signals at low temperatures (Figs. 6 and 7). The Na^+ - Ca^{2+} exchanger is also partly responsible for Ca^{2+} removal in ES at higher temperatures. Thus, the Na^+ - Ca^{2+} exchanger inhibitors slightly but significantly prolonged the decay time only at higher temperatures (Figs. 6A and 7). Therefore, Ca^{2+} released by ES at low temperatures was primarily transported by the SR although the transport rate was slow [35]. Since sufficient Ca^{2+} was transported by the SR and partly by the Na^+ - Ca^{2+} exchanger in ES, the mitochondrial inhibitors did not show a significant effect on the decay time of the light signal.

In ferret ventricular muscles, the sarcolemmal Ca^{2+} pump is suggested to contribute to the decay of the Ca^{2+} signal [36]. We made a preliminary investigation of the effect of the inhibitors for the sarcolemmal Ca^{2+} pump on the decay time of the light signals. Although there are no specific inhibitors for the sarcolemmal Ca^{2+} pump, high concentrations of extracellular Ca^{2+} and vanadate are known to inhibit the sarcolemmal Ca^{2+} pump. The decay time of the light signals induced by ES at 30°C was not significantly altered by the inhibitors of the sarcolemmal Ca^{2+} pump (8 mM Ca^{2+} in extracellular solution or 10^{-5} M sodium vanadate). In addition, the decay time of the light signal in RC was not significantly influenced by the inhibitors. Therefore, the contribution of the sarcolemmal Ca^{2+} pump is not significant and we did not examine the effect of the inhibitors of the sarcolemmal Ca^{2+} pump in this experiment.

Significance of Ca^{2+} uptake by mitochondria. Mitochondria occupy a large volume in the myoplasm of the mammalian ventricular muscle [4] and have a uniport system for Ca^{2+} transport which is driven by the electrochemical gradient of protons

across the mitochondrial membrane [9, 10]. The inhibition of metabolism by NN diminishes the proton gradient and inhibits Ca²⁺ transport by mitochondria [28]. Thus, prolongation of the decay time of the light signals induced by Caf application or RC by NN indicates inhibition of the mitochondrial Ca²⁺ transport at low temperatures (Figs. 3B and 5). On the other hand, RR more directly inhibits the mitochondrial uniport for Ca²⁺ [27]. According to Emerson *et al.* [37], the mitochondrial Ca²⁺ uniport is actually inhibited by a contaminant in RR, μ -O-[(HCO₂)(NH₃)₄Ru]₂Cl₃. In this experiment, we used RR which probably contained a sufficient amount of the chemical which inhibits the mitochondrial Ca²⁺ uniport. The penetration of RR into the intact cell is suggested to be difficult [38]. However, we confirmed the penetration of RR into intact mammalian cardiac cells with proper treatment of the preparation [39]. Thus, the effect of RR on the decay time of the Caf- and RC-induced light signals further supports the hypothesis that mitochondria can transport sufficient Ca²⁺ under the condition in which the net Ca²⁺ uptake by the SR and Na⁺-Ca²⁺ exchanger are inhibited (Figs. 3B, 4, and 5). However, the decay of the Ca²⁺ signal during RC slightly declined even in the presence of the mitochondrial inhibitors. This suggests that other Ca²⁺ transport mechanisms might be responsible for Ca²⁺ removal during RC (see Fig. 8).

A large Ca²⁺ signal induced by RC in the presence of RR suggests that the SR has a full accumulation of Ca²⁺ if Ca²⁺ transport by mitochondria is inhibited and the accumulated Ca²⁺ can be released by RC. RR inhibits Caf-induced Ca²⁺ release from the SR [15]. RC can release Ca²⁺ by a different mode of Ca²⁺ release channel opening from either Caf application or ES [21], which is related to Ca²⁺-induced Ca²⁺ release [12]. Therefore, the peak [Ca²⁺]_i during RC in the presence of the mitochondrial inhibitor is larger than that in Caf application and ES.

The Ca²⁺ uniport of mitochondria is influenced by various intracellular factors (ionic strength, pH, and Mg²⁺ concentration) [9] and temperature (although the *Q*₁₀ of the rate of Ca²⁺ transport is low) [32, 33]. However, the Ca²⁺ uniport of mitochondria can transport sufficient Ca²⁺ when other Ca²⁺ removal mechanisms (SR and Na⁺-Ca²⁺ exchanger) cannot significantly operate, and [Ca²⁺]_i continues to rise with a slower time course. Thus, the contribution of Ca²⁺ transport by mitochondria becomes more significant under pathophysiological conditions in which the functions of the SR and Na⁺-Ca²⁺ exchanger are suppressed and intracellular Ca²⁺ is overloaded.

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