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# Species- and Temperature-Dependency of the Decrease in Myofilament $Ca^{2+}$ Sensitivity Induced by $\beta$-Adrenergic Stimulation

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ABSTRACT—Although $\beta$-adrenergic stimulation has been shown in many studies to decrease myofilament $Ca^{2+}$ sensitivity in various types of cardiac muscle such as rat and rabbit ventricles, other studies disagree with this conclusion. In the present study, we aimed to explain these contradictory findings. We examined the effect of $\beta$-adrenoceptor stimulation on $Ca^{2+}$ sensitivity using guinea pig and rat ventricles. We performed the experiment at two different temperatures and compared the results. In guinea pig ventricles, isoproterenol and forskolin did not alter the relationship between $[Ca^{2+}]_i$ and muscle force during the relaxation phase of tetanic contraction at either 24°C or 30°C. In rat ventricles, in contrast, isoproterenol shifted the $[Ca^{2+}]_i$-force curve to the right at 24°C, but not at 30°C. In guinea pig ventricles permeabilized by $\alpha$-toxin, in which the cAMP/PK-A system is intact, the addition of cAMP did not decrease $Ca^{2+}$ sensitivity. These results suggest that there are species- and temperature-dependent differences in the regulation of myofilament $Ca^{2+}$ sensitivity by $\beta$-adrenergic stimulation.

*Keywords*: Isoproterenol, Protein kinase A, $Ca^{2+}$ transient, Permeabilized fiber

The positive inotropic effect of $\beta$-adrenergic stimulation results from the increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), which is mediated by the phosphorylation of proteins such as phospholamban and sarcolemmal $Ca^{2+}$ channels and which results from the activation of adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase A (PK-A) (1 – 3). Stimulation of the $\beta$-adrenoceptor also induces an abbreviation of the time-course of contraction. In this abbreviated time-course, the phosphorylation of phospholamban contributes to the acceleration of $Ca^{2+}$ reuptake into the sarcoplasmic reticulum (SR). On the other hand, the decrease in $Ca^{2+}$ sensitivity of the contractile apparatus by means of $\beta$-adrenergic stimulation has been reported in various preparations, including rat ventricle (4 – 6), rabbit ventricle (7, 8) and ferret papillary muscle (9). It has been suggested that phosphorylation of troponin-I is involved in the PK-A-mediated decrease in $Ca^{2+}$ sensitivity (10). Such a mechanism is also considered to contribute to the abbreviated time-course of contraction upon $\beta$-adrenoceptor stimulation (11).

Although many reports suggest the presence of a mechanism that decreases myofilament $Ca^{2+}$ sensitivity by means of $\beta$-adrenoceptor stimulation in various types of cardiac preparations, O'Rourke et al. (12) reported that there was no obvious $Ca^{2+}$ desensitizing effect when stimulated by isoproterenol in rat myocytes at 37°C. We also did not observe such an effect in the guinea pig ventricles at 30°C in our preliminary experiments (13). In contrast, studies showing the presence of myofilament $Ca^{2+}$ desensitization due to $\beta$-adrenergic stimulation in the rat hearts were performed at lower temperatures (4 – 6). In addition, a recent study in human cardiomyocytes showed the $Ca^{2+}$ desensitizing effect of PK-A in failing human heart, but not in donor heart (14), suggesting that the PK-A-mediated myofilament desensitization is important under pathophysiological conditions.

In another study involving guinea pig hearts, Talosi et al. (15) reported that mechanical response and cAMP level closely correlated with the dephosphorylation of phospholamban, but did not correlate with the dephosphorylation of troponin-I during the washing out of isoproterenol. This result suggests that the change in $Ca^{2+}$ mobilization but not in $Ca^{2+}$ sensitivity is modulated by cAMP. Furthermore, isoproterenol failed to enhance the rate of myocardial relaxation in phospholamban-deficient mouse hearts (16). These analyses contradict the studies that show a PK-A-mediated desensitization of the contractile apparatus. Until now, the

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reason for these contradictory findings has not been found. To address this problem, we have examined the effects of PK-A activation on $Ca^{2+}$ sensitivity in different animal species and under the different experimental conditions employed in the above-mentioned papers.

## MATERIALS AND METHODS

### *Langendorff's method of loading fura-PE3 into papillary muscle*

Papillary muscle was loaded with fura-PE3 as previously described (17). Briefly, male guinea pigs (350 – 500 g) or Wistar rats (250 – 350 g) were killed under anesthesia with gaseous diethyl ether. The hearts were rapidly removed and washed in ice-cold (4°C) normal physiological salt solution (PSS) (145.0 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 10.0 mM glucose, 10.0 mM HEPES, and 1.6 mM $CaCl_2$ in guinea pigs and 1.0 mM in rats). Each heart was perfused in Langendorff's mode with PSS warmed to 30°C and gassed with 100% $O_2$. The coronary flow was normalized to 8 ml /min. Changes in $[Ca^{2+}]_i$ were monitored with fura-PE3, a fluorescent $Ca^{2+}$ indicator (an analog of fura-2). After a 20-min equilibrium, the perfusion was switched to recirculating PSS (20 ml) containing 4 $\mu$M fura-PE3/AM and 0.025% cremophor EL for 0.5 – 1.0 h. Upon completion of the fura-PE3 loading, the perfusion was switched to fura-PE3-free PSS for 15 min to wash the uncleaved fura-PE3 /AM from the tissue. Papillary muscles with a diameter less than 1 mm were excised from the fura-PE3-loaded heart, and one end of the muscle was fixed to the siliconized floor of a specialized perfusion chamber with stainless steel pins.

We have previously shown that 100 $\mu$M $Mn^{2+}$ preferentially quenched cytosolic fura-PE3, and residual fluorescent signals (background fluorescence) were not affected by various factors that affect $[Ca^{2+}]_i$ (17). These results suggest that, under our loading conditions, most of the fura-PE3 molecules distribute in cytoplasm and the fura-PE3 signal is a good indicator of $[Ca^{2+}]_i$. Because of the large background fluorescence and an inaccurate $K_d$ for $Ca^{2+}$ of fura-PE3 in the cytoplasm, we have not calculated the absolute $Ca^{2+}$ concentration in this experiment.

### *Measurements of cytosolic $Ca^{2+}$ concentration and muscle force*

Fluorescent signals were directed to and obtained from a surface portion of the papillary muscle through a single quartz fiber optic (800 $\mu$m diameter). Fura-PE3 fluorescence was measured with a dual excitation fluorimeter (CAM230; Jasco, Tokyo). Fluorescent intensity (F340, F380) excited at 340 nm or 380 nm was measured, and the ratio of the fluorescence (F340/F380) was used as an indicator of $[Ca^{2+}]_i$.

The muscle was maintained throughout the experiments by perfusing the chamber continuously (12 ml/min) with a pre-oxygenated PSS solution. The force of contraction was recorded isometrically. The resting tension applied to each preparation was adjusted to give 90% of the maximum developed tension. During the equilibration period, the muscles were stimulated electrically by square pulses of 5-ms duration at 0.5 Hz with a voltage 1.2-fold greater than the threshold intensity, using an electronic stimulator (SEN-3201; Nihon Kohden, Tokyo). Tetanic contraction was induced by square pulses of 40-ms duration at 10 Hz with a voltage threefold greater than the threshold intensity in the presence of ryanodine (5 $\mu$M) and cyclopiazonic acid (10 $\mu$M). To evaluate the force-$[Ca^{2+}]_i$ relationship in a wide range of $[Ca^{2+}]_i$, submaximal tetanic contractions were induced in 10 mM extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$) in guinea pigs and in 4 mM $[Ca^{2+}]_o$ in rats. $[Ca^{2+}]_i$ and contractile force were digitized with an A/D converter (A Burr-Brown Company, Tucson, AZ, USA) and an IBM PC/AT computer at a sampling rate of 110 points/s. To avoid the interference with the $\alpha$-adrenoceptor-mediated effect of isoproterenol, 1 $\mu$M phentolamine was added 10 min before the experiments were started. Ascorbic acid (0.5 mM) was added to isoproterenol stock solution (1 mM) to prevent oxidation. Developed tension was $1.9 \pm 0.5$ mN /$mm^2$ in guinea pigs (n = 12) at 1.6 mM $[Ca^{2+}]_o$ and $3.1 \pm 0.7$ mN/$mm^2$ in rats (n = 8) at 1.0 mM $[Ca^{2+}]_o$ (at 0.5-Hz stimulation frequency at 30°C).

### *Permeabilized muscle preparations*

Small bundles of trabeculae (120 – 200 $\mu$m in diameter and 1 – 2 mm in length) were dissected from the right ventricle or teased from the papillary muscle of guinea pigs and rats, as described by Miyamoto et al. (18). Bundles were permeabilized with 80 $\mu$g/ml *Staphylococcus aureus* $\alpha$-toxin for 30 min or 50 $\mu$g/ml saponin for 40 min in a relaxing solution (see below). Both ends of the bundles were tied with monofilament silk thread. One end of each bundle was connected to an isometric transducer, and the other end was fixed to the siliconized floor of the chamber with a stainless steel pin under a resting tension of 0.5 mN and equilibrated for 60 – 90 min. During this period, 10 $\mu$M $Ca^{2+}$ was repeatedly applied until the peak force became reproducible. The amplitude of contraction induced by 10 $\mu$M $Ca^{2+}$ was $20.4 \pm 2.6$ mN/$mm^2$ (n = 14). Experiments were carried out at room temperature (22 – 24°C).

The relaxing solution contained 5.25 mM $Mg^{2+}$-(methansulphonate)$_2$, 40 mM $K^+$-methansulphonate, 5.3 mM ATP, 20 mM piperazine-*N*,*N*′-bis 2-ethansulfonic acid (PIPES), 10 mM phosphocreatine, 20 unit/ml creatine phosphokinase, 1 $\mu$M carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), 1 $\mu$g/ml E-64 and 10 mM EGTA. The ionic strength was adjusted to 0.2 M, and the pH was adjusted to 7.1. The free $Ca^{2+}$ concentration was changed by adding an

appropriate amount of $Ca^{2+}$-(methansulphonate)$_2$. The low EGTA solution was identical to the relaxing solution except that the EGTA concentration was 0.05 mM. The SR loading solution was adjusted to be pCa 7 by adding $Ca^{2+}$-(methansulphonate)$_2$ to the low EGTA solution. The ionic composition of these solutions was calculated by a computer program originally developed by Fabiato (19).

*Protocols in permeabilized preparation*

The $Ca^{2+}$-uptake experimental protocol was basically the same as that described by Nosek et al. (20), with slight modifications. The protocol consisted of five steps: 1) adding 50 mM caffeine to the relaxing solution to deplete the SR of $Ca^{2+}$, 2) washing the caffeine with relaxing solution for 3 min, 3) washing with low EGTA solution (0.05 mM EGTA) for 2 min to remove EGTA, 4) adding SR-loading solution for 15 min (pCa 7, 0.05 mM EGTA), and 5) adding 50 mM caffeine to the SR-loading solution to release $Ca^{2+}$ from SR. cAMP (100 $\mu$M) was added to the loading solution at step 4).

In $\alpha$-toxin permeabilized preparations, we first observed the potentiation of SR $Ca^{2+}$ uptake by cAMP to discern whether the activity of the cAMP/PK-A system was intact. Then 100 $\mu$M ryanodine and 10 $\mu$M cyclopiazonic acid were added for 25 min to inhibit the functions of the SR. Subsequently, 100 $\mu$M cAMP was added for 20 min to the relaxing solution and various pCa-solutions were cumulatively applied in the presence of 100 $\mu$M cAMP. The data were normalized with the force elicited by 10 $\mu$M $Ca^{2+}$, which was applied at the beginning of the experiment. The data were fitted to the Hill equation:

$$F = F_{Max} \times [Ca^{2+}]^{nH} / ([EC_{50}]^{nH} + [Ca^{2+}]^{nH}),$$

where $F_{Max}$ is the maximal force, F is the developed force due to the application of various pCa, $EC_{50}$ is the concentration of $Ca^{2+}$ that induces the half-maximal activation, and $n_H$ is the Hill coefficient, which is an index of cooperativity.

In this study, we also used permeabilized preparations with saponin, which makes the cell membrane permeable to larger molecular solutes of biological interest such as PK-A. To inhibit the functions of SR, 100 $\mu$M ryanodine and 10 $\mu$M cyclopiazonic acid were added for 25 min. PK-A (1000 unit/ml) was added for 45 min in the relaxing solution.

*Chemicals*

Chemicals used were acetoxymethyl ester fura-PE3 (fura-PE3/AM; Texas Fluorescent Laboratories, Austin, TX, USA); PK-A, ryanodine, cyclopiazonic acid, phentolamine hydrochloride, saponin, isoproterenol hydrochloride (Sigma Chemicals, Tokyo); and cremophor EL (Nacalai Tesque, Kyoto). cAMP (Boehringer Mannheim, Tokyo), staurosporine (Kyowa Medex, Tokyo), fura-PE3/AM, and ryanodine were dissolved in dimethyl sulfoxide. The final concentration of the solvent was less than 0.01%. The solvent alone had no effects on $[Ca^{2+}]_i$ or the contractile force. The $\alpha$-toxin was kindly supplied by Dr. Iwao Kato, Chiba University.

*Statistics*

The numerical data were expressed in terms of the mean ± S.E.M. Differences between the mean values were evaluated by a paired Student's *t*-test (unless otherwise stated), and a probability of less than 0.05 was taken as a statistically significant difference.

## RESULTS

*Effects of isoproterenol on twitch contraction and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)*

An increase in extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$) and stimulation frequency increased the amplitudes of $[Ca^{2+}]_i$ transients and force in the guinea pig ventricle, as shown in Fig. 1A. Isoproterenol (1 $\mu$M), which elicited the maximal positive inotropy at 0.2 Hz, augmented both the $[Ca^{2+}]_i$ transient and the force to 135.7 ± 3.5% and to 425.4 ± 57.4%, respectively. In the presence of isoproterenol, we often observed the humps in $[Ca^{2+}]_i$ transient and contraction during the rising phase, as has been reported previously in guinea pig ventricle (21, 22). Ryanodine (300 nM) abolished the first but not the second component of twitch contraction (data not shown), suggesting that the first component is induced by the release of $Ca^{2+}$ from the SR and the second component is induced by the influx of $Ca^{2+}$ (22). In rat papillary muscle, however, only a single component of $[Ca^{2+}]_i$ transient or contraction was observed.

The decreases in duration of both the $[Ca^{2+}]_i$ transient and force were also observed. In the absence of isoproterenol, increase in $[Ca^{2+}]_o$ to 3.2 mM and stimulation frequency to 1 Hz also decreased the durations of both the $[Ca^{2+}]_i$ transient and force and increased the amplitude of both the $[Ca^{2+}]_i$ transient and force ($[Ca^{2+}]_i$ transient, 131.6 ± 5.4%; force, 343.4 ± 22.3%). These values were almost the same as those obtained in the presence of 1 $\mu$M isoproterenol at 0.2 Hz.

The trajectory between $[Ca^{2+}]_i$ and force in the relaxation phase has been utilized to represent the $Ca^{2+}$ sensitivity of the myocardial contractile element (5, 8, 23). As shown in Fig. 1B, isoproterenol did not change the trajectory between $[Ca^{2+}]_i$ and force in the relaxation phase.

*Relationship between contraction and $[Ca^{2+}]_i$ during the relaxation phase of tetanic contraction*

The disability of SR functions slowed the decay rate of $[Ca^{2+}]_i$, which allowed the force to be in a steady-state with $[Ca^{2+}]_i$. Therefore, the force-$[Ca^{2+}]_i$ relationship during the relaxation of twitch contractions in the absence of SR func-

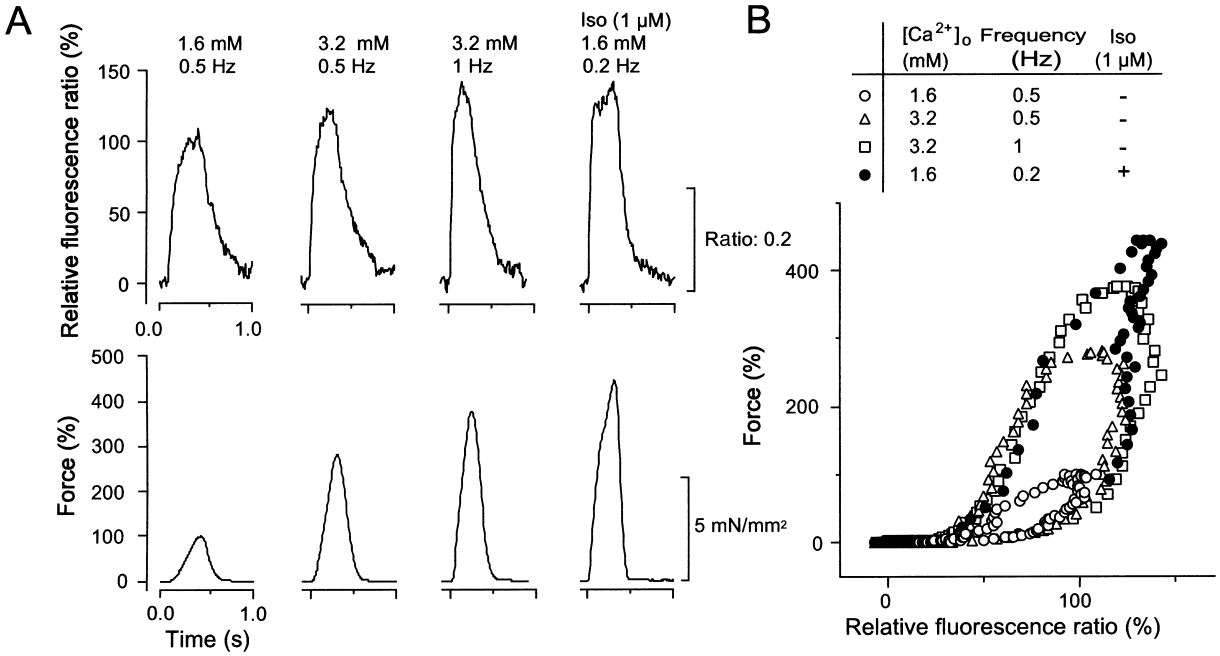


**Fig. 1.** Effects of isoproterenol (Iso, 1 $\mu$M) on twitch contraction and $[Ca^{2+}]_i$ transient (indicated as the relative fluorescence ratio) in guinea pig papillary muscle. The values obtained at a stimulation frequency of 0.5 Hz in the presence of 1.6 mM $Ca^{2+}$ were taken as 100% (Control). Experiments were conducted at 30°C. A: Typical traces. B: Phase-plane diagrams of the relative increases in fluorescence ratio and force in panel A.

tions has been widely used as an indicator of the steady-state relationship between $[Ca^{2+}]_i$ and force in cardiac muscles (6, 24). In addition, the force-$[Ca^{2+}]_i$ relationship during the relaxation of tetani in the absence of SR function has also been utilized for the same purpose (25). In this study, tetanic stimulation was introduced to the muscles pretreated with ryanodine (5 $\mu$M) and cyclopiazonic acid (10 $\mu$M) for 25 min at 0.5 Hz as shown in Figs. 2 and 3. Using this experimental protocol, we first examined the effect of isoproterenol (1 $\mu$M) in rat papillary muscle at 24°C. Figure 2A shows the representative results, and the inset shows that the phase-plane diagrams of $[Ca^{2+}]_i$ and force during the relaxation phase in the presence of 4.0 mM $[Ca^{2+}]_o$ were shifted rightward by the application of isoproterenol. This result suggests that isoproterenol decreased $Ca^{2+}$ sensitivity. Figure 2B shows the phase-plane diagrams of averaged $[Ca^{2+}]_i$ and averaged force signals at 24°C and 30ºC. On the diagrams, 100% represents the amplitude of the signals obtained by tetanic stimulation in the presence of 4.0 mM $[Ca^{2+}]_o$ at each temperature. The data show that, in contrast to the results at 24ºC, isoproterenol failed to alter the trajectory between $[Ca^{2+}]_i$ and force at 30ºC.

We then examined the effects of isoproterenol on guinea pig papillary muscle in the presence of 10 mM $[Ca^{2+}]_o$ at 24ºC. Figure 3 shows the averaged signals in which 100% represents the amplitudes of the signals obtained by tetanic stimulation. Isoproterenol did not alter the relationship between $[Ca^{2+}]_i$ and force during the relaxation phase of tetanic contraction (Fig. 3A). We also found that forskolin (1 $\mu$M) failed to alter this relationship (Fig. 3B). Also, at 30ºC, neither isoproterenol nor forskolin changed the relationship between $[Ca^{2+}]_i$ and force in guinea pig papillary muscle (Fig. 3: C and D).

*Effects of PK-A on the steady-state pCa-force relationship in permeabilized preparations*

To evaluate the role of PK-A on myofilament $Ca^{2+}$ sensitivity in guinea pig ventricles, we examined how exogenously applied PK-A affected the contraction that was induced by 1 $\mu$M $Ca^{2+}$ in the saponin-permeabilized preparations (Fig. 4). Preliminary experiments showed that 1 $\mu$M $Ca^{2+}$ induced a contraction of approximately half the maximum force (46.6 ± 3.0% of the contraction induced by pCa 5, n = 7). Taking the contraction induced by the first application of 1 $\mu$M $Ca^{2+}$ as a reference response (100%), the second application of 1 $\mu$M $Ca^{2+}$ elicited a contraction that was 97.6 ± 4.1% (n = 4) of the first contraction. Pretreating the muscle with 1000 unit/ml PK-A and 100 $\mu$M cAMP for 45 min decreased the second contraction to 80.3 ± 2.2% ($P$<0.05, n = 3).

It has been suggested that the pores formed by $\alpha$-toxin in the plasma membrane do not permeate high molecular weight (>4,000) solutes (26, 27), and thus a preparation permeabilized with $\alpha$-toxin retains endogenous protein ki-

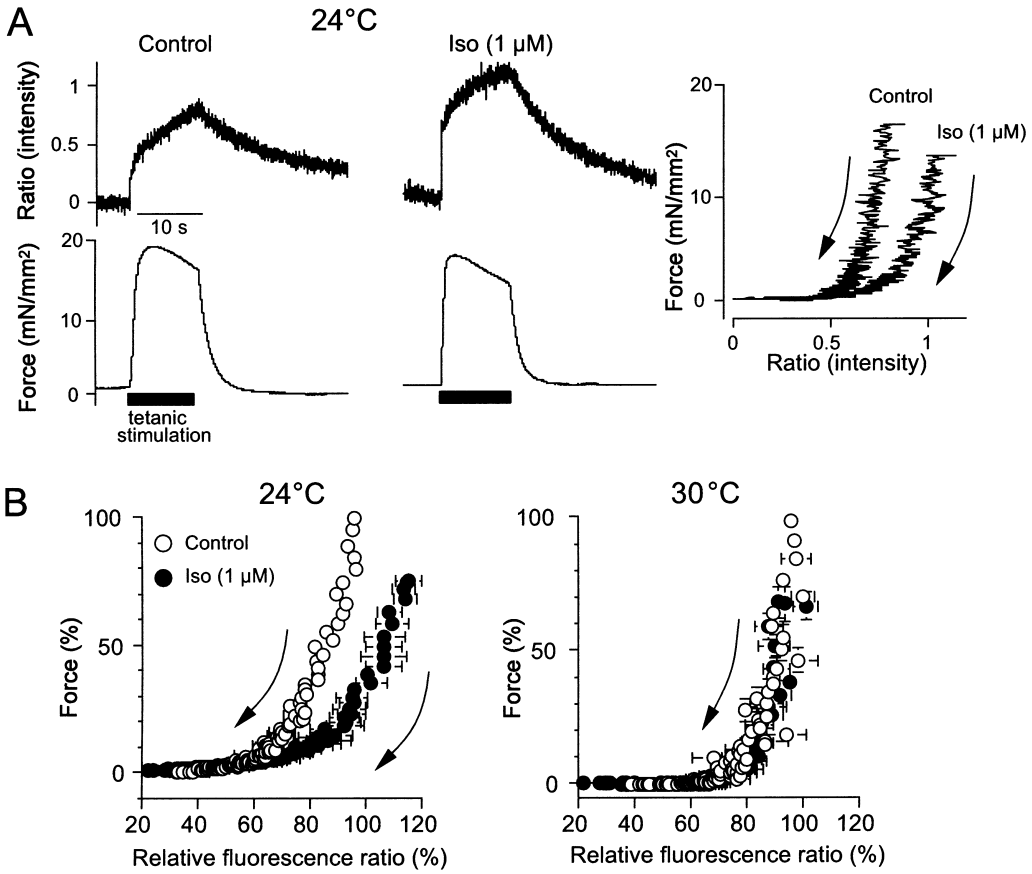


**Fig. 2.** Effects of isoproterenol (Iso) on the trajectory between $[Ca^{2+}]_i$ transient (indicated as the relative fluorescence ratio) and force during the relaxation phase of tetanic contractions in rat papillary muscles. A: Typical trace of the changes in the fluorescence ratio and force elicited by tetanic stimulation in the presence of 4.0 mM $[Ca^{2+}]_o$ at 24°C. Inset: Trajectory between the relative fluorescence ratio and force. B: Summary of the results obtained in rat papillary muscle at 24°C (left panel, n = 4) and at 30°C (right panel, n = 4). The averaged force signal was plotted against the averaged fluorescence ratio signal. The 100% level represents the amplitudes of the increases in the fluorescence ratio and force that were obtained by tetanic stimulation in the presence of 4.0 mM $[Ca^{2+}]_o$. Arrows indicate the direction of the signals.

nases such as PK-A and protein kinase C. We examined the effect of endogenous PK-A activation on the pCa-force relationship in this preparation. To determine whether PK-A is retained in the guinea pig papillary muscle permeabilized by $\alpha$-toxin, we first examined the effect of PK-A activation on SR $Ca^{2+}$ uptake. As shown in Fig. 5 (A and B), cAMP (100 $\mu$M), added during the $Ca^{2+}$ loading into SR (see Methods), augmented the caffeine-induced contracture by 30.6 ± 5.6%. This augmentation was inhibited by 3 $\mu$M staurosporine, a nonselective protein kinase inhibitor (n = 3, data not shown). These results suggest that the cAMP/PK-A system remains in the $\alpha$-toxin permeabilized muscle. As shown in Fig. 5C, however, the pCa-force relationship and Hill coefficient were not affected by 100 $\mu$M cAMP ($EC_{50}$ of $Ca^{2+}$, control: 1.06 ± 0.026 $\mu$M vs cAMP: 0.94 ± 0.054 $\mu$M, $P$>0.05; Hill coefficient, control: 6.5 ± 1.01 vs cAMP: 6.2 ± 0.96, $P$>0.05). The maximum contraction induced by 10 $\mu$M $Ca^{2+}$ was similarly unaffected by 100 $\mu$M cAMP (control: 99.1 ± 1.16% vs cAMP: 95.7 ± 2.53%, taking the pCa 5-induced contraction performed just before the testing as 100%).

## DISCUSSION

In intact guinea pig papillary muscle, an increase in $[Ca^{2+}]_o$ and/or an increase in stimulation frequency resulted in an increase in the amplitude of $[Ca^{2+}]_i$ transients and twitch contractions. The addition of isoproterenol produced effects similar to those of the increase in $[Ca^{2+}]_o$ and/or stimulation frequency. However, we did not observe any change in the relationship between the amplitudes of the twitch contractions and $[Ca^{2+}]_i$ transients under these conditions (Fig. 1A). In addition, isoproterenol did not change the force-$[Ca^{2+}]_i$ relationship during the twitch contraction's relaxation stage (Fig. 1B). This relationship at this stage has been used as an index of myofilament $Ca^{2+}$ re-

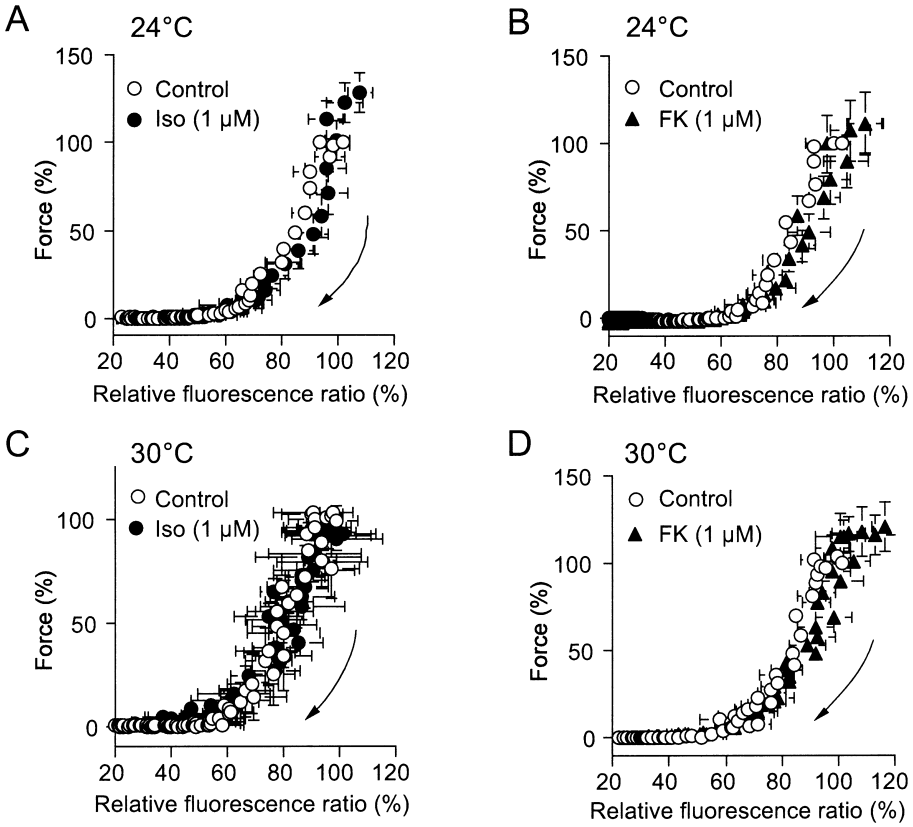


**Fig. 3.** Effects of isoproterenol (Iso) and forskolin (FK) on the trajectory between $[Ca^{2+}]_i$ transient (indicated as the relative fluorescence ratio) and force during the relaxation phase of tetanic contractions in guinea pig papillary muscles in the presence of 10 mM $[Ca^{2+}]_o$. The averaged force signal was plotted against the averaged fluorescence ratio. The 100% level represents the amplitudes of the increases in the fluorescence ratio and force that were obtained by tetanic stimulation in the presence of 10 mM $[Ca^{2+}]_o$ at each temperature. A: 24°C, 1 $\mu$M isoproterenol, n = 6; B: 24ºC, 1 $\mu$M forskolin, n = 5; C: 30ºC, 1 $\mu$M isoproterenol, n = 3; D: 30°C, 1 $\mu$M forskolin, n = 5. Arrows indicate the direction of the signals.

sponsiveness (23). These results imply that in guinea pig papillary muscle, $\beta$-adrenergic stimulation does not change the myofilament $Ca^{2+}$ responsiveness. To further evaluate the effects of isoproterenol, we evaluated the force-$[Ca^{2+}]_i$ relationship during relaxation after the tetanic contraction that was obtained in the presence of ryanodine and cyclopiazonic acid (Fig. 3). However, we again found that isoproterenol and forskolin seemed to have no effect on the relationship between $[Ca^{2+}]_i$ and force (either at 24°C or 30ºC) (Fig. 3). These results suggest that, in guinea pig papillary muscle, activation of the $\beta$-adrenoceptors does not change the $Ca^{2+}$ sensitivity of the myofilament.

As shown in Fig. 5, cAMP (100 $\mu$M), added during the $Ca^{2+}$ loading, augmented the caffeine-induced contracture in the guinea pig ventricles permeabilized with $\alpha$-toxin. This result might be due to an augmentation of SR $Ca^{2+}$ uptake that occurs via PK-A induced phosphorylation of phospholamban (1). In any case, this result suggests that the cAMP/PK-A system is intact in this preparation. However, 100 $\mu$M cAMP failed to change the maximum contraction, the Hill coefficient and the level of $EC_{50}$ in the pCa-force relationship, a result that supports the implication obtained in studies of intact muscle, namely, that activation of PK-A by $\beta$-adrenergic stimulation has no effect on myofilament $Ca^{2+}$ sensitivity. On the contrary, the addition of PK-A together with cAMP decreased the contractile force elicited by 1 $\mu$M $Ca^{2+}$ in saponin-permeabilized muscles (Fig. 4), as has been demonstrated by Johns et al. (28) in guinea pig trabeculae. These results suggest that, although guinea pig ventricles possess a cAMP/PK-A-dependent mechanism to decrease the $Ca^{2+}$ sensitivity of the contractile element, the intrinsic activity of PK-A in guinea pig ventricles is not sufficient to modulate the $Ca^{2+}$ sensitivity. An alternative explanation may be that in our skinned preparation, troponin (Tn)-I phosphorylation was not sufficient to decrease the myofilament $Ca^{2+}$ sensitivity, whereas phospholamban phosphorylation was sufficient to augment $Ca^{2+}$ uptake by the SR. However, it has been suggested that the time-courses of phosphorylation of these two proteins are similar in response to isoproterenol (29), and that there is about a 20

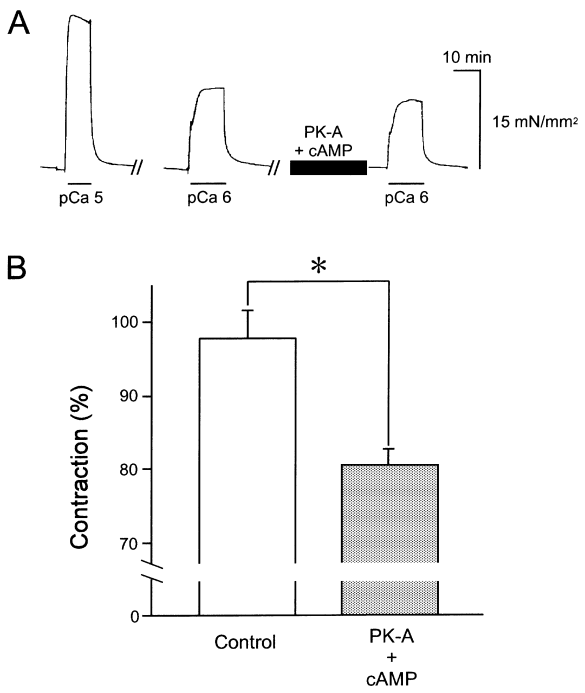


**Fig. 4.** Effects of PK-A on contractions elicited by 1 $\mu$M $Ca^{2+}$ in saponin-permeabilized guinea pig ventricles. In PK-A treated muscles (n = 3), 1000 units/ml PK-A was applied with 100 $\mu$M cAMP in a relaxing solution for 45 min. Control muscles (n = 4) were incubated without PK-A and cAMP for 45 min. The 100% level represents the level of contraction induced by the first application of 1 $\mu$M $Ca^{2+}$. A: A typical trace in PK-A treated muscles. B: Summary of the results in panel A. *Significantly different from the value in the control, with $P<0.05$. Differences between mean values were evaluated by the non-paired Student's *t*-test.

times higher sensitivity in Tn-I phosphorylation towards isoproterenol (and the increase in intracellular cAMP content) compared with phospholamban phosphorylation (30).

In contrast to the findings in guinea pig ventricles, isoproterenol was observed in rat papillary muscle to elicit a rightward shift of the relationship between $[Ca^{2+}]_i$ and force during the relaxation phase after a tetanic contraction at 24°C (Fig. 2). This result agrees with previous observations that $\beta$-adrenoceptor stimulation results in apparent myofilament $Ca^{2+}$ desensitization in intact rat tissue (6), isolated cells (5) and permeabilized preparations (31, 32). Our results from guinea pig and rat ventricles suggest that there are species differences in the effect of $\beta$-adrenoceptor stimulation on the $Ca^{2+}$ sensitivity of the myofilament.

Even in rat papillary muscle, isoproterenol did not alter the relationship between $[Ca^{2+}]_i$ and force at 30°C (Fig. 2B). Similarly, O'Rourke et al. (12) did not observe the isoproterenol-induced decrease in myofilament $Ca^{2+}$ sensitivity in rat single myocytes at 37°C. These results suggest that the decrease in myofibrillar $Ca^{2+}$ sensitivity elicited by $\beta$-adrenergic stimulation may be diminished by raising the temperature. In support of this suggestion, previous studies showing myofilament $Ca^{2+}$ desensitization in rat hearts were performed at lower temperatures (<25°C) (5, 6). On the other hand, there is a report suggesting that $\beta$-adrenergic stimulation decreases myofilament $Ca^{2+}$ sensitivity of rat ventricular muscle at 30°C, in which $Na^+$ deficient contracture was decreased by isoproterenol (4). The methodological differences to evaluate $[Ca^{2+}]_i$ may be re-

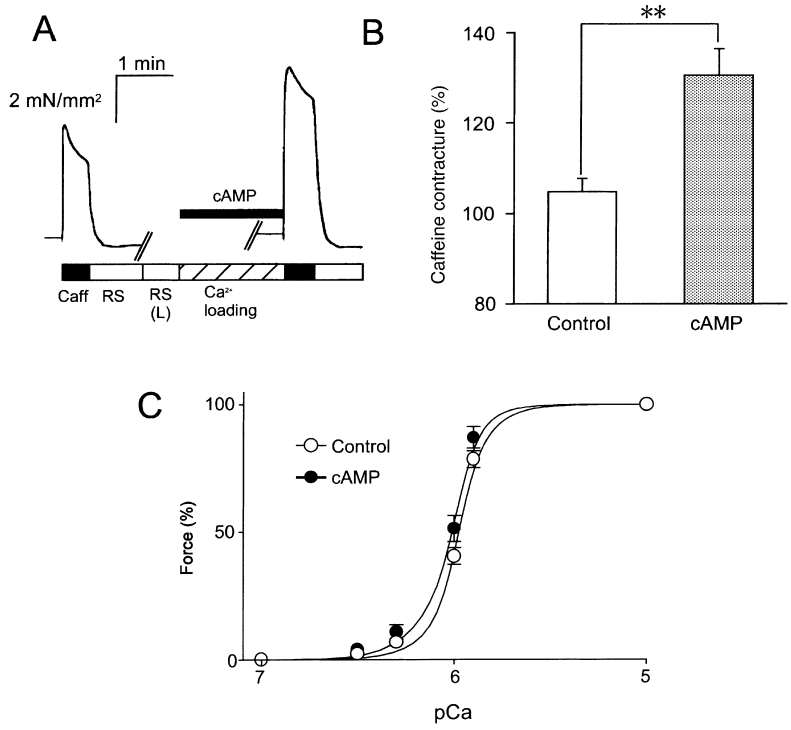


**Fig. 5.** Effects of cAMP on contractions in ventricles permeabilized with $\alpha$-toxin. A: A typical trace of the effects of 100 $\mu$M cAMP on $Ca^{2+}$ loading of SR. Caff = caffeine, RS = relaxing solution, RS(L) = low EGTA relaxing solution. B: Summary of the results in panel A. In cAMP treated muscles (cAMP, n = 7), 100 $\mu$M cAMP was applied to the muscles during the SR $Ca^{2+}$ loading period for 15 min. Control: cAMP untreated muscles (n = 4). **Significantly different from the value in the control, with $P<0.01$. Differences between mean values were evaluated by the non-paired Student's *t*-test. C: Effects of cAMP on the pCa-force relationship. Force was normalized to the peak force at pCa 5. Muscles were pretreated with 100 $\mu$M cAMP (n = 7) in a relaxing solution for 20 min. Control: cAMP untreated muscles (n = 7).

sponsible for this discrepancy; fluorescent indicators were used in the present study and by O'Rourke et al. (12), while aequorin was used by Kurihara and Konishi (4). Further studies are needed to clarify this point.

It has been reported that the increase in myofilament $Ca^{2+}$ sensitivity in skinned mammalian ventricular muscle is temperature-dependent (33, 34), in which the $Ca^{2+}$ sensitivity of Tn-C may play an important role (35). Therefore, it may be possible that increase in $Ca^{2+}$ sensitivity of Tn-C with the increase in temperature competes with the effect of Tn-I phosphorylation. Because the temperature-dependency of Tn-I phosphorylation by PK-A has not been evaluated, further studies are needed to clarify this possibility.

The difference in temperature-dependency between kinase and phosphatase has been reported in smooth muscle, in which increases in temperature augmented the activity of myosin phosphatase more strongly than the activity of myosin light chain kinase (36). Thus, one possible explanation for the effect of temperature on myofilament $Ca^{2+}$ sensitivity is the difference in temperature-dependency between PK-A and phosphatase for Tn-I. On the other hand, it has been reported that the level of myosin light chain phosphorylation is increased by $\beta$-adrenergic stimulation and enhances the $Ca^{2+}$ responsiveness of the contractile apparatus (37). Therefore, the temperature-dependency of myosin light chain phosphorylation should also be taken into account for the temperature dependency of the $\beta$-adrenergic effect. In such a case, net effects of $\beta$-adrenergic stimulation on $Ca^{2+}$ sensitivity of myofilament may be determined by the balance between the effect on Tn-I phosphorylation (desentitization) and the effect on myosin light chain phosphorylation (sensitization). In the present study, we attempted to demonstrate the temperature-dependency of the $Ca^{2+}$ desensitization mechanism in permeabilized rat ventricles. However, after being permeabilized with $\alpha$-toxin, the tissue lost its PK-A-mediated functions by an unknown mechanism. Further study is therefore needed to clarify the precise mechanism of the temperature dependency.

In conclusion, there are species- and temperature-dependent differences in the regulation of myofilament $Ca^{2+}$ sensitivity by $\beta$-adrenergic stimulation.

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