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ALTERATIONS IN CONTRACTILE PROPERTIES AND Ca^{2+} TRANSIENTS BY β- AND MUSCARINIC RECEPTOR STIMULATION IN FERRET MYOCARDIUM

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SUMMARY

1. To clarify the mechanism which regulates the time course of twitch tension when β- and muscarinic receptors are stimulated, intracellular Ca^{2+} transients, Ca^{2+} sensitivity of the contractile element and the cross-bridge cycling rate (CCR) were measured in ferret ventricular muscles.

2. Isoprenaline (Iso; 0·1 μM) increased peaks of Ca^{2+} transients measured with aequorin and tension, and abbreviated the time courses of both signals. Addition of acetylcholine (ACh; 0·01–1 μM) to the Iso-treated preparation dose dependently decreased the peaks of both signals and restored the time course of Ca^{2+} transients. However, the time course of tension was not recovered by the addition of ACh, and the relaxation time in particular, was further shortened by ACh. Carbachol (1 μM) applied to the Iso-treated preparation yielded similar results.

3. $[Ca^{2+}]_i$ and tension at a quasi-steady level of tetanic contraction, which was produced by ryanodine (5 μM) and repetitive stimulation, were measured and Ca^{2+} sensitivity of the contractile element was estimated. Iso (0·1 μM) decreased the Ca^{2+} sensitivity and the addition of ACh (1 μM) completely recovered it to the control level.

4. In order to measure CCR, the perturbation analysis method was applied to steady-state tension of tetanic contraction. The CCR was not altered even when the tetanic tension level was decreased to 50 % by decreasing $[Ca^{2+}]_o$. Iso (0·1 μM) slightly decreased the tetanic tension level and increased the CCR from 2·73 to 3·25 Hz. The effect of Iso was observed when the Iso-decreased tension was recovered by an increase in $[Ca^{2+}]_i$. The addition of ACh (1 μM) recovered the CCR which was increased by Iso, to the control level. Atropine (10 μM) blocked the effect of ACh, and carbachol (1 μM) restored the CCR increased by Iso to the control level.

5. The time course of Ca^{2+} transients, Ca^{2+} sensitivity and CCR were antagonistically regulated by β- and muscarinic receptor stimulation, but the time course of tension did not parallel the changes in these parameters. Therefore, these results suggest that the time course of tension, particularly the relaxation time, is not determined by the time course of Ca^{2+} transients, Ca^{2+} sensitivity and the CCR, and that other factors might be involved in the regulation of the time course of tension when β- and muscarinic receptors are stimulated.

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INTRODUCTION

Contractile properties of cardiac muscle are regulated by neurotransmitters of the autonomic nervous system. Stimulation of β-adrenoceptor increases the magnitude of intracellular Ca^{2+} transients and tension in twitch contraction and shortens the time courses of both signals (Allen & Kurihara, 1980; Kurihara & Konishi, 1987; Endoh & Blinks, 1988; Okazaki, Suda, Hongo, Konishi & Kurihara, 1990). The effects of β-adrenoceptor stimulation which alter the time courses of twitch tension and Ca^{2+} transients are considered to be induced by the phosphorylation of phospholamban in sarcoplasmic reticulum (SR) and troponin-I (Tada, Kirchberger, Repke & Katz, 1974; Ray & England, 1976). The phosphorylation of phospholamban in the SR quickly removes Ca^{2+} from the myoplasm and the decrease in Ca^{2+} sensitivity of the contractile element, induced by the phosphorylation of troponin-I, dissociates Ca^{2+} from its binding sites on the myofilament at a faster rate. Therefore, the time course of contraction is considered to be regulated by the time course of intracellular Ca^{2+} transients and Ca^{2+} sensitivity of the contractile element when β-adrenoceptor is stimulated.

However, there are controversial reports that the time course of Ca^{2+} transients and Ca^{2+} sensitivity cannot explain the time course of twitch tension, particularly the relaxation time (Kurihara & Allen, 1982; McIvor, Orchard & Lakatta, 1988; Okazaki *et al.* 1990). Hoh, Rossmanith, Kwan & Hamilton (1988) indicated that the change in the time course of twitch tension induced by β-adrenoceptor stimulation is due to the faster cross-bridge cycling rate (CCR) and it is prominent in the heart with the fast-type myosin isozyme (V_1). Therefore, the CCR is considered to be a factor which determines the time course of twitch contraction. In addition, the synchronization of each myocyte in contraction and relaxation might be related to the time course of twitch contraction (Okazaki *et al.* 1990).

On the other hand, there is not sufficient evidence on the mechanism of muscarinic receptor stimulation to confirm alteration of the contractile properties of cardiac muscle, particularly when β-adrenoceptor is stimulated, although muscarinic receptor stimulation is known to inhibit twitch tension and Ca^{2+} current in mammalian atrial and ventricular muscles (Ten Eick, Nawrath, McDonald & Trautwein, 1976; Hino & Ochi, 1980). McIvor *et al.* (1988) simultaneously measured Ca^{2+} transients and tension in ferret ventricular muscles and showed a dissociation of the time course of relaxation in twitch contraction and Ca^{2+} sensitivity when acetylcholine (ACh) was applied to preparations treated with isoprenaline (Iso). However, if the time courses of twitch contraction and Ca^{2+} transients are altered, the assessment of change in the Ca^{2+} sensitivity of the contractile element by measuring the peaks of tension and Ca^{2+} transients is difficult (Allen & Kurihara, 1980). Therefore, measurement of the relation between tension and $[Ca^{2+}]_i$ at a steady state is necessary to observe the change in the Ca^{2+} sensitivity of the contractile element. However, there is no direct evidence regarding the effect of muscarinic receptor stimulation on the Ca^{2+} sensitivity of the contractile element measured at steady state, although Winegrad (1984) suggests that muscarinic receptor stimulation antagonizes the decreased Ca^{2+} sensitivity induced by Iso in hyperpermeable cells. In addition, the CCR has not been measured when the

muscarinic receptor is stimulated in preparations treated with β-adrenoceptor stimulant, which is thought to alter the time course of twitch tension.

In the present study, we investigated the mechanism which alters the time course of twitch tension, particularly the relaxation time when β- and muscarinic receptors are stimulated. For this purpose, intracellular Ca^{2+} transients, Ca^{2+} sensitivity and the CCR were measured in intact ferret ventricular muscles.

The preliminary results of this study were presented at the annual meeting of the Japanese Physiological Society (Hongo, Tanaka, Suda & Kurihara, 1990).

METHODS

Preparations

Ferrets (body weight, 600–1000 g) were anaesthetized by intraperitoneal injection of pentobarbitone (150 mg kg^{-1}), and hearts were quickly removed. The right ventricular wall was opened and the thin papillary muscles were dissected out. The diameter of the preparations was $0{\cdot}80 \pm 0{\cdot}04$ mm (mean ± S.E.M., less than 1·03 mm, $N = 30$) and the length was $4{\cdot}01 \pm 0{\cdot}17$ mm (mean ± S.E.M., less than 6·5 mm, $N = 30$). Both ends of the preparation were tied with silk threads. The preparation was transferred to an experimental chamber and mounted horizontally between a length driver (JOCX-101A, General Scanning Co. Inc., CA, USA), which is capable of imposing length changes within 4 ms, and a tension transducer (BG-10, Kulite, New Jersey, USA, compliance 2·5 μm g^{-1}, unloaded resonant frequency 0·6 kHz). Electrical field stimulation was applied to the preparation using a pair of platinum electrodes placed parallel to the preparation. Muscle length (L) was adjusted to L_{max} at which developed tension was maximal. To evoke twitch tension, the preparation was regularly stimulated by a 5 ms duration single square pulse at 0·2 Hz, and the strength of the stimulation was 1·5-fold threshold. To produce tetanic contraction, the muscle was stimulated by repetitive square pulses with 40 ms duration at 10 Hz in the presence of ryanodine (5 μM), and tetanic tension level was altered by changing the concentration of Ca^{2+} in solutions from 1 to 20 mM. The strength of the stimulation was 3-fold threshold; the threshold was measured in each solution (see Yue, Marban & Wier, 1986 and Okazaki *et al.* 1990).

Solutions

Normal Tyrode solution used for dissection of preparations 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 l^{-1}; pH, 7·34 at 30 °C when equilibrated with 5% CO_2 + 95% O_2. Tyrode solution buffered with Hepes (*N*-2-hydroxy ethyl-piperazine-*N'*-2-ethanesulphonic acid) (Hepes–Tyrode solution) used in the experiments 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, 10; and insulin, 5 units l^{-1}; pH was adjusted to 7·35 with NaOH at 30 °C and was equilibrated with 100% O_2. When $[Ca^{2+}]_o$ was changed, $CaCl_2$ was added or decreased without compensating osmotic pressure. In the experiments to increase $[Ca^{2+}]_o$ to 40 mM however, the concentration of NaCl was decreased to 78 mM. The temperature of the solution was monitored with a thermocouple and maintained at 30 ± 0·5 °C.

Aequorin injection and light detection

Aequorin (purchased from Dr J. R. Blinks) was dissolved in the 150 mM KCl and 5 mM Hepes solution at pH 7·0, with a final concentration of 50–100 μM. For aequorin injection, microelectrodes with a resistance of 30–50 MΩ were made and filled with aequorin. Aequorin was injected into 50–100 superficial cells of each preparation by applying high pressure nitrogen gas. Aequorin light signals were detected with a photomultiplier placed just above the preparation and recorded simultaneously with tension. Data were stored on a magnetic tape (NFR-3515W, Sony Magnescale Inc., Japan) and a computer for later analysis. In twitch response, light signals were recorded through a 500 Hz low-pass filter and 32–128 signals were averaged to improve the signal-to-noise ratio. In the figures in the present study, the light signal is expressed as the fractional luminescence (FL, logarithm of light signal normalized to the maximum light; Blinks, Wier, Hess & Prendergast, 1982).

In the tetanic experiment, the light signals were recorded through a 1 Hz low-pass filter and two to four signals were averaged. In order to obtain the relation between $[Ca^{2+}]_i$ and tension, the light signals were converted to $[Ca^{2+}]_i$ using the calibration curve of a method similar to that of Blinks *et al.* (1982). The calibration curve was made at 30 °C and the composition of the solution used for the calibration curve was similar to that described in the paper of Kurihara & Konishi (1987). The constants used for the conversion of the light signals to $[Ca^{2+}]_i$ in the present study were as follows: n, 3·14; K_R, $4·025 \times 10^6$; K_{TR}, 114·6 (see Blinks *et al.* 1982).

Measured parameters

For evaluation of the effects of β- and muscarinic receptor stimulation, the following parameters were measured in twitch response: (1) peaks of light and tension, (2) time-to-peak light (TPL) and time-to-peak tension (TPT), which represent the time measured from the onset of stimulus to the peaks of light and tension, respectively, (3) half-decay time of light (T_{L50}) which is the time for the light to decay from the peak to 50%, (4) decay time of light (T_{L75-25}) which is the time for the light to decay from 75 to 25% of the peak, (5) half-relaxation time (T_{R50}) which is the time for the tension to fall from the peak to 50%, and (6) relaxation time (T_{R75-25}) which is the time for the tension to fall from 75 to 25% of the peak.

To analyse the relationship between $[Ca^{2+}]_i$ and tension in the tetanized preparation, the Hill equation was used, and two constants were calculated: $K_{\frac{1}{2}}$, $[Ca^{2+}]_i$ required for half-maximal tension development, and n, slope of the relationship between $[Ca^{2+}]_i$ and tension (see Results section).

Perturbation analysis

In order to measure the cross-bridge cycling rate (CCR), a method similar to that used by Saeki, Sagawa & Suga (1978) for steady tension in Ba^{2+} contracture, was applied to tetanic contraction. Tetanic contraction was produced in Hepes-Tyrode solution containing 20 mM $[Ca^{2+}]_o$ and ryanodine (5 μM). At steady-state tension level in the tetanic contraction, sinusoidal length changes with a constant amplitude were applied to the preparation using the length driver mentioned above. The amplitude of the length change was less than 1% of L_{max} and the frequency was between 0·7 and 10 Hz. The temperature of the solution was kept at 30 °C. Peak-to-peak amplitude of force in response to length changes was measured and the frequency at which amplitude became a minimum (dip) was determined as the CCR (Saeki *et al.* 1978; Hoh *et al.* 1988).

Drugs

The following drugs were used: L-isoprenaline D-bitartrate (Nacalai Tesque Inc., Tokyo), acetylcholine chloride (Daiichi Pharmaceutical Co. Ltd, Tokyo), ryanodine (Agri-Systems, Inc., Pennsylvania), and Bay K 8644 (a gift from Bayer, AG). A stock solution of isoprenaline (1 mM) was prepared by dissolving in double-distilled water containing 50 μM EDTA and 30 μM ascorbic acid to prevent oxidation, and kept at a low temperature. Bay K 8644 (1 mM) was dissolved in ethanol for the stock solution. The direct effects of the drugs on aequorin luminescence were checked and were found to be negligible.

Statistical analysis

Measured values were expressed as mean ± standard error of the mean (S.E.M.) and Student's paired t test was employed for statistical analysis. Statistical significance was verified at the 5% level of the P value in the two-sided test unless otherwise specified. N indicates the number of experiments.

RESULTS

Effects of acetylcholine on peaks of Ca^{2+} transients and tension modulated by β-adrenoceptor stimulation

In twitch response, isoprenaline (Iso) increased the peaks of Ca^{2+} transients and tension and abbreviated the time courses of both signals (Fig. 1*A*, *B*, *F* and *G*) (Okazaki *et al.* 1990). Since the effects of Iso on the peaks of Ca^{2+} transients and tension were almost maximal at a concentration of 0·05 μM, as reported by Okazaki

et al. (1990), 0·1 μM Iso was used in the present study. In some preparations (four of seven experiments), a slight increase in the light signal (extra-light) was observed during the relaxation phase (Fig. 1*B* and *C*, indicated by an arrow). The addition of acetylcholine (ACh) to the preparation treated with Iso decreased the peaks of Ca^{2+}

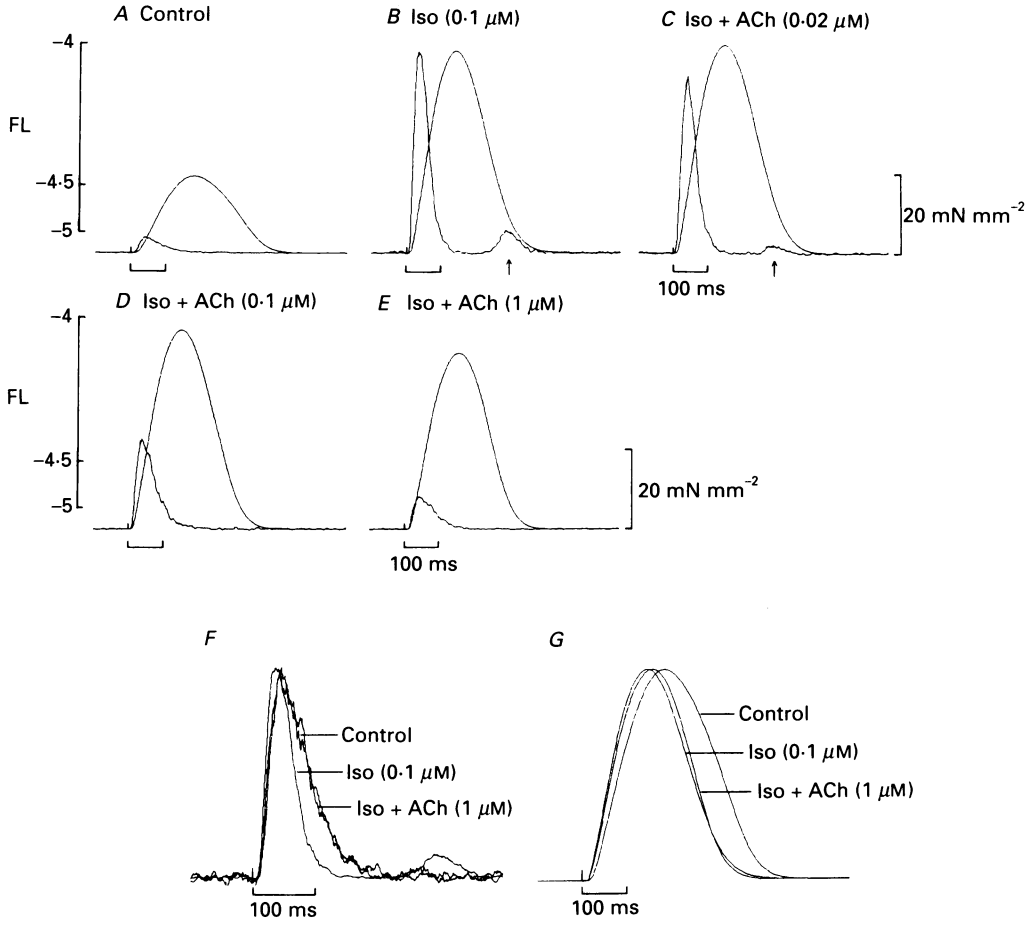

Fig. 1. Effects of acetylcholine (ACh) on light transients and tension in isoprenaline (Iso)-treated preparations. Iso (0·1 μM) increased peaks of light transients and tension (*B*). ACh dose dependently decreased peaks of both signals increased by Iso (*C–E*). Noisy and fast traces are light signals, and the concentrations of the drugs are indicated in each panel. The extra-light is observed in *B* and *C* (indicated by arrows). In *F* peaks of light signals in control (*A*), in Iso (*B*) and in addition of ACh (1 μM) (*E*) are normalized and superimposed. In *G*, tension traces from *A*, *B* and *E* are normalized and superimposed. Small vertical marks just before both signals indicate application of stimuli.

transients and tension which were increased by Iso (Fig. 1*C–E*), and the inhibitory effect of ACh on the Ca^{2+} transients was almost maximal at 1 mM (six experiments). The peaks of Ca^{2+} transients and tension decreased shortly after the addition of ACh and then partially recovered after a few minutes, as was observed in the preparation which was not treated with Iso (fade) (see Boyett, Kirby, Orchard & Roberts, 1988).

Since the time courses of Ca^{2+} transients and tension were slightly different in the early and later phases after the addition of ACh, we measured the time courses of both signals at a nearly steady level, 10 min after the addition of ACh. The peak of Ca^{2+} transients recovered to the control level after the addition of ACh and no significant difference compared to that in control was observed at 1 μM ACh. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) calculated from the peak of Ca^{2+} transients in the control was $0{\cdot}61 \pm 0{\cdot}15$ μM ($N = 7$). Iso (0·1 μM) increased $[Ca^{2+}]_i$ to $2{\cdot}18 \pm 0{\cdot}31$ μM ($N = 7$) and the addition of ACh (1 μM) decreased it to $0{\cdot}88 \pm 0{\cdot}08$ μM ($N = 6$). In the presence of Iso, time-to-peak light (TPL) was shortened (Fig. 1*F*) and the gradient of $[Ca^{2+}]_i$ might be increased. Since the aequorin light signal relates to $[Ca^{2+}]_i$ in the highest Ca^{2+} concentration region, the calculated $[Ca^{2+}]_i$ in the presence of Iso might be overestimated. However, it is not essential in the following experiments. $[Ca^{2+}]_i$ in the presence of Iso and ACh (1 μM) was not significantly different from that in the control. Therefore, we compared the time courses of Ca^{2+} transients in the control and those in Iso + ACh (1 μM) at nearly the same amplitude (see below). The peak tension in the presence of Iso (0·1 μM) was 235% of the control. In the presence of both Iso and ACh (1 μM), the peak tension was still 60% higher than that of the control, although the difference compared to that of control was not significant. The extra-light observed in some preparations treated with Iso disappeared with application of a higher concentration of ACh (Fig. 1*D* and *E*).

Effects of ACh on the time courses of Ca^{2+} transients and tension

Iso significantly shortened the time-to-peak light (TPL) and the time-to-peak tension (TPT) (Okazaki *et al.* 1990). The addition of ACh dose dependently recovered both parameters. A higher concentration of ACh (1 μM) completely restored the TPL. The TPL in the presence of Iso and ACh (1 μM) was not significantly different from that of the control ($43{\cdot}2 \pm 2{\cdot}0$ ms, $N = 7$ in the control; $44{\cdot}2 \pm 2{\cdot}3$ ms, $N = 6$ in the presence of Iso and ACh). However, TPT partially recovered when ACh (1 μM) was applied to the Iso-treated preparation ($193{\cdot}4 \pm 4{\cdot}0$ ms, $N = 7$ in the control; $171{\cdot}9 \pm 6{\cdot}7$ ms, $N = 6$ in the presence of Iso and ACh). The TPT in the presence of Iso and ACh (1 μM) was significantly prolonged compared to that in Iso alone, but was still significantly shorter than that in the control.

Iso also shortened the time course of the light signal decay (T_{L75-25}, T_{L50}) and the relaxation time (T_{R75-25}, T_{R50}). T_{L75-25} and T_{L50} were both markedly shortened by Iso, and these were restored in a dose-dependent manner by the addition of ACh. T_{R75-25} in the control was $96{\cdot}17 \pm 5{\cdot}90$ ms ($N = 7$) and Iso significantly shortened it to $87{\cdot}32 \pm 9{\cdot}03$ ms ($N = 7$). However, the addition of ACh (1 μM) to the Iso-treated preparation further shortened T_{R75-25} to $71{\cdot}93 \pm 3{\cdot}88$ ms ($N = 6$) which was significantly shorter than that in Iso alone or in control. T_{R50} in the control was $145{\cdot}25 \pm 13{\cdot}30$ ms ($N = 7$) and Iso shortened it to $120{\cdot}87 \pm 11{\cdot}72$ ms ($N = 7$). Similarly to T_{R75-25}, T_{R50} was significantly shortened by the addition of ACh (1 μM) to the Iso-treated preparation ($103{\cdot}22 \pm 7{\cdot}23$ ms, $N = 6$). Therefore, there was a clear dissociation between the decay time of the light signal and the relaxation time when the preparation was exposed to Iso and ACh. In addition, the effects of ACh (1 μM) on the light signal and tension were completely blocked by atropine (10 μM) (data not shown).

Effects of carbachol on Ca^{2+} transients and tension modulated by β-adrenoceptor stimulation

Carbachol, a potent muscarinic agonist, decreased the peaks of light transients and tension which were potentiated by Iso (0·1 μM) (Fig. 2*A*, *B* and *C*). The peaks of light transients and tension in the presence of both Iso and carbachol were similar to those in the control (Fig. 2*A* and *C*). The changes in the time courses of both signals were

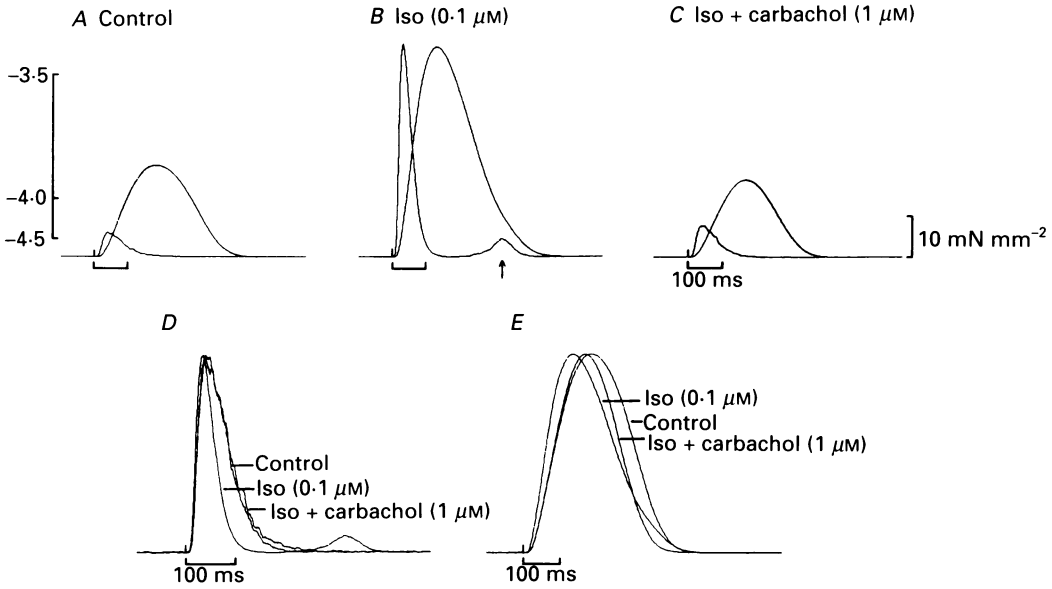

Fig. 2. Effects of carbachol on light transients and tension in Iso-treated preparations. Carbachol (1 μM) decreased the peaks of light signals and tension which were increased by Iso (0·1 μM) (*C*). The extra-light shown in *B* (indicated by an arrow) was attenuated by the addition of carbachol. In *D* and *E*, peaks of light signals and tension, respectively, are normalized and superimposed.

qualitatively similar to those observed with ACh (three experiments). The time course of Ca^{2+} transients recovered but the relaxation time was further shortened; this change was much more obvious than that occurring with ACh (Fig. 2*D* and *E*). In one experiment to study the effects of choline on the time courses of Ca^{2+} transients and tension, choline chloride at 1 mM did not alter the time courses of either parameter shortened by Iso. Therefore, muscarinic receptor stimulation in the presence of Iso is necessary for the abbreviation of the relaxation time.

Changes in Ca^{2+} sensitivity of the contractile element by Iso and ACh in the tetanized preparations

As the change in the relaxation time (T_{R75-25}, T_{R50}) did not parallel that of the decay time of the light transients (T_{L75-25}, T_{L50}), the change in the Ca^{2+} sensitivity of the contractile element was observed by measuring the relation between $[Ca^{2+}]_i$ and

tension in tetanic contraction. Repetitive stimulation (40 ms duration, 10 Hz) produced tonic tension in the ryanodine (5 μM)-treated preparation (Fig. 3*A*); the relationship between $[Ca^{2+}]_i$ and tension at a quasi-steady level (not transient as in twitch) could be measured (Yue *et al.* 1986; Okazaki *et al.* 1990). The measured light

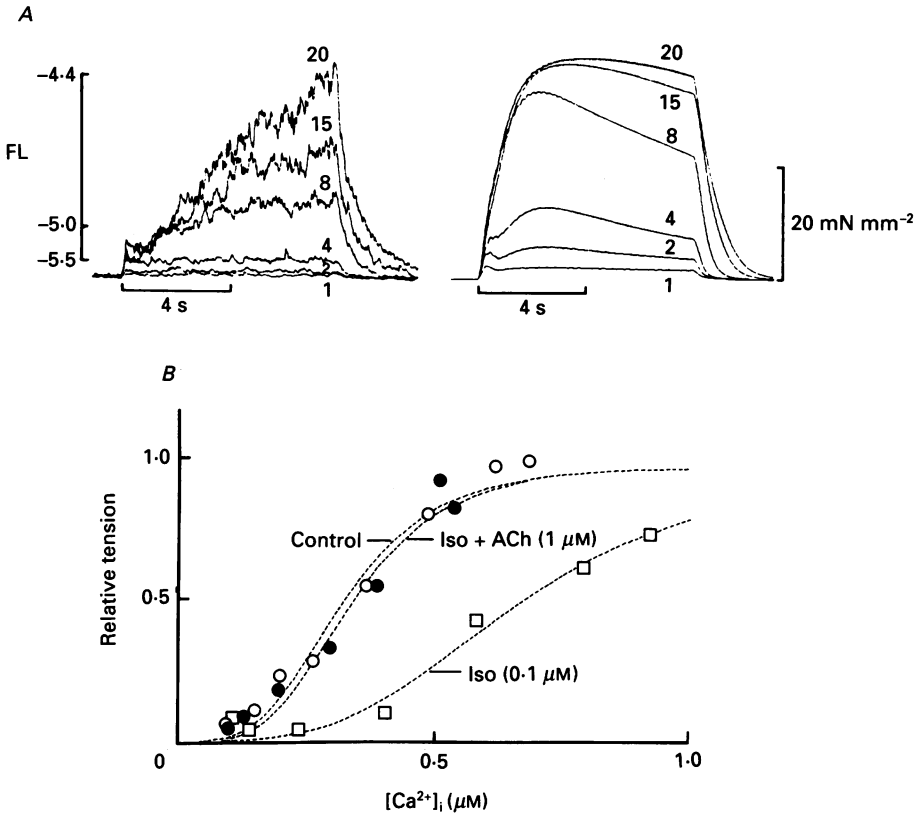

Fig. 3. Effects of Iso and ACh on the relation between $[Ca^{2+}]_i$ and tension. *A* shows the original records of light signals (left) and tension (right) in tetanic stimulation without drugs. Numbers beside each trace represent $[Ca^{2+}]$ in solutions (mM). Light signals and tension were measured at 6 s, and the light signal was converted to $[Ca^{2+}]_i$ using the calibration curve. *B* shows the relation between $[Ca^{2+}]_i$ and tension. The ordinate is relative tension and the abscissa is $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ and tension were fitted using the Hill equation (see text). Iso (0·1 μM) shifted the relationship to the right, and the addition of ACh (1 μM) completely restored the relationship to the control level.

signal was converted to $[Ca^{2+}]_i$ using the calibration curve (see Methods) and the relationship between $[Ca^{2+}]_i$ and tension (t) was fitted by the Hill equation which is expressed as follows:

$$t = [Ca^{2+}]_i^n / (K_{\frac{1}{2}}^n + [Ca^{2+}]_i^n).$$

Since $K_{\frac{1}{2}}$ and n (Hill coefficient) varied according to the time after the onset of stimulation, both parameters were measured at 6 s after the onset of stimulation as

in a previous report (Okazaki *et al.* 1990). At 6 s, tetanic tension did not reach a complete steady level; a quasi-steady state was obtained between 4 s and 8 s (see Fig. 8 in Okazaki *et al.* 1990) and the relation between $[Ca^{2+}]_i$ and tension was measured.

Iso (0·1 μM) significantly shifted the relationship between $[Ca^{2+}]_i$ and tension to the right (Okazaki *et al.* 1990), and the addition of ACh (1 μM) completely restored the

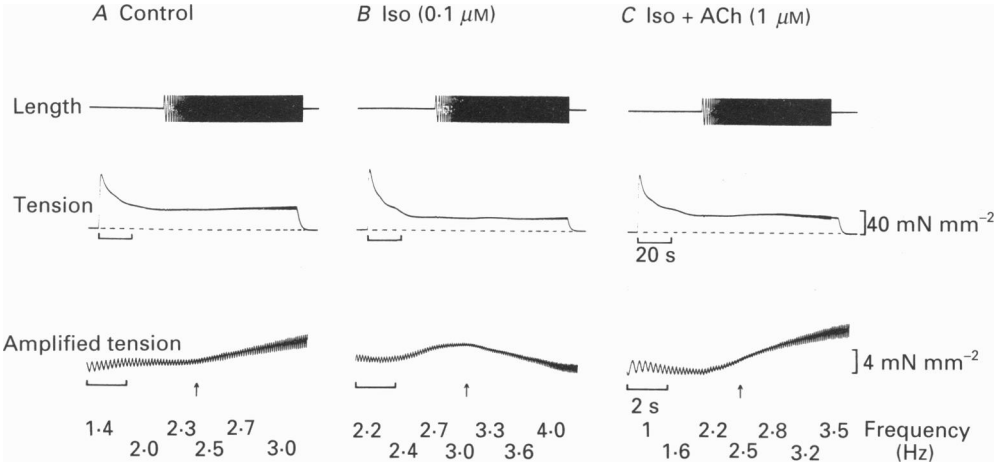

Fig. 4. Measurement of the cross-bridge cycling rate (CCR). Upper traces show the command signal of the sinusoidal length change at various frequencies. Middle traces show tension of tetanic contraction which is produced in the solution containing ryanodine (5 μM) and 20 mM $[Ca^{2+}]_o$. Lower traces show the amplified tension response to the length change. Frequency of sinusoidal length change is indicated at the bottom of the figure. Upper and middle traces have the same time scale. Note the different time scale in the lower trace. The peak-to-peak amplitude of tension response at each frequency was measured to determine dip. The frequency of the dip is indicated by arrows under the lower traces.

altered relationship to that of the control (Fig. 3*B*; $N = 5$). In one preparation of five experiments, the curve was shifted to the left beyond the control curve by ACh. In addition, the increased $K_{\frac{1}{2}}$ and the decreased n by Iso were also restored to the control level by ACh. In the control, $K_{\frac{1}{2}}$ was $0{\cdot}48 \pm 0{\cdot}05$ μM ($N = 5$) and was increased to $0{\cdot}75 \pm 0{\cdot}05$ μM ($N = 5$) by Iso (0·1 μM). ACh (1 μM) applied to the Iso-treated preparation restored $K_{\frac{1}{2}}$ to $0{\cdot}51 \pm 0{\cdot}05$ μM ($N = 5$), which was not significantly different from that in the control. Similarly, the Hill coefficient (n) was decreased from $4{\cdot}18 \pm 0{\cdot}69$ ($N = 5$) to $3{\cdot}65 \pm 0{\cdot}52$ ($N = 5$) by Iso, and the addition of ACh restored the decreased n to $4{\cdot}59 \pm 0{\cdot}56$ ($N = 5$). No statistically significant difference was observed in the Hill coefficient in the control and in the presence of Iso and ACh. These results suggest that the Ca^{2+} sensitivity of the contractile element is antagonistically regulated by β- and muscarinic receptor stimulation.

Changes in cross-bridge cycling rate by Iso and ACh

Since the decreased Ca^{2+} sensitivity of the contractile element by Iso was restored by ACh, the alteration in the CCR by Iso and ACh was observed using a perturbation method similar to that applied in studies of Ba^{2+} contracture (Saeki *et al.* 1978; Hoh

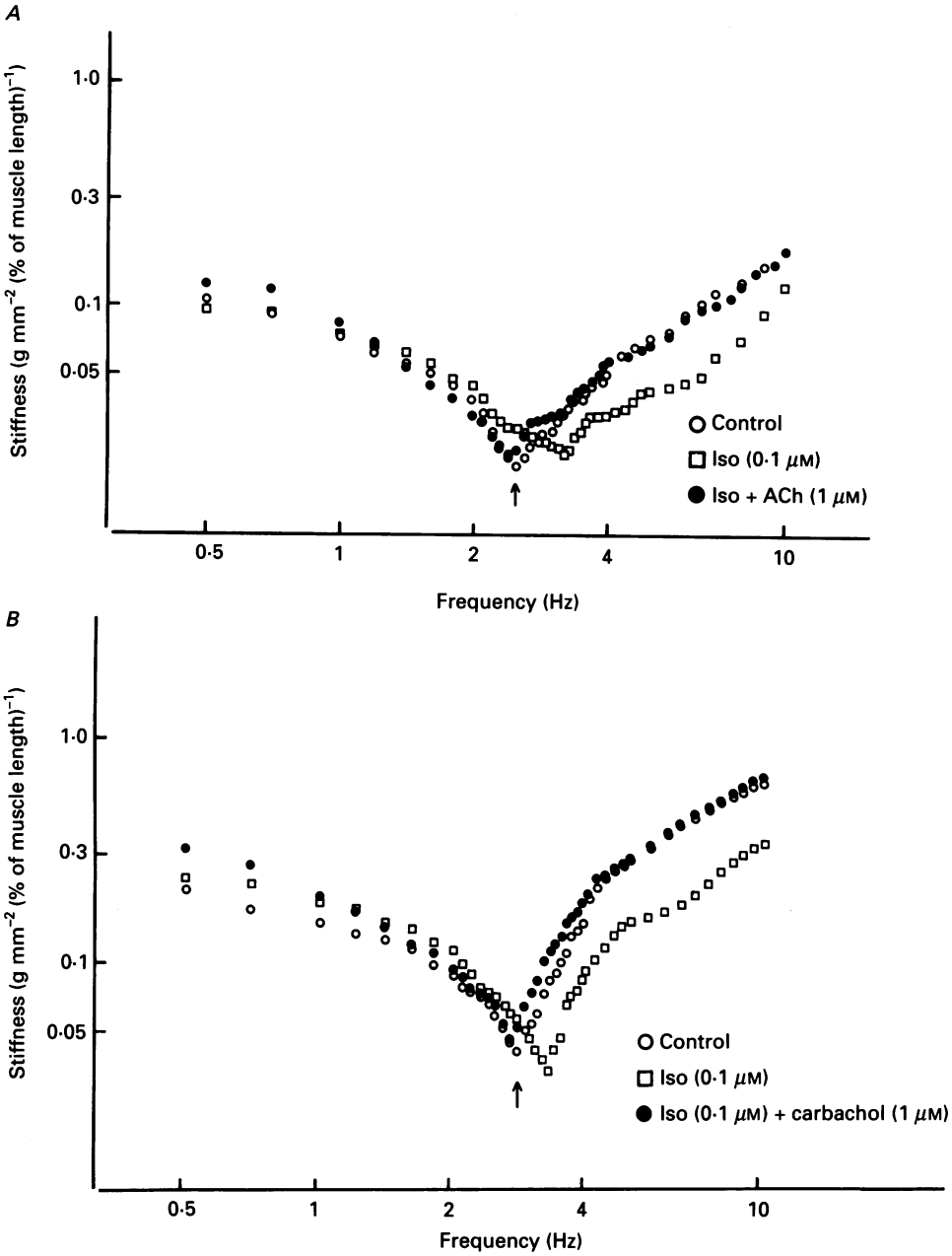

Fig. 5. Effects of Iso and ACh (*A*) or carbachol (*B*) on the cross-bridge cycling rate (CCR). The ordinate is muscle stiffness which is calculated from the amplitude of tension response (see Fig. 4), and the abscissa is frequency of sinusoidal length change. Both scales are logarithmic. The frequency at which the muscle stiffness is minimal (dip) was determined (indicated by an arrow in control). Iso (0·1 μM) shifted the frequency of dip to a higher frequency. Addition of ACh (1 μM) restored the frequency of dip to the control level (*A*). Similarly, carbachol (1 μM) also restored the frequency of dip, increased by Iso, to the control level (*B*).

et al. 1988; Berman, Peterson, Yue & Hunter, 1988). In these studies, the stiffness during a steady-state tension level of Ba^{2+} contracture was measured, and the frequency at which the muscle stiffness becomes minimal (dip) was considered as the CCR. They also indicated that the CCR is independent of the activation level and the

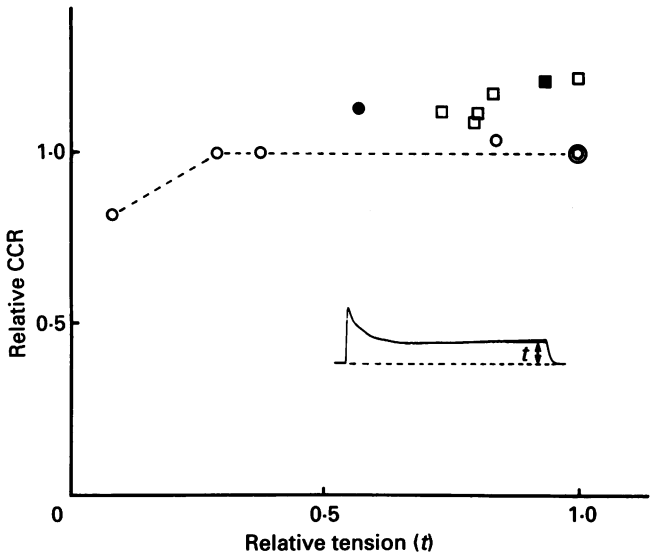

Fig. 6. The dependence of the cross-bridge cycling rate (CCR) on the tension level in tetanic contraction and the effect of Iso on the CCR at different tension levels. The ordinate is relative CCR and abscissa is relative tension in long tetanus. The inset shows the measured tetanic tension level (t). Measured CCR and steady-state tension are normalized to those in 20 mM $[Ca^{2+}]_o$ without drugs (◎). ○, $[Ca^{2+}]_o$ was changed from 4 to 20 mM to produce varying tension levels. ●, Iso (0·1 μM) was added to 20 mM $[Ca^{2+}]_o$ solution. ■, Iso (0·1 μM) was added to 40 mM $[Ca^{2+}]_o$ solution with 78 mM NaCl to compensate osmotic pressure. □, Iso (0·1 μM) and various concentrations of Bay K 8644 (0·1–0·5 μM) were added to 20 mM $[Ca^{2+}]_o$ solution. Combined results of seven experiments.

activators which produce tension (Ca^{2+} or Ba^{2+}). Here, to measure the CCR, we applied the perturbation analysis method with a steady-state tension level of tetanic contraction, produced using ryanodine and repetitive stimulation, as in the determination of Ca^{2+} sensitivity (Fig. 4). Repetitive stimulation, applied to the preparation treated with ryanodine (5 μM) and 20 mM $[Ca^{2+}]_o$, produced an initial large tension which then gradually declined. Finally, the preparation showed a sustained tension 20–30 s after the onset of the stimulation (Fig. 4, middle traces). The length (L) change applied to the preparation was less than 1 % of L_{max} (usually 0·6–0·7 %) and the tension response to the length changes at various frequencies were measured (Fig. 4, lower traces). In the control (at 30 °C), the frequency of dip which showed minimal stiffness was $2·73 \pm 0·11$ Hz ($N = 4$). Iso (0·1 μM) increased the frequency of dip to $3·25 \pm 0·10$ Hz ($N = 4$) (Figs 4*B* and 5*A*). These results are compatible with those of previous reports which suggested an increase in the CCR by β-adrenoceptor stimulation (Hoh *et al.* 1988; Berman *et al.* 1988; Saeki, Shiozawa, Yanagisawa & Shibata, 1990). The addition of ACh (1 μM) restored the Iso-altered

frequency of dip to the control level ($2{\cdot}75 \pm 0{\cdot}13$ Hz, $N = 4$) (Figs 4*C* and 5*A*). The effect of ACh was completely blocked by atropine (10 μM), and carbachol (1 μM) showed an antagonistic effect similar to that observed with ACh (Fig. 5*B*).

There is a possibility that the changes of the CCR in Iso and in Iso + ACh were due to a change of the tension level in tetanic contraction. Therefore, the dependence of the CCR on the tension level in tetanic contraction was observed by changing $[Ca^{2+}]_o$. The CCR was not significantly altered when the change in tension level was less than 50% (Fig. 6). As Iso (0·1 μM) decreased the tension level, we measured the CCR at a tension level similar to the control, which was produced by the addition of Bay K 8644 or by increasing $[Ca^{2+}]_o$ to 40 mM. An increase in the CCR was also observed at a tension level similar to that of the control (Fig. 6). Therefore, the increase in the CCR caused by Iso and the recovery of the CCR caused by ACh were not the result of a change in the tension level of tetanic contraction.

DISCUSSION

Effects of β- and muscarinic receptor stimulation on the magnitude of Ca^{2+} transients and tension

The present study confirmed changes similar to those of a previous study (Allen & Kurihara, 1980; Endoh & Blinks, 1988; Okazaki *et al.* 1990) regarding the magnitudes and the time courses of the Ca^{2+} transients and tension induced by β-adrenoceptor stimulation in mammalian ventricular muscles. ACh applied to the Iso-treated preparation decreased the peaks of Ca^{2+} transients and tension as in previous reports (Fig. 1) (McIvor *et al.* 1988; Endoh, 1989). The effects of ACh on both signals are not a time-dependent change induced by Iso, because (1) the immediate application of ACh (1 μM) in the Iso-treated preparation caused a similar fade phenomenon and (2) long-term treatment of Iso (more than 1 h) did not cause fade (data not shown). The reduction in the peak tension by ACh was less than that of the peak of the Ca^{2+} transients (Fig. 1). There are two possible explanations for the above result: (1) the developed tension in the presence of Iso is saturated and a decrease in Ca^{2+} transients causes only a slight reduction in peak tension; (2) ACh recovers the Iso-decreased Ca^{2+} sensitivity of the contractile element (Fig. 3).

Alterations in the time courses of Ca^{2+} transients and tension by β- and muscarinic receptor stimulation

ACh restored the Iso-shortened time-to-peak light (TPL) to the control level. Similarly, the time course of the falling phase of the Ca^{2+} transients (T_{L75-25}, T_{L50}) in the presence of Iso and ACh was not significantly different from that of the control (Fig. 1*A*, *E* and *F*). Therefore, the Iso-shortened time course of the Ca^{2+} transients is restored to that of the control by ACh. Similar results were obtained when carbachol was added to the Iso-treated preparation (Fig. 2). The report by Lindemann & Watanabe (1985) stating that ACh reverses the enhanced Ca^{2+} uptake by Iso in the isolated SR supports our result.

However, McIvor *et al.* (1988) reported that ACh (1 μM) does not affect the Iso (1 μM)-altered time course of Ca^{2+} transients. The reason for these differing results is not clear, but the concentration of Iso in the present study differs from that in their

study. In the present study, 0·1 μM Iso was used which produced nearly maximal effects on the Ca^{2+} transients and tension, but McIvor *et al.* (1988) employed a higher concentration of Iso (1 μM). Thus, in their study, the concentration of ACh (1 μM) might not be sufficient to cause an alteration in the time course of the Ca^{2+} transients.

The ACh-induced recovery of the time course of tension did not parallel that of the Ca^{2+} transients. The addition of a higher concentration of ACh (1 μM) did not completely restore the Iso (0·1 μM)-abbreviated time-to-peak tension (TPT), and TPT in the presence of both Iso (0·1 μM) and ACh (1 μM) was still shorter than that in the control. On the other hand, the Iso-shortened relaxation time ($T_{R75\ 25}$, T_{R50}) was further abbreviated by ACh, and 1 μM ACh significantly shortened both parameters compared to those in 0·1 μM Iso. The ACh-induced abbreviation of the relaxation time of the Iso-treated preparation was not a time-dependent artifactual phenomenon, because the immediate application of a higher concentration of ACh (1 μM) (after observation of the effects of Iso) also clearly shortened the relaxation time. Carbachol, applied immediately after confirming the effects of Iso, also caused a faster relaxation and recovery of the time course of the Ca^{2+} transients (Fig. 2). These results indicate that the time course of Ca^{2+} transients is not a determinant of the time course of twitch contraction, and more specifically, that the relaxation time and the falling phase of the Ca^{2+} transients are not altered in parallel.

Since the dissociation of the relaxation time and the time course of the falling phase of the Ca^{2+} transients was not recognized when muscarinic receptor stimulation was blocked by atropine (data not shown), it appears that muscarinic receptor stimulation or the removal of β-stimulant (Okazaki *et al.* 1990) is necessary for the dissociation. If the signal transduction process of muscarinic receptor stimulation was blocked, the time courses of Ca^{2+} transients and tension were not changed, and the inhibition of adenylate cyclase was thought to be an important mechanism in the regulation of muscarinic receptor stimulation (Hongo, Tanaka & Kurihara, 1993). ACh, applied in normal Tyrode solution, did not cause any significant changes in the time course of tension (data not shown). In addition, the application of choline chloride (1 mM) or the simultaneous application of Iso (0·1 μM) and ACh (1 μM) (data not shown) did not show the same dissociation as mentioned above. These results indicate that prestimulation of the β-adrenoceptor before muscarinic receptor stimulation is essential for the dissociation.

Alteration in Ca^{2+} sensitivity of the contractile element by Iso and ACh

During tonic contraction of tetanus, Marban & Kusuoka (1987) reported no drastic change in intracellular ionic conditions 6–10 s after the start of tetanic stimulation. Therefore, we thought that it would be suitable to estimate the Ca^{2+} sensitivity by comparing $[Ca^{2+}]_i$ and tension 6 s after the start of the tetanic contraction. One of the problems in the use of tetanic contraction is the release of endogenous catecholamines by strong repetitive stimulation, as discussed in a previous report (Okazaki *et al.* 1990). The fact that the application of bupranolol (1 μM) or ACh (1 μM) without Iso caused a leftward shift of the relationship between $[Ca^{2+}]_i$ and tension suggests that endogenous catecholamines might be released during tetanic contraction. In addition, in some preparations in the present study, the addition of ACh to the Iso-treated preparation caused a leftward shift of the relation between $[Ca^{2+}]_i$ and

tension beyond the control curve. Thus we used the same stimulation strength throughout one tetanic experiment to minimize the change in the release of endogenous catecholamines.

It has been hypothesized that the rapid relaxation induced by β-adrenoceptor stimulation is due to rapid Ca^{2+} removal from the myoplasm by SR (Tada & Katz, 1982; Lindemann, Jones, Hathaway, Henry & Watanabe, 1983) and the rapid dissociation of Ca^{2+} from troponin which is caused by decreased Ca^{2+} sensitivity of the contractile element (Robertson, Johnson, Holroyde, Kranias, Potter & Solaro, 1982; England, Pask & Mills, 1984). However, a dissociation of the change in the Ca^{2+} sensitivity and relaxation time is observed when β-adrenoceptor is blocked after treatment of the preparation with Iso (Okazaki *et al.* 1990). The present study also indicates that the relaxation time is not altered in parallel with the Ca^{2+} sensitivity when the muscarinic receptor is stimulated in the Iso-treated preparation (Figs 1, 2 and 3). Therefore, the alteration of the Ca^{2+} sensitivity of the contractile element is not critical for the determination of the relaxation time when β- and muscarinic receptors are stimulated.

Alteration of the cross-bridge cycling rate in β- and muscarinic receptor stimulation

Hoh *et al.* (1988) showed an increase in CCR in rat ventricular muscle treated with adrenaline and suggested that an increase in CCR is a factor in faster contraction. In the present study, a similar perturbation method to that employed by Saeki *et al.* (1978) was applied to a tonic contraction of tetanus to measure the CCR. The two main reasons for the application of the perturbation analysis method to a tonic contraction of tetanus rather than a Ba^{2+} contracture are as follows: (1) it was intended to initiate sustained contraction by Ca^{2+} as in other experiments, and (2) although Ba^{2+} contracture can be sustained for 30–40 min at 20–25 °C, at 30 °C with repeated perturbation the contracture cannot be maintained for a long time period. Tetanic contraction was reproducible; the same CCR was observed in the repeat trials, with a preparation rest interval of 15–25 min between each tetanus. A clearly observable dip, which disappears in the rigor state with a low ATP concentration, suggests that the dip reflects active cross-bridge attachment and detachment.

Kusuoka, Weisfeldt, Zweier, Jacobus & Marban (1986) measured intracellular metabolites during tetanic contraction using NMR and observed an increase in intracellular hydrogen (H^+) and phosphate (P_i) concentration during long tetanic contraction. They noted a correlation between a decrease in maximal tension and an increase in P_i. Therefore, changes in the intracellular ionic condition during long tetanic contraction should be taken into consideration; the measured CCR might be influenced by the intracellular ionic condition. In skinned preparations of skeletal and cardiac muscle, P_i is known to increase the CCR but H^+ has no significant effect on the CCR of cardiac muscle (Mekhfi & Ventura-Clapier, 1988; Kawai & Halvorson, 1991; Kentish, 1991). On the other hand, when the concentration of P_i increases, due to a breakdown of ATP, the concentration of creatine phosphate (CP) is expected to decrease (Kusuoka *et al.* 1986). A decrease in the CP concentration is known to decrease the CCR of cardiac skinned preparations (Mekhfi & Ventura-Clapier, 1988). Therefore, the CCR measured during a steady-state long tetanic contraction might be influenced by both an increase in P_i and a decrease in CP which have opposite effects on the CCR. Since, in the present study, the effects of Iso and ACh on the CCR

were observed at a similar stage of tetanic contraction, the measured CCR is probably influenced by P_i and CP to a similar extent (see below).

The measured CCR was $2{\cdot}73 \pm 0{\cdot}11$ Hz ($N = 4$) at 30 °C, and was accordingly compatible with the CCR at 20 °C in Ba^{2+} contracture (1·0 Hz; Saeki, Kawai & Zhao, 1991), based on the fact that the temperature coefficient, Q_{10}, of CCR is considered to be 3·3 (18–31 °C) in cardiac muscle containing myosin isozyme V_3 (Rossmanith, Hoh, Kirman & Kwan, 1986), and ferret cardiac muscle predominantly consists of myosin isozyme V_3 (MacKinnon, Gwathmey, Allen, Briggs & Morgan, 1988). β-Adrenoceptor stimulation increased the CCR, as reported by Hoh *et al.* (1988) and others (Berman *et al.* 1988; Saeki *et al.* 1990), and muscarinic receptor stimulation by ACh and carbachol restored the increased CCR (Fig. 5). The tonic tension in the Iso-treated preparation was lower than that of the control, and there is a possibility that a change in the tension level influenced the CCR. However, the change in tension level brought about by altering $[Ca^{2+}]_o$ had no substantial influence on the CCR when tension was not changed more than 50% (Fig. 6). The results showing that an Iso-induced increase in the CCR is observed at a tension level similar to that in the control support the view that the Iso-induced increase in the CCR is not due to a change in the tension level of tetanic contraction. In addition, a decrease in tonic tension caused by Iso might not be due to an increase in P_i concentration alone which suppresses maximal tension. The observation that Iso shifted the relation between $[Ca^{2+}]_i$ and tension to the right (a decrease in Ca^{2+} sensitivity) suggests that tonic tension in the presence of Iso might not have reached the maximum. Furthermore, the fact that the Iso-induced tension decrease can be restored by increasing $[Ca^{2+}]_i$ with Bay K 8644 and a higher $[Ca^{2+}]_o$ support this view. Thus, the change in the CCR caused by β- and muscarinic receptor stimulation is not due to an alteration in tension level and the CCR is antagonistically regulated by β- and muscarinic receptor stimulation.

Possible mechanism involved in the determination of the relaxation time

In the presence of Iso, an increase in the Ca^{2+} transients causes extra-light (Figs 1 and 2; Allen & Kurihara, 1980; Okazaki *et al.* 1990) which appears at the tail of relaxation. As shown in Fig. 1, the magnitude of the extra-light in the presence of Iso (*B*) was similar to that of the light signal without Iso (*A*) but substantial tension development was not observed. This is probably due to asynchronous contraction of each cell and/or to a decrease in Ca^{2+} sensitivity of the contractile element at the tail of relaxation. In a Ca^{2+}-overloaded condition each cell contracts asynchronously and the developed tension is small although $[Ca^{2+}]_i$ is increased (Wier, Kort, Stern, Lakatta & Marban, 1983); therefore it seems feasible that the asynchronous contraction might occur during relaxation when $[Ca^{2+}]_i$ is increased. Thus, the relaxation time is influenced by Ca^{2+} removal, Ca^{2+} sensitivity, the CCR and the synchronization of each cell. In the presence of Iso, all the factors except synchronization shorten the relaxation time, but an increase in $[Ca^{2+}]_i$ which causes asynchronous contraction may retard relaxation. In the presence of ACh all factors which influence the relaxation time changed by Iso are restored to control levels, but the relaxation time becomes faster than in the presence of Iso. Therefore, some factors which accelerate relaxation by β-adrenoceptor stimulation are not antagonized by muscarinic receptor stimulation but rather are promoted.

One possible explanation is that dephosphorylation of a phosphorylated protein is related to the phenomenon. For example, the recovery of peak tension and the phosphorylation of troponin-I after removal of Iso do not show the same time course (England, 1976). Similarly, cholinergic stimulation of an amphibian heart preparation treated with Iso decreases phosphorylation of C-protein which is related to relaxation and developed tension, but the decrease in tension is more marked than that in dephosphorylation of C-protein (Hartzell & Titus, 1982). Thus, the prolongation of phosphorylation of some proteins which are related to the relaxation time but not to the other factors measured by us (the time course of Ca^{2+} transients, Ca^{2+} sensitivity and the CCR) might be involved in the alteration of the relaxation time.

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