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European Journal of Pharmacology 375 (1999) 177–196

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Review

Muscarinic regulation of Ca^{2+} signaling in mammalian atrial and ventricular myocardium

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Accepted 30 April 1999

Abstract

The differential regulation of the contractility of mammalian atrial and ventricular myocardium upon activation of muscarinic receptors can be ascribed, for the most part, to alterations in intracellular Ca^{2+} transients. However, alterations in myofibrillar sensitivity to Ca^{2+} ions also contribute to such regulation. In atrial muscle, the following actions are all associated with the corresponding alterations in the amplitude of Ca^{2+} transients in the same direction as those in the strength of the contractile force: (1) the direct inhibitory action on the basal force of contraction; (2) the increase (recovery) in force that is induced during the prolonged stimulation of muscarinic receptors; and (3) the rebound increase in force induced by washout of muscarinic receptor agonists. In addition, for a given decrease in force induced by muscarinic receptor stimulation in atrial muscle, the amplitude of Ca^{2+} transients is decreased to a smaller extent than the decrease in amplitude induced by reduction of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), an indication that muscarinic receptor stimulation might increase myofibrillar sensitivity to Ca^{2+} ions simultaneously with the reduction in the amplitude of Ca^{2+} transients during induction of the direct inhibitory action. In mammalian ventricular myocardium, the direct inhibitory action of muscarinic receptor stimulation exhibits a wide range of species-dependent variation. A pronounced direct inhibitory action is induced in ferret papillary muscle, which is also associated with a definite increase in myofibrillar sensitivity to Ca^{2+} ions. By contrast, in the ventricular myocardium of other species including the rabbit and the dog, muscarinic receptor stimulation scarcely affects the baseline Ca^{2+} transients and the force, but it results in a pronounced decrease in Ca^{2+} transients and force when applied in the presence of β-adrenoceptor stimulation, a phenomenon known as 'accentuated antagonism' or the 'indirect inhibitory action' of muscarinic receptor stimulation in mammalian ventricular myocardium. During induction of the indirect inhibitory action in mammalian ventricular myocardium, muscarinic receptor stimulation reverses all the effects induced by β-adrenoceptor stimulation, including the increase in Ca^{2+} transients, the positive inotropic and lusitropic effects, and the decrease in myofibrillar sensitivity to Ca^{2+} ions. The relationship between the amplitude of Ca^{2+} transients and force is unaffected during induction of the indirect inhibitory action in rabbit and dog ventricular myocardium. The direct and indirect inhibitory actions of muscarinic receptor stimulation on Ca^{2+} transients have clearly different dependences on frequency: the former is more pronounced at a higher rate of stimulation, while the latter is more pronounced at a lower rate. The more complex interaction of muscarinic receptor and β-adrenoceptor stimulation in mammalian atrial muscle and ferret ventricular muscle might be explained by the contribution of both the direct and the indirect regulatory mechanisms to the interaction.

Keywords: Muscarinic receptor stimulation; Ca^{2+} transient; Atrial muscle; Ventricular muscle, ferret; Ventricular muscle, mammalian; Direct inhibitory action; Indirect inhibitory action; β-Adrenoceptor stimulation; cAMP; cGMP; Phosphoinositide hydrolysis

1. Introduction

The heart is able to alter its pump function considerable in order to adapt to the hemodynamic requirements of the body. Cardiac contractile regulation can be achieved via the intrinsic contractile properties of cardiac muscle and via external regulatory mechanisms that involve neuronal and humoral interventions. External regulatory mechanisms under physiological conditions can be classified as facilitatory regulation and inhibitory regulation: the former is primarily achieved by β-adrenoceptor stimulation subsequent to sympathetic nerve activation, while the latter involves muscarinic and adenosine receptors. Intimate cross-talk occurs between the two regulatory systems.

The regulation of the contractility of mammalian cardiac muscle that is induced by stimulation of muscarinic

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00231-9

receptors is complex and varies among species of experimental animals (e.g., ferret vs. other mammals), among regions of the heart (e.g., atrium vs. ventricle) and with the physiological conditions (e.g., frequency of contraction) and the biochemical or metabolic conditions (e.g., cellular cyclic AMP (cAMP) level) under which the muscarinic receptor stimulation is applied (for reviews, see Blinks and Koch-Weser, 1963; Higgins et al., 1973; Löffelholz and Pappano, 1985). At least four different components can be distinguished in the overall inotropic response to muscarinic receptor stimulation, although some of the components might be mutually related and traceable to a common signal-transduction process. Since the early 1950s, intracellular signaling processes involved in these components have been analyzed in relation to the alterations of membrane electrophysiology induced by muscarinic receptor stimulation and a key role for the modulation of intracellular Ca^{2+} signaling has been proposed in contractile regulation. The variability of the inotropic response to muscarinic receptor stimulation can be attributed to differences in the relative prominence of four components, which can be specified as described in the following paragraphs.

1.1. A direct negative inotropic effect that is unrelated to the level of cAMP in the muscle and is exerted on the basal force of contraction

This effect is prominent in mammalian and amphibian atrial musculature and in ferret ventricular muscle but is scarcely present in the ventricular myocardium of most other mammals (Löffelholz and Pappano, 1985; Endoh, 1987; Boyett et al., 1988). This direct inhibitory action is strongly influenced by the frequency of contraction, that is to say, it becomes less pronounced when the interval between individual contractions is prolonged and it is absent when the interval between contractions is long enough (Blinks and Koch-Weser, 1963). The direct inhibitory action occurs in association with a substantial reduction in the duration of action potentials (Burgen and Terroux, 1953; Trautwein and Dudel, 1958; Baumann et al., 1963; Gertjegerdes et al., 1979). It probably reflects a cumulative depletion of intracellular stores of Ca^{2+} ions subsequent to curtailment of the entry of Ca^{2+} ions due to the abbreviation of successive action potentials that is induced by stimulation of muscarinic receptors. The direct inhibitory action of muscarinic receptor stimulation is also associated with a substantial abbreviation of contraction (Burgen and Terroux, 1953).

1.2. A positive inotropic effect that is elicited with a more gradual onset and offset than the direct inhibitory action during prolonged exposure to or after washout of muscarinic receptor agonists

It has been proposed that the positive inotropic effect during the recovery phase might result from accumulation of Na^+ ions in myocardial cells, which leads, in turn, to increased storage of Ca^{2+} ions in stores in the sarcoplasmic reticulum through the Na^+–Ca^{2+} exchanger (Korth and Kühlkamp, 1985; Saeki et al., 1997; Protas et al., 1998). The onset of this effect might be manifested as a 'recovery' from the direct inhibitory action during prolonged exposure to muscarinic receptor agonists. Because its offset is slower than that of the direct inhibitory action, the effect might lead to a 'rebound' or 'overshoot' in contractility above the baseline control level during washout of the muscarinic receptor agonist or after cessation of vagal nerve stimulation. Under such circumstances, this type of positive inotropic effect of muscarinic receptor stimulation is present in a relatively pure form and contractions are somewhat prolonged, even though the duration of action potentials is not altered significantly (Baumann et al., 1963; Gertjegerdes et al., 1979). While the receptor kinase-dependent desensitization of muscarinic receptors during prolonged exposure to muscarinic receptor agonists might contribute, in part, to the recovery phenomenon (Shui et al., 1995), there appears to be a definite positive inotropic component that reverses the direct inhibitory action, which is responsible for the rebound phenomenon, as will be discussed below.

1.3. A pronounced negative inotropic effect due to muscarinic receptor stimulation that scarcely affects the basal force of contraction in most mammalian ventricular myocardium (ferret is an exception) but occurs when muscarinic receptor stimulation is applied during activation of β-adrenoceptors

This negative inotropic effect, associated with a characteristic electrophysiological effect, is assumed to result from attenuation of previously promoted, cAMP-mediated, intracellular signal-transduction processes (Watanabe and Besch, 1975; Endoh, 1979; Biegon and Pappano, 1980; Rardon and Pappano, 1986). This effect can be readily investigated in mammalian ventricular myocardium, in general, where the direct inhibitory action is scarcely present, but the effect is present in atrial muscle also (for reviews, see Higgins et al., 1973; Löffelholz and Pappano, 1985; Endoh, 1987). This inhibitory action is termed an 'indirect inhibitory action' (secondary to inhibition of facilitated cAMP-mediated processes), an 'anti-adrenergic effect', or an 'accentuated antagonism' because the effect has been observed most often as an antagonistic effect on the β-adrenoceptor-mediated positive inotropic effect (Hollenberg et al., 1965; Levy, 1971).

1.4. A positive inotropic effect elicited by muscarinic receptor stimulation, which results from the release of norepinephrine from adrenergic nerve terminals within the myocardium (Dempsey and Cooper, 1969; Endoh et al., 1970; Higgins et al., 1973)

This effect is not prominent at standard experimental concentrations of muscarinic receptor agonists but is ob-

served at relatively high concentrations of muscarinic receptor agonists. It does not represent an effect of the stimulation of muscarinic receptors on cardiac muscle cells. Therefore, we shall not consider further in relation to the regulation of Ca^{2+} signaling in myocardial cells in the present study. Characteristics of this effect in the regulation of cardiac Ca^{2+} signaling are similar to those of the β-adrenoceptor stimulation that is mediated by the accumulation of cAMP (Kurihara and Konishi, 1987; Endoh and Blinks, 1988; Okazaki et al., 1990).

While these various effects of the stimulation of muscarinic receptors on myocardial contractility are transformed to yield a change in the mobilization of intracellular Ca^{2+} ions and/or a change in the sensitivity of myofilaments to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the final step of the signal transduction process, information on such changes in intact myocardial cells has been rather fragmentary and controversial because of the contributions of the various diverse components to the regulation of Ca^{2+} signaling that is induced by muscarinic receptor stimulation.

The present descriptions of the alterations of Ca^{2+} signaling that might be responsible for the regulation of myocardial contractility that is induced by stimulation of muscarinic receptors will be described on the basis of the findings in atrial and ventricular myocardium that has been loaded with the Ca^{2+}-sensitive bioluminescent protein aequorin (Blinks et al., 1978, 1982).

2. Methods

Hearts were excised from rabbits, guinea pigs and ferrets that had been lightly anesthetized with chloroform or ether. Pectinate muscle from rabbit and guinea-pig left atria and papillary muscles from rabbit or ferret right ventricles were dissected out in bicarbonate-buffered Krebs–Henseleit solution bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs–Henseleit solution used throughout the experiments was as follows (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 11.1. The pH was 7.4 when the solution had equilibrated with the mixture of 95% O_2 and 5% CO_2. Each muscle was mounted horizontally in an organ bath constructed for injection of aequorin. Freshly oxygenated solution was forced to circulate rapidly through an open-topped extension that contained the muscle, and bubbling occurred in another sidearm (Blinks and Endoh, 1986). The muscle was stimulated through punctate electrodes with square pulses of 5-ms duration at an intensity just above the threshold. The muscle was stretched to a length that gave an active tension of 50% of the tension at L_{max} in the case of atrial muscles and 90% in the case of papillary muscles. Aequorin was dissolved at approximately 2 mg/ml in a solution of 150 mM KCl and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.5, and microinjected into superficial cells of muscle preparations through fine-tipped micropipettes with a resistance of 35 to 50 MΩ in 150 mM KCl at 30°C (Blinks et al., 1978, 1982). After the injection, the muscle was transferred to an experimental apparatus constructed for simultaneous detection of aequorin light signals and changes in the force of contraction at 37.5°C (Blinks and Endoh, 1986). The concentration–response curve for carbachol in rabbit left atria was determined at a lower temperature (32.5°C). The experimental temperature in each series of experiments is given in the respective legend to the figures. The light emitted by the injected aequorin was detected with a photomultiplier (EMI 9635A; Thorn-EMI, Plainview, NY). For the records shown in this report, between 16 and 256 signals were signal-averaged. Atropine or bupranolol was allowed to act for more than 20 min before the administration of carbachol.

Drugs used were as follows: carbamylcholine chloride (carbachol, Sigma, St. Louis, MO); (−)-isoproterenol bitartrate and (−)-epinephrine bitartrate (Sterling-Winthrop, Rensselaer, NY); (−)-norepinephrine bitartrate hydrate (Calbiochem, San Diego, CA); (±)-bupranolol hydrochloride (Sanol, Monheim, Germany); and atropine sulfate (Merck, Rahway, NJ). Catecholamines were dissolved at 10^{-2} M in 1% ascorbic acid with 0.025 mM EDTA (ethylenediaminetetraacetic acid, disodium salt) as stock solutions and diluted on the day of each experiment with a 0.9% solution of NaCl (plus 0.025 mM EDTA). EDTA was included at 0.025 mM to retard the autoxidation of catecholamines.

3. Results

3.1. Direct inhibitory action on rabbit and guinea-pig atrial muscle and ferret ventricular muscle

In rabbit atrial muscle, a muscarinic receptor agonist, carbachol, administered in a cumulative manner, decreased the amplitude of aequorin signals and that of isometric contractions in a concentration-dependent manner and, apparently, in parallel (Fig. 1). The maximum direct inhibitory action of carbachol on atrial muscle was reached at a concentration of 10^{-6} M. The EC_{50} values for the inhibition of aequorin signals and isometric contractions were 1.5×10^{-9} and 3.0×10^{-9} M, respectively.

Injection of aequorin into atrial muscle cells was less successful than that into ventricular myocytes, probably because of the smaller size of individual atrial cells (Taniguchi et al., 1981) and the more abundant connective tissue on the endocardial surface of atrial muscle than on papillary muscle as observed macroscopically during injection of aequorin. Nevertheless, it was evident that a definite abbreviation of the duration of aequorin signals and of isometric contractions was associated with a reduction in

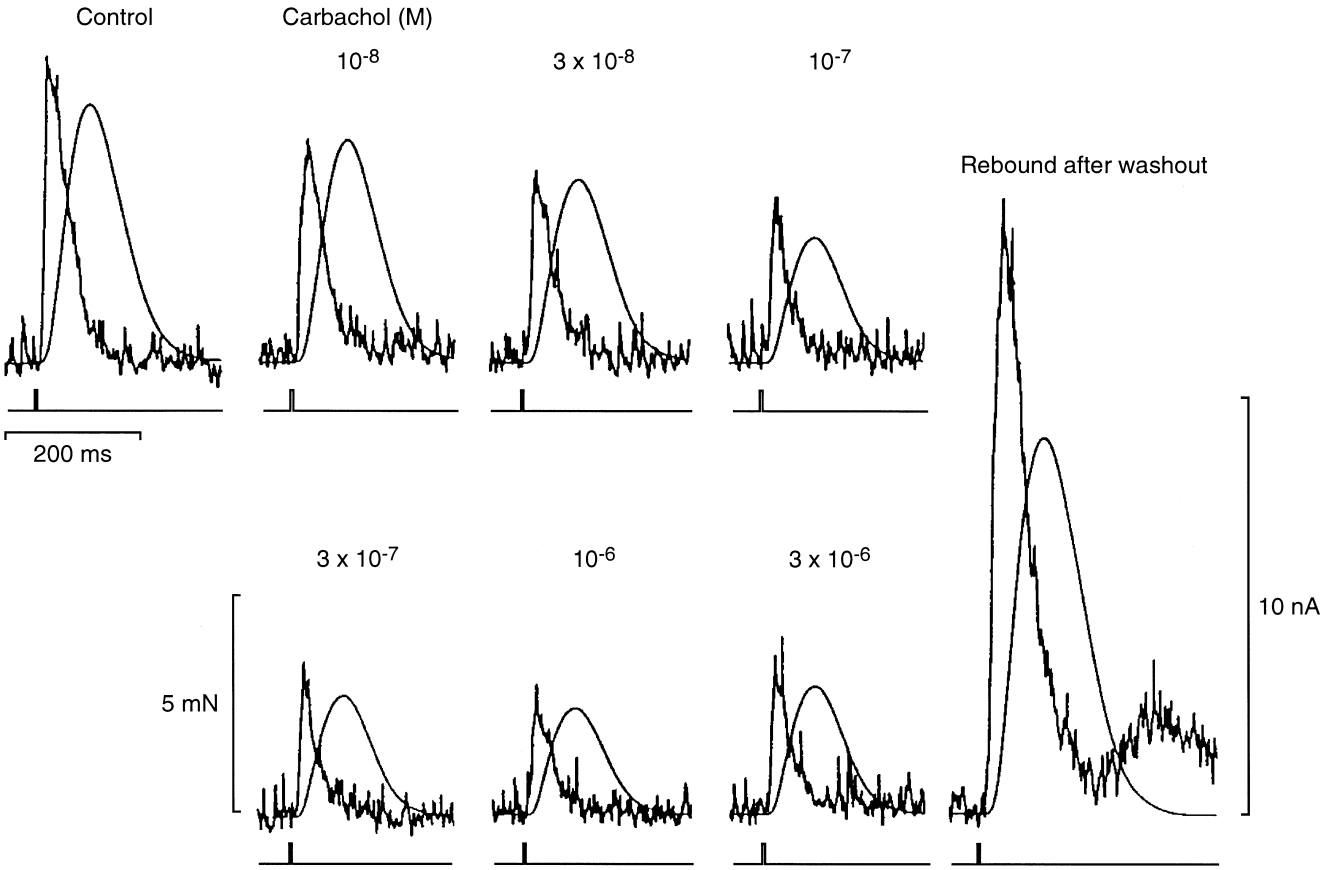


Fig. 1. Effects of carbachol administered in a cumulative manner on aequorin signals and isometric contractions in isolated rabbit left atrial muscle (11 mm long, with a cross-sectional area of 0.67 mm^2). Stimulation frequency, 1.25 Hz; temperature, 32.5°C. Signal-averaged records of 256 successive contractions with aequorin signals (noisy tracings) and isometric contractions (smooth tracings) are superimposed. The bottom tracing associated with each signal shows the timing of stimulation pulses. Tracings on the right side (rebound phenomenon) show aequorin signals and isometric contractions after washout of carbachol at 3×10^{-6} M, the highest concentration administered. Temperature was lowered for this series of experiments because of difficulty in determining the concentration–response curve for carbachol over a wide range of concentrations at 37.5°C.

force when the atrial muscle was exposed to increasing concentrations of carbachol, as shown on actual tracings and on peak-adjusted and superimposed tracings (Figs. 1 and 2), which were characteristic of activation of muscarinic receptors in atrial muscle.

A decrease in Ca^{2+} transients might be causally related to the direct inhibitory action of muscarinic receptor stimulation on the basal force of contraction. In addition, to clarify whether the myofibrillar sensitivity to Ca^{2+} ions was modified during induction of the direct inhibitory action, we compared the effect of carbachol on the relationship between the amplitude of Ca^{2+} transients and force with the effect observed during alteration of $[Ca^{2+}]_o$.

While carbachol abbreviated the duration of Ca^{2+} transients (Fig. 2A), elevation or lowering of $[Ca^{2+}]_o$ did not affect the duration of Ca^{2+} transients (Fig. 2B). In atrial muscle, when $[Ca^{2+}]_o$ was decreased or increased to yield a reduction or an elevation in force similar to that induced by carbachol or during its washout, the duration of Ca^{2+} transients and contractions was not appreciably affected (Fig. 2B).

During twitch contraction in intact cardiac muscle, the duration of Ca^{2+} transients does not last long enough to allow complete activation of myofibrils. This statement implies that, for a given elevation of $[Ca^{2+}]_i$, the amplitude of twitch contraction is lower than the level that is achieved during tetanic contraction in an equilibrium state (Yue, 1987; Yue et al., 1986). The abbreviation of Ca^{2+} transients produced by muscarinic receptor stimulation should, therefore, result in further dissociation of the force from the amplitude of Ca^{2+} transients in a direction that apparently indicates a decrease in myofibrillar sensitivity to Ca^{2+} ions.

It was evident, however, that, in spite of a definite abbreviation of Ca^{2+} transients by muscarinic receptor stimulation, for a given decrease in the amplitude of Ca^{2+} transients, the force was decreased less by carbachol than it was decreased by lowering $[Ca^{2+}]_o$ (Fig. 3A).

A similar result was obtained during induction of the direct inhibitory action in ferret ventricular myocardium (Fig. 3B). Our findings imply that muscarinic receptor stimulation has a paradoxical effect on Ca^{2+} signaling that

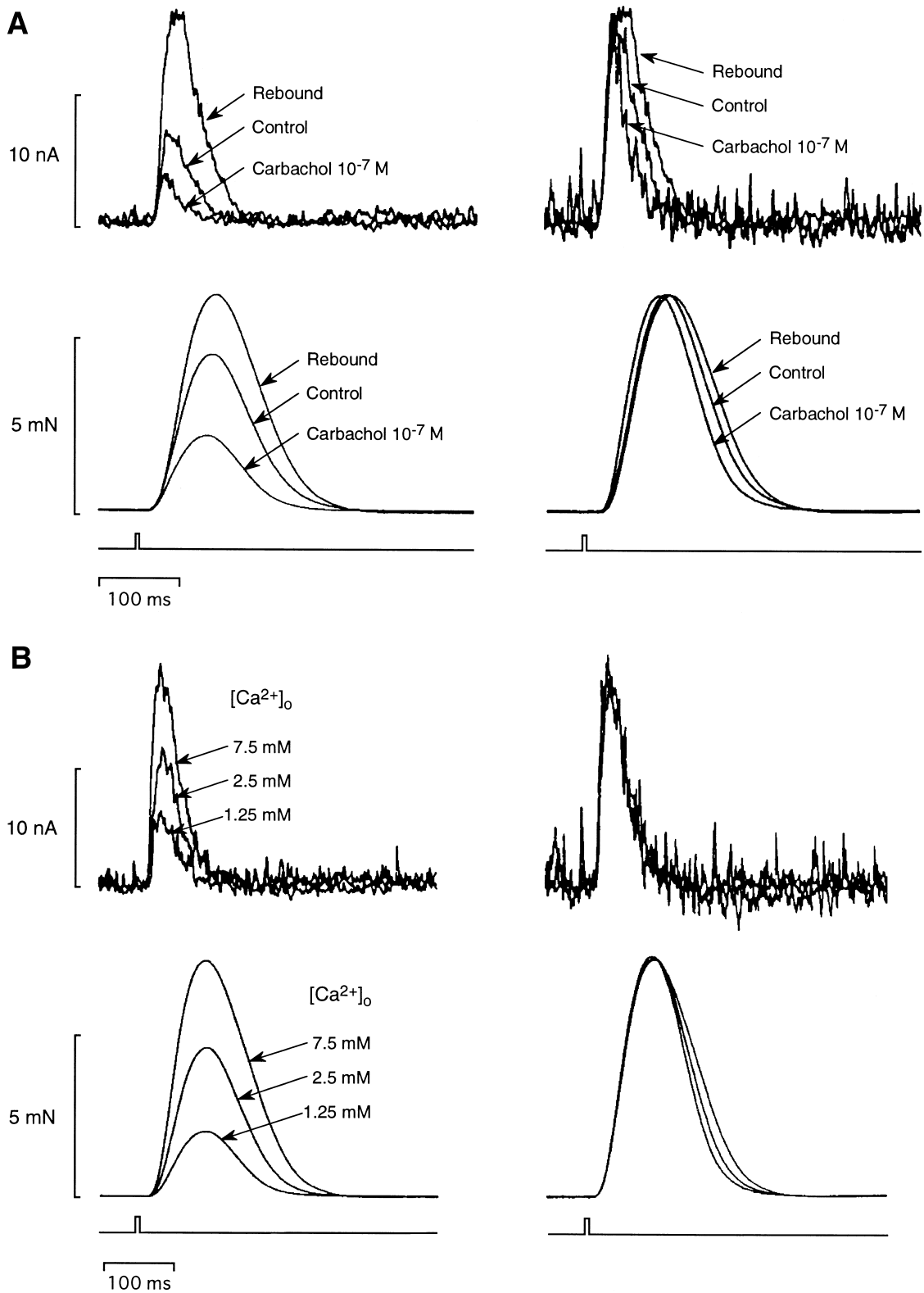


Fig. 2. Changes in time courses of aequorin signals and isometric contractions induced by 10^{-7} M carbachol, during the rebound increase after washout of 3×10^{-6} M carbachol, and upon changes in $[Ca^{2+}]_o$ in isolated rabbit left atrial muscle. Tracings were obtained from the same muscle as that for which results are shown in Fig. 1. (A) Left panels: actual tracings superimposed; right panels: the amplitude of signals has been adjusted to facilitate comparisons of the time courses of aequorin signals and isometric contractions. (B) Actual tracings recorded at 1.25, 2.5 and 7.5 mM $[Ca^{2+}]_o$; right panels: amplitudes of signals have been adjusted to facilitate comparisons of time courses. The temperature was 32.5°C. Signal-averaged records of 256 successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented.

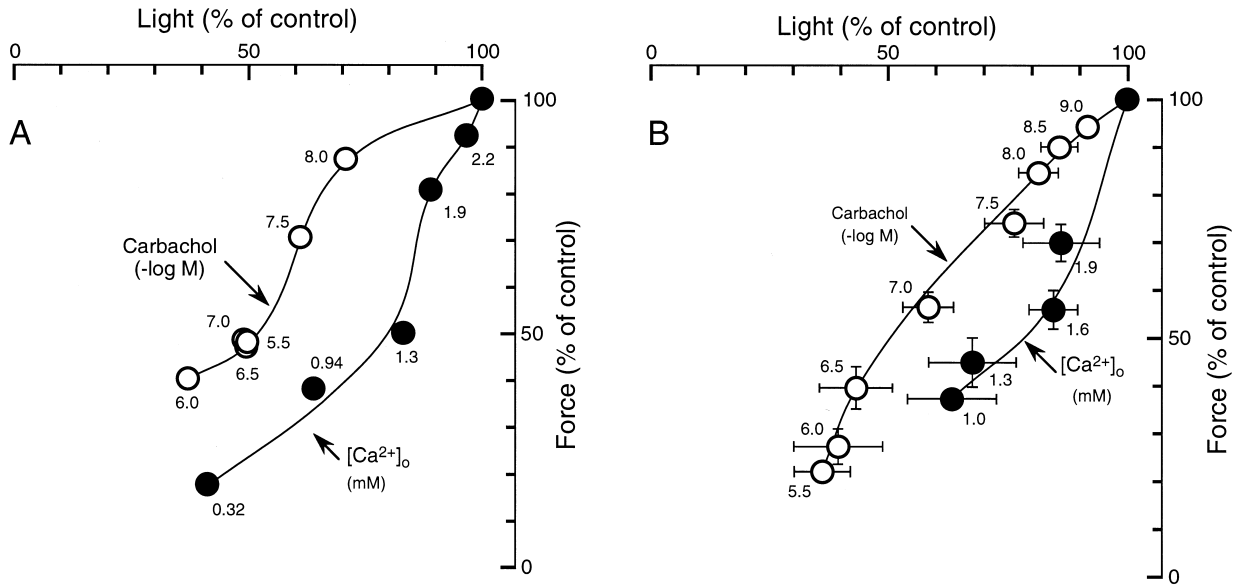


Fig. 3. (A) The relationship between changes in the amplitude of aequorin signals and isometric contractions during cumulative administration of carbachol and during changes in $[Ca^{2+}]_o$ in isolated rabbit left atrial muscle. Data are from the same muscle as that for which results are shown in Figs. 1 and 2. (B) The relationship between changes in the amplitudes of aequorin signals and isometric contractions during cumulative administration of carbachol and during changes in $[Ca^{2+}]_o$ in papillary muscles isolated from the ferret right ventricular myocardium electrically driven at 0.33 Hz. Experiments were performed at 37.5°C. Paired data for carbachol and $[Ca^{2+}]_o$ were obtained from each muscle ($n = 5$). Open circles: carbachol; closed circles: $[Ca^{2+}]_o$.

is the opposite of that produced by β-adrenoceptor stimulation, namely, attenuation of the mobilization of Ca^{2+} ions in association with an increase in myofibrillar sensitivity to Ca^{2+} ions.

As noted in Section 1, the direct inhibitory action of muscarinic receptor stimulation is markedly influenced by the frequency of contraction. It was evident that the direct inhibitory actions of carbachol on Ca^{2+} transients and force were more pronounced at high as compared to low frequencies of contraction (Fig. 4). At the steady state, in a strip of rabbit atrial muscle that was electrically stimulated at the markedly different frequencies of 0.06 and 2 Hz (with stimulation intervals of 16 and 0.5 s, respectively), carbachol at 10^{-6} M scarcely affected aequorin signals and isometric contractions at the lower frequency (Fig. 4, left panels), whereas at the same concentration carbachol caused a prominent reduction in aequorin signals and isometric force at the higher frequency (Fig. 4, right panels).

3.2. Ca^{2+} signaling during recovery and the rebound positive inotropic effect

Carbachol at 3×10^{-6} M produced a slight increase in the force of contraction as compared with carbachol at 10^{-6} M (Fig. 1). This slight increase in force was accompanied by a slight increase in the amplitude of aequorin signals. When carbachol at 10^{-6} M was administered initially as a single bolus, the inotropic response was biphasic: the force decreased and then increased. The increase in force was associated with a small but definite increase in the amplitude of aequorin signals (Fig. 5). At longer stimulation intervals, when the direct inhibitory action became less, the recovery increase became more pronounced as shown in Fig. 5.

After determination of the concentration–response relationship, when carbachol at the final concentration (3×10^{-6} M) had been washed out, we observed a large rebound increase in the force of contraction (Fig. 1). The aequorin signals recorded during this rebound response had a prominently large amplitude, and they were often followed by a second component (after-glimmer) that was similar to the rebound increase in force associated with after-contraction (Fig. 1), as also observed with cardiotonic agents, such as catecholamines and digitalis, that increase the intracellular mobilization of Ca^{2+} ions in myocardial cells.

The duration of aequorin signals and isometric contractions was increased during the rebound increases in aequorin signals and force (Fig. 2A). The prolongation of aequorin signals and force during the rebound increases was the opposite of the decrease produced by stimulation of β-adrenoceptors (Allen and Kurihara, 1980; Blinks et al., 1982; Morgan and Blinks, 1982; Kurihara and Konishi, 1987; Endoh and Blinks, 1988), an indication that the rebound increases were not due to stimulation of β-adrenoceptors by released catecholamines.

In strips of atrial muscle that were contracting at a low rate of stimulation, carbachol had a slight but unequivocal positive inotropic effect. The extent of this positive in-

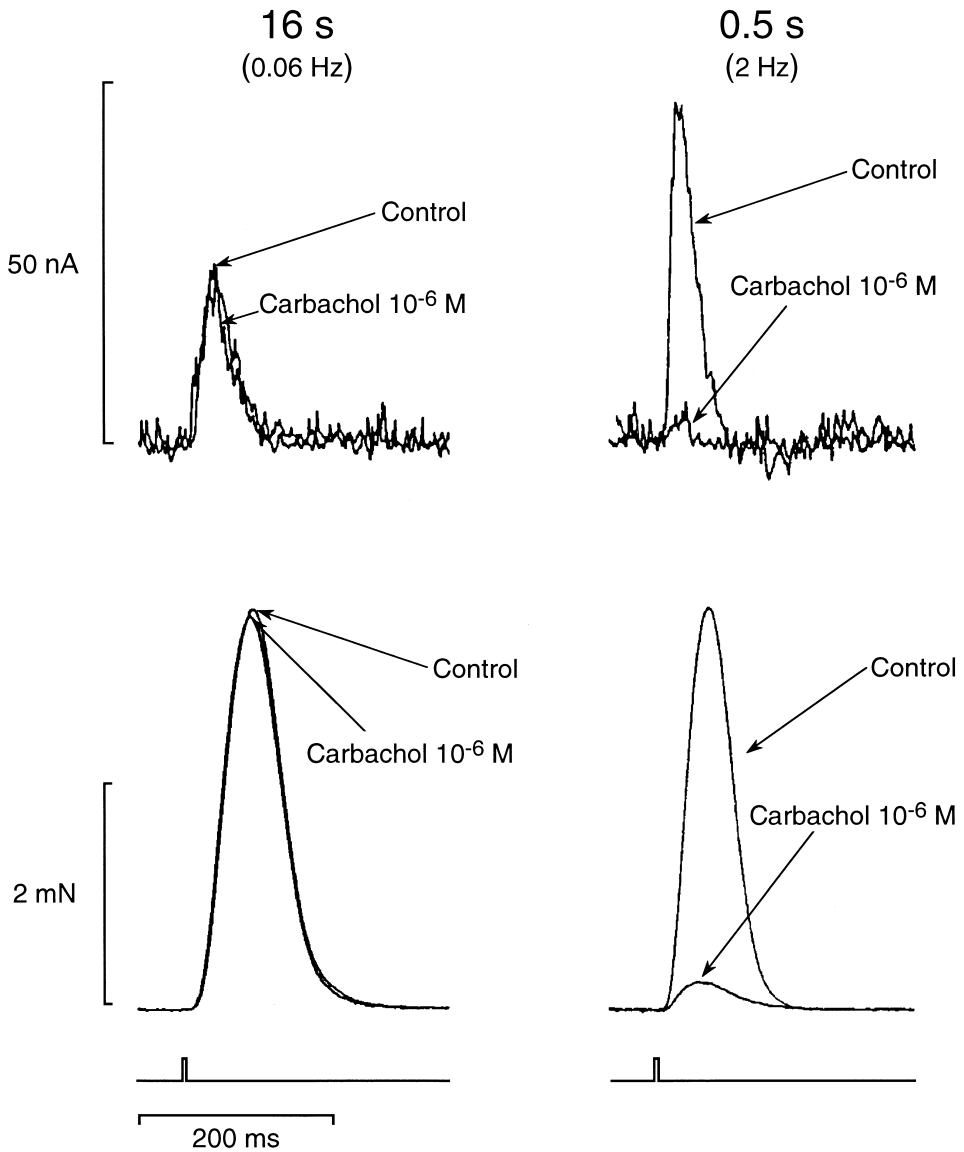


Fig. 4. Influence of the frequency of stimulation on the direct inhibitory action of 10^{-6} M carbachol on aequorin signals and isometric contractions in isolated rabbit left atrial muscle (11 mm long, with a cross-sectional area of 0.35 mm^2). Stimulation intervals were 16 s (left panels) and 0.5 s (right panels); the temperature was 37.5°C. Signal-averaged records of 64 successive contractions with aequorin signals (upper tracings) and isometric contraction (lower tracings) are presented.

otropic effect on basal contraction in atrial muscle varied among individual preparations of muscle, a result that indicates that the development of the positive inotropic effect might be counteracted by the weak direct inhibitory action at low rate of stimulation. The positive inotropic effect was associated with a concomitant increase in the amplitude of aequorin signals. The increase in the amplitude of Ca^{2+} transients and force induced by carbachol at a low rate of stimulation was unaffected by 10^{-7} M bupranolol, which blocks β-adrenoceptors, but it was abolished by atropine at 10^{-6} M (Fig. 5). It should be noted that, during the course of this series of experiments, aequorin signals declined gradually. This decline might have interfered partially with the ability of carbachol to produce a clear-cut increase in aequorin signals after administration of the receptor antagonists, as compared with that in the control trial, while the potential increase in the sensitivity of contractile proteins to Ca^{2+} ions might have also contributed to a more pronounced increase in force than the increase in Ca^{2+} transients (Fig. 5).

The rebound increase in aequorin signals and force that was produced after washout of carbachol was also not inhibited by bupranolol but it was abolished by atropine when the direct inhibitory action was inhibited by the muscarinic receptor antagonist.

Muscarinic receptor stimulation increases myofibrillar sensitivity to Ca^{2+} ions under certain experimental conditions (Horowits and Winegrad, 1983). The positive in-

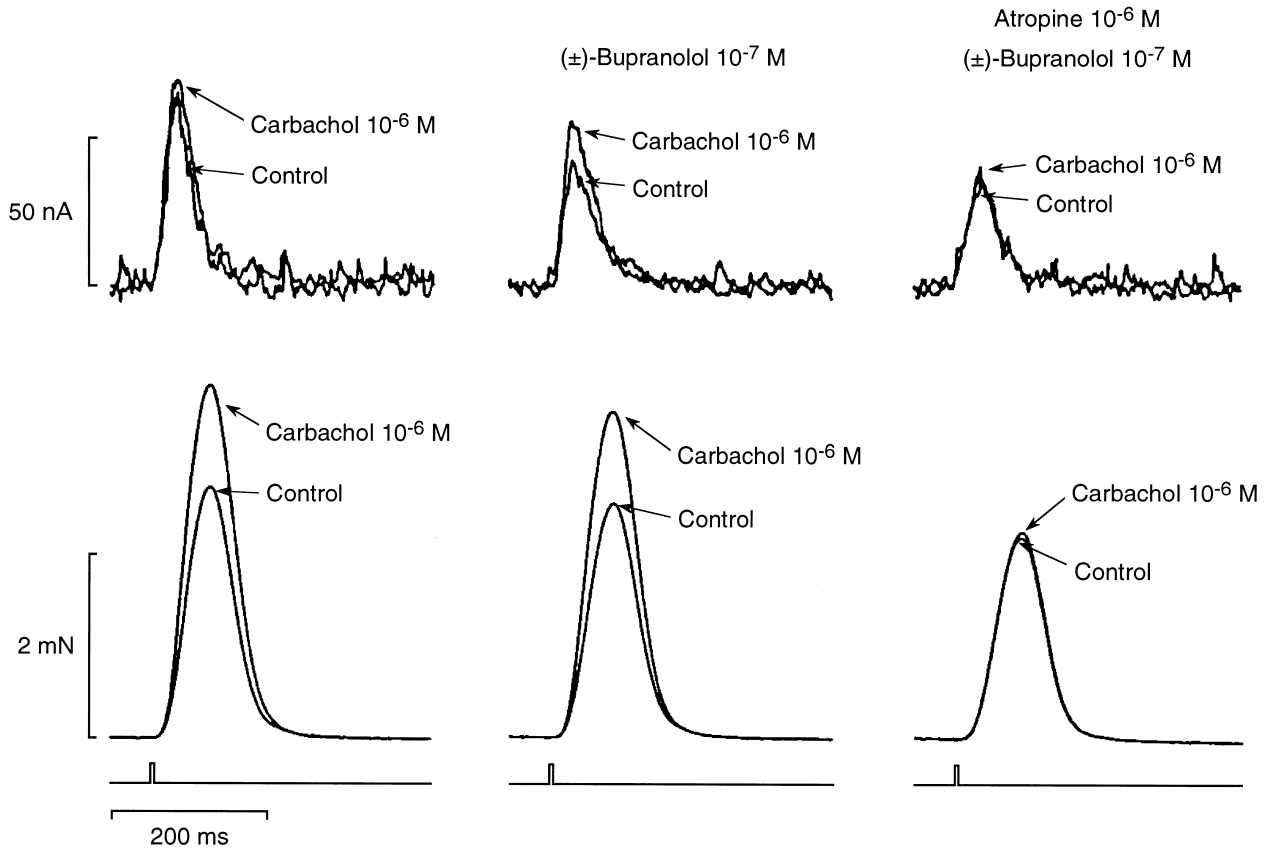


Fig. 5. Influence of (±)-bupranolol (10^{-7} M) and atropine (10^{-6} M) on the positive inotropic effect of 10^{-6} M carbachol at a long stimulation interval (16 s) in isolated rabbit left atrial muscle (11 mm long, with a cross-sectional area of 0.35 mm^2). Temperature, 37.5°C. Signal-averaged records of 128 successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented. During the course of experiments, aequorin signals declined gradually. This decline might have interfered partially with the ability of carbachol to produce a clear-cut increase in aequorin signals, as compared with the increase in the control trial, while the potential increase in the sensitivity of contractile proteins to Ca^{2+} ions might have contributed to the results.

otropic effect of 3×10^{-6} M carbachol (5.5 in Fig. 3A) was not associated with any modulation of the relationship between the amplitude of Ca^{2+} transients and force, as compared with the relationship at lower concentrations, an indication that a similar increase in the sensitivity to Ca^{2+} ions continues during the recovery phenomenon. In other words, the primary cause of recovery might have been an increase in the amplitude of Ca^{2+} transients that had been masked by the direct inhibitory action during the early phase after the application of the muscarinic receptor agonist.

3.3. Indirect inhibitory action

3.3.1. Atrial muscle

In atrial muscle, in the presence of stimulation of β-adrenoceptors, muscarinic receptor stimulation had a pronounced inhibitory effect on Ca^{2+} transients and force, even at a low rate of stimulation. Carbachol at 10^{-6} M decreased the amplitude of Ca^{2+} transients and force in the presence of 3×10^{-5} M norepinephrine in guinea-pig atrium (Fig. 6), an indication that, in atrial muscle, the indirect inhibitory action of muscarinic receptor stimulation was consistently more pronounced than the direct inhibitory action over a wide range of stimulation frequencies, namely, from 0.03 to 2.5 Hz. It is evident, therefore, that muscarinic receptor stimulation exerts an additional indirect inhibitory effect on both Ca^{2+} transients and force by antagonizing β-adrenoceptor-mediated responses in atrial muscle.

The direct inhibitory action of muscarinic receptor stimulation is more pronounced at higher rates of contraction (Blinks and Koch-Weser, 1963; Friedman et al., 1967), an observation that was reflected by the frequency-dependent changes in Ca^{2+} transients (Fig. 4). By contrast, the indirect inhibitory action of muscarinic receptor stimulation is more pronounced at lower frequencies of stimulation (Fig. 9). This phenomenon might be due, in part, to dependence on frequency of the accumulation of cAMP. The activities of adenylyl cyclase and phosphodiesterase are regulated by Ca^{2+} ions (Teo and Wang, 1973; Tada et al., 1975). The accumulation of cAMP is, therefore, regu-

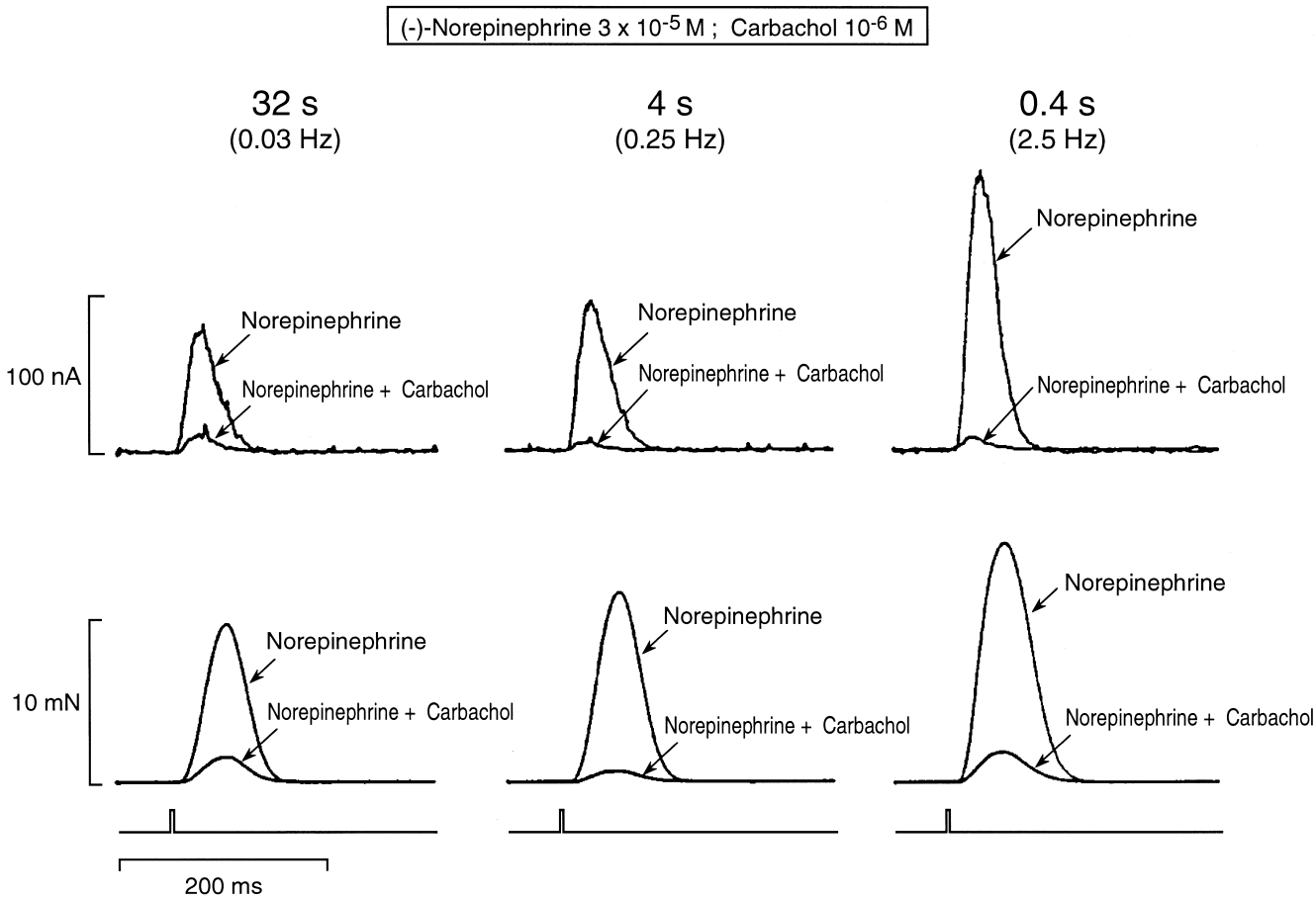


Fig. 6. Influence of the frequency of stimulation (stimulation interval) on the inhibitory action of 10^{-6} M carbachol on aequorin signals and isometric contractions in the presence of 3×10^{-5} M norepinephrine in the isolated guinea-pig left atrial muscle (9.5 mm long, with a cross-sectional area of 0.46 mm^2). The stimulation intervals were 32 s (left), 4 s (middle) and 0.4 s (right panels); the temperature was 37.5°C. Signal-averaged records of 32 successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented.

lated by frequency-dependent alterations in $[Ca^{2+}]_i$ in intact myocardial cells. The accumulation of cAMP that is induced by β-adrenoceptor stimulation in rabbit ventricular muscle is more pronounced at higher frequencies (Schümann et al., 1977). This situation might contribute to some extent to the resistance of β-adrenoceptor stimulation to muscarinic receptor-mediated inhibition at higher rates of contraction. In atrial muscle, the inhibitory action of muscarinic receptor stimulation in the presence of stimulation of β-adrenoceptors is most pronounced at intermediate frequencies of stimulation because of the differential dependence on frequency of the direct and indirect inhibitory actions: the former is more pronounced at higher frequencies, whereas the latter is more effectively exerted at lower frequencies (Fig. 6).

3.3.2. Ventricular muscle

In isolated rabbit papillary muscle, carbachol at 10^{-6} M and at 3×10^{-6} M scarcely affected the Ca^{2+} transients and the force of isometric contractions: there was usually only a small decrease, of less than 10% of the baseline level, in the force, which was followed by a small increase that was associated with a slight increase in the time to peak force. During these small alterations in contractile parameters that were induced by muscarinic receptor stimulation, the aequorin signals were almost unchanged (Fig. 7). This result indicates the absence of a direct inhibitory action on rabbit ventricular myocardium.

By contrast to above results, carbachol in rabbit papillary muscle antagonized the increase in Ca^{2+} transients and the positive inotropic effect induced by β-adrenoceptor stimulation. Fig. 8A shows the influence of carbachol at 3×10^{-6} M (right panels) on the concentration–response relationship for isoproterenol (left panels, control response), with increases in Ca^{2+} transients (upper panels) and in the force of isometric contractions (lower panels). The response of Ca^{2+} transients and isometric contractions to isoproterenol was markedly reduced in the presence of carbachol. The maximum responses of Ca^{2+} transients and force were unchanged but the concentration–response curve for isoproterenol was shifted to the right and in parallel to a similar extent (Fig. 8B).

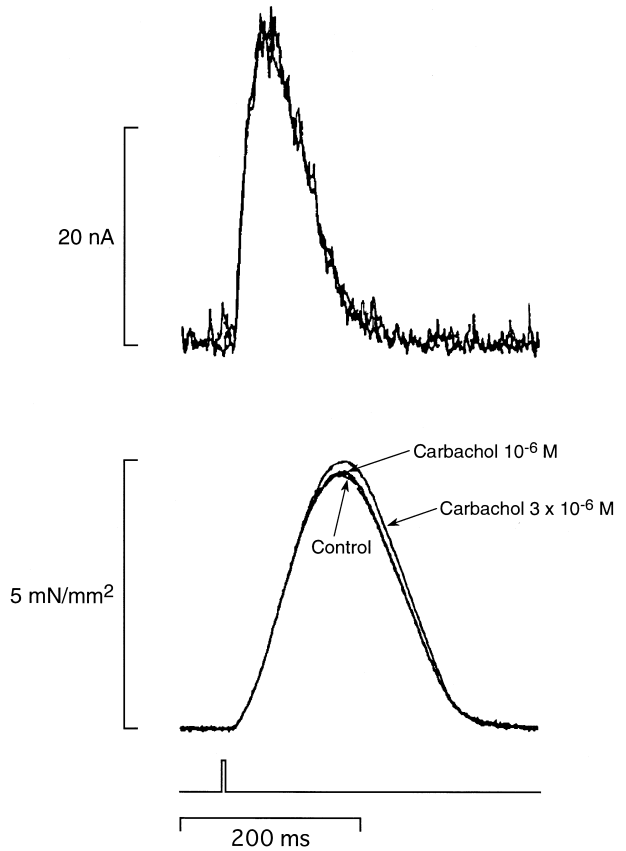
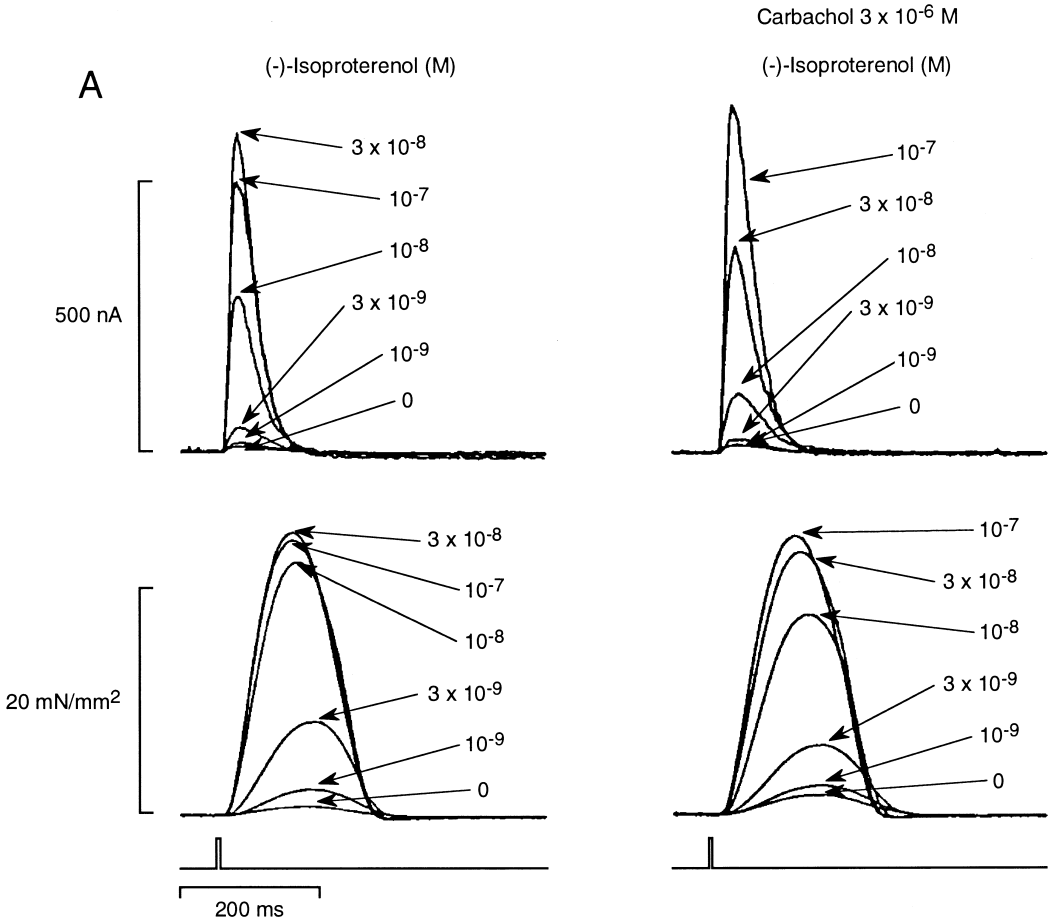


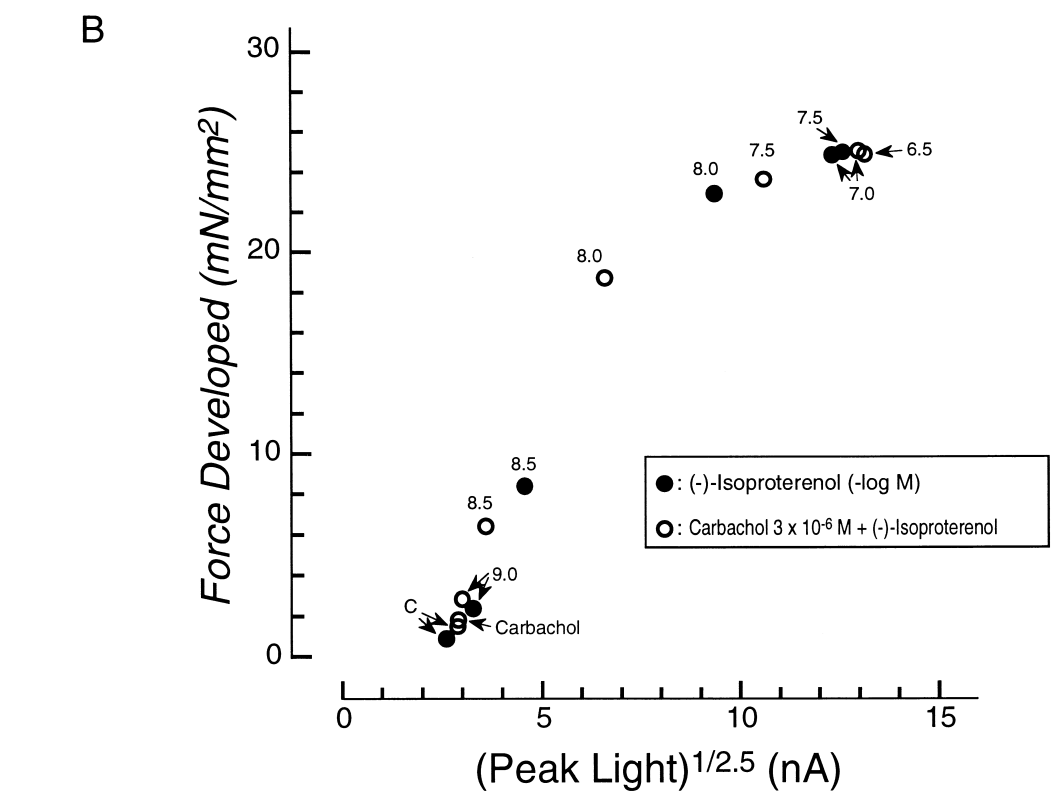
Fig. 7. Effects of 10^{-6} and 3×10^{-6} M carbachol on aequorin signals and isometric contractions in a rabbit papillary muscle (5.5 mm long, with a cross-sectional area of 0.50 mm^2). Stimulation interval, 1 s; temperature, 37.5°C. Signal-averaged records of 64 successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented.

In order to analyze in further detail whether or not the relationship between the amplitude of Ca^{2+} transients and force can be modified by muscarinic receptor stimulation, we examined the relationship in the presence of isoproterenol alone and in the presence of isoproterenol plus carbachol in rabbit papillary muscle. In studies with methacholine in hyperpermeable rat ventricular muscle (Horowits and Winegrad, 1983) and with acetylcholine in the ferret papillary muscle (McIvor et al., 1988), it has been reported that this relationship is modified by muscarinic receptor stimulation. In the rabbit papillary muscle, by contrast, we found that the relationship between the amplitude of Ca^{2+} transients and isometric contractions in the presence of isoproterenol alone was unaltered by the additional presence of carbachol, an indication that Ca^{2+} transients and force were attenuated in parallel by carbachol (Fig. 8). The time course of Ca^{2+} transients and isometric contractions was scarcely altered by carbachol from that induced by isoproterenol alone. For example, isoproterenol at 10^{-8} M alone increased the amplitude of Ca^{2+} transients and force to a similar extent to that induced by isoproterenol at 3×10^{-8} M plus carbachol at 3×10^{-6} M (Fig. 8). Under these experimental conditions, the duration of Ca^{2+} transients and contractions, respectively, measured at the level of 50% of maximum, were 42 and 125 ms with isoproterenol alone and 42 and 128 ms with isoproterenol plus carbachol.

The indirect inhibitory action of muscarinic receptor stimulation in rabbit papillary muscle was more pronounced at lower frequencies, in direct contrast to the direct inhibitory action in atrial muscle which was more pronounced at higher frequencies. When the frequency of contraction was increased from 0.06 to 0.5 Hz in the presence of isoproterenol at 10^{-7} M, the force increased in a frequency-dependent manner and the amplitude of Ca^{2+} transients also increased but to a relatively smaller extent (Fig. 9A, left panels). When carbachol at 3×10^{-6} M was administered in the presence of isoproterenol at 10^{-7} M, it decreased the isoproterenol-induced response more effectively at lower frequencies of stimulation (Fig. 9A, right panels). The amplitudes of Ca^{2+} transients in the presence of isoproterenol plus carbachol, with stimulation at frequencies of 0.06, 0.13, 0.25 and 0.5 Hz (intervals between stimulation were 16, 8, 4 and 2 s, respectively), were 65%, 69%, 80% and 100%, respectively, of the corresponding values with isoproterenol alone, an indication that the inhibition by carbachol was more pronounced at lower frequencies. The amplitudes of isometric contractions at

Fig. 8. (A) Influence of 3×10^{-6} M carbachol on the effects of isoproterenol that was administered cumulatively on aequorin signals and isometric contractions in isolated rabbit papillary muscle (5.5 mm long, with a cross-sectional area of 0.51 mm^2). Stimulation interval, 1 s; temperature, 37.5°C. Signal-averaged records of 32 (10^{-7} M isoproterenol), 64 (10^{-8} to 3×10^{-8} M) and 128 (3×10^{-9} M and lower) successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented. The concentration–response relationships for isoproterenol were determined first in the presence of carbachol (right panels) and then in the absence of carbachol (left panels) in order to minimize the deterioration of both parameters, due to repetition of determinations of the relationships, that might lead to an overestimation of the true inhibitory action of carbachol on responses to isoproterenol. (B) Relationships between the amplitude of aequorin signals and isometric contractions during cumulative administration of isoproterenol in the absence (closed circles) and in the presence (open circles) of 3×10^{-6} M carbachol in isolated rabbit papillary muscle. The values presented were calculated from the data in (A). Numbers attached to each symbol are $-\log$ M of isoproterenol. A total of four experiments of this type (determination of full concentration–response curves in the presence and absence of carbachol) was performed: three with isoproterenol, and one with dopamine as the β-adrenoceptor agonist. The influence of carbachol on the effects of single concentrations of norepinephrine, epinephrine and dopamine was also investigated. In all of these experiments with the rabbit papillary muscle, carbachol did not modify the relationship between the amplitude of Ca^{2+} transients and force induced by the β-adrenoceptor agonists. Carbachol did not affect the increase in the amplitude of aequorin signals and force induced by phenylephrine, which is mediated by α-adrenoceptors, in the presence of bupranolol (data not shown).


A
(-)-Isoproterenol (M)
Carbachol 3 x 10-6 M
(-)-Isoproterenol (M)
500 nA
20 mN/mm2
3 x 10-8
10-7
10-8
3 x 10-9
10-9
0
200 ms


B
Force Developed (mN/mm2)
(Peak Light)1/2.5 (nA)
8.0
7.5
6.5
7.0
8.5
9.0
C
Carbachol
●: (-)-Isoproterenol (-log M)
○: Carbachol 3 x 10-6 M + (-)-Isoproterenol

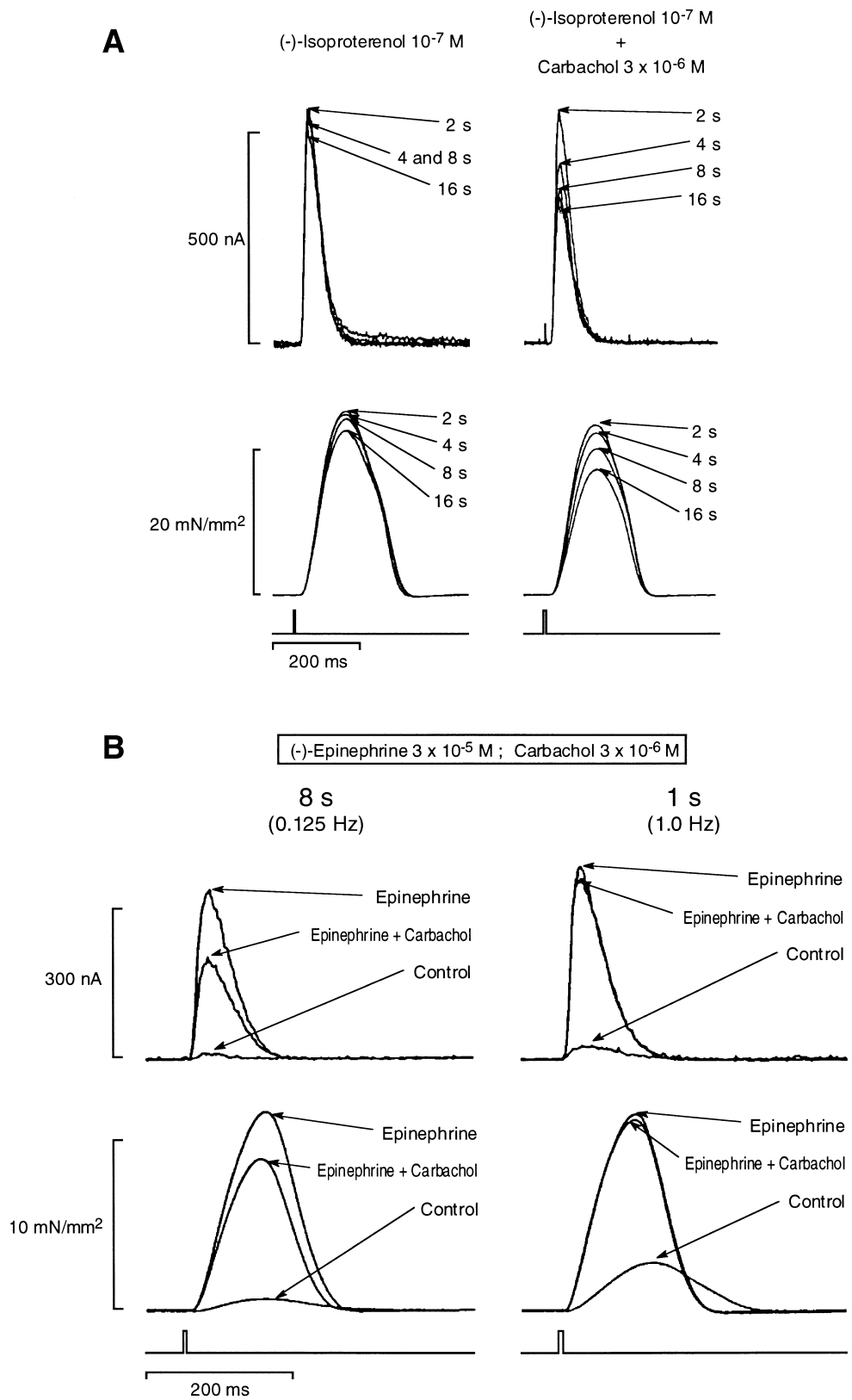


Fig. 9. Influence of the frequency of stimulation on the inhibitory action of carbachol (3×10^{-6} M) on aequorin signals and isometric contractions in the presence of 10^{-7} M isoproterenol (A) and 3×10^{-5} M epinephrine (B) in isolated rabbit papillary muscle [5.5 mm long, with a cross-sectional area of 0.51 (A) and 0.50 (B) mm^2]. (A) Left panels, isoproterenol alone; right panels, isoproterenol plus carbachol. Signal-averaged records of 16 (16 s) and 32 (2, 4 and 8 s) successive contractions with aequorin signals (upper tracings) and isometric contractions (lower tracings) are presented. (B) Left panels: stimulation interval, 8 s; right panels: stimulation interval, 1 s. Signal-averaged records of 64 successive contraction are presented. Temperature, 37.5°C.

corresponding frequencies were 76%, 83%, 90% and 93%, respectively (Fig. 9A, lower right panels).

It was evident that carbachol at 3×10^{-6} M inhibited the β-mediated response to epinephrine at 3×10^{-5} M more prominently at a lower frequency of stimulation in rabbit papillary muscle. The attenuation of the amplitude of Ca^{2+} transients and isometric contractions induced by carbachol in the presence of epinephrine was 41% and 24%, respectively, at 0.13 Hz (interval, 8 s) and 5% and 3%, respectively, at 1 Hz (Fig. 9B).

4. Discussion

4.1. Direct inhibitory action of muscarinic receptor stimulation on rabbit and guinea-pig atrial muscle and ferret ventricular muscle

In mammalian atrial muscle, the direct negative inotropic effect induced by muscarinic receptor stimulation can essentially be explained in terms of changes in Ca^{2+} transients, as shown first by means of electrical field stimulation and the resultant action of endogenously released acetylcholine in pectinate muscle dissected from the endocardial surface of rabbit left atrium that was electrically stimulated at 1.25 Hz (Endoh and Blinks, 1984). In rabbit atrial muscle, the muscarinic receptor agonist carbachol, when administered in a cumulative manner, decreased the amplitude of aequorin signals and isometric contractions in a concentration-dependent manner and, apparently, in parallel (Fig. 1). These changes in Ca^{2+} transients might reflect changes in $[Ca^{2+}]_i$ that resulted from the electrophysiological effects of muscarinic receptor stimulation of atrial muscle, namely an abbreviation of action potential duration due to activation of G protein-coupled $I_{K,ACh}$ (Pfaffinger et al., 1985; Kurachi et al., 1986) may result in a reduction of slow channel conductance (Ten Eick et al., 1976) and an attenuation of slow inward current (Burgen and Terroux, 1953; Trautwein and Dudel, 1958).

What might be the mechanism for the regulation of Ca^{2+} signaling that is responsible for the direct inhibitory action of muscarinic receptor stimulation? It has been suggested that the accumulation of cyclic GMP (cGMP) upon muscarinic receptor stimulation and the subsequent activation of a cGMP-dependent protein kinase might play a crucial role in the induction of the direct inhibitory action of muscarinic receptor stimulation in atrial muscle (Brooker, 1977; Mirro et al., 1979; Lincoln and Keeley, 1980; Endoh and Yamashita, 1981). By contrast, however, an increase in the Ca^{2+} sensitivity of myofibrils in relation to the accumulation of cGMP has been proposed in hyperpermeable rat ventricular trabeculae (Winegrad, 1984; Winegrad et al., 1983). Hence, the role of cGMP in the muscarinic receptor-mediated regulation of cardiac contractility is controversial. There is quite a large discrepancy between tissue levels of cGMP and contractile function or electrophysiological changes induced by muscarinic receptor stimulation (Brooker, 1977; Daimond et al., 1977; Linden and Brooker, 1979; Mirro et al., 1979). Nevertheless, since nitroprusside, which markedly increases levels of cGMP, and 8-bromo-cGMP have a negative inotropic effect on atrial muscle but not on ventricular muscle (Endoh and Shimizu, 1979; Endoh and Yamashita, 1981; Endoh, 1983), it is possible that cGMP might contribute, to some extent, to the direct inhibitory action and the decrease in the amplitude of Ca^{2+} transients that are induced by muscarinic receptor stimulation of atrial muscle. Shah et al. (1994) have demonstrated that 8-bromo-cGMP decreases the myofibrillar sensitivity to Ca^{2+} in intact ventricular myocytes of the rat, which might partly contribute to the direct inhibitory action. In addition, cGMP plays an important role in the indirect inhibitory action, in particular, in the adrenergic-cholinergic interaction through modulation of the process of cAMP hydrolysis by cAMP-phosphodiesterase (Méry et al., 1997), as will be discussed below.

Other candidates for the intracellular second messengers in the muscarinic receptor-mediated regulation of cardiac Ca^{2+} signaling are products of the hydrolysis of phosphoinositide, which has been shown to be stimulated by activation of muscarinic receptors in cardiac muscle. Muscarinic receptor stimulation accelerates the hydrolysis of phosphoinositide in cardiac myocytes, with production of inositol 1,4,5-trisphosphate and diacylglycerol in cardiac myocytes (Brown et al., 1985; Poggioli et al., 1986), which might be involved in muscarinic receptor-mediated signal transduction in the heart. Various agents, such as α-adrenoceptor agonists, endothelin and angiotensin II, have been shown to stimulate likewise the hydrolysis of phosphoinositide, which has been postulated to be involved in the induction of the positive inotropic effect. It has been proposed that these agents induce a small increase in $[Ca^{2+}]_i$ and an increase in myofibrillar sensitivity to Ca^{2+} ions (Endoh and Blinks, 1988; Wang et al., 1991; Fujita and Endoh, 1996). It appears, therefore, that the products of the hydrolysis of phosphoinositide might contribute to a positive inotropic effect rather than to the negative inotropic effect of muscarinic receptor stimulation.

It is very likely that a reduction in the rate of release of Ca^{2+} ions from the sarcoplasmic reticulum, due to a decrease in the influx of Ca^{2+} ions that results from abbreviation of the duration of the action potential might be responsible for the decrease in the amplitude of Ca^{2+} transients, leading to the direct inhibitory action. Activation of an $I_{K,ACh}$ current subsequent to the muscarinic receptor-coupled activation of a G protein, G_o, might be a primary cause of abbreviation of the action potential in atrial muscle (Soejima and Noma, 1984; Iijima et al., 1985; Pfaffinger et al., 1985; Kurachi et al., 1986). It has been demonstrated that the negative inotropic effect of

acetylcholine is markedly dependent on the regional distribution of the $I_{K,ACh}$ current in dog ventricular myocardium. In sub-epicardial cells, acetylcholine activates $I_{K,ACh}$, leading to a shortening of the action potential and, thus, a negative inotropic effect. By contrast, in sub-endocardial cells, where no $I_{K,ACh}$ current is detectable, a negative inotropic effect is not produced (Yang et al., 1996). The above mentioned possibility is supported by the fact that, in ferret ventricular muscle, which has relatively abundant $I_{K,ACh}$ channels (Ito et al., 1995), a pronounced direct inhibitory action is elicited by muscarinic receptor and by adenosine receptor agonists (Boyett et al., 1988; Endoh et al., 1993; Komukai and Kurihara, 1994). The $I_{K,ACh}$ current was 6.3 times smaller in rat ventricular than in rat atrial cells, which may explain why the negative inotropic effect of acetylcholine is more pronounced in atrial cells than in ventricular cells (McMorn et al., 1993). In their study, this conclusion was further supported by the fact that acetylcholine had no effect on the contractile force when the contractions were triggered by the voltage-clamp pulses with constant duration.

4.2. Ca^{2+} signaling during recovery and the rebound positive inotropic effect

Muscarinic receptor stimulation has long been known to produce an increase in the force of atrial contraction that is unrelated to the release of catecholamines, subsequent to its direct inhibitory action (Baumann et al., 1960). Since the positive inotropic effect of muscarinic receptor stimulation, which is known as recovery or the rebound phenomenon is not associated with a detectable change in the duration of action potential (Baumann et al., 1963; Gertjegerdes et al., 1979), the increase in the amplitude of Ca^{2+} transients observed during the phenomenon might be due to a mechanism other than facilitation of the opening of L-type Ca^{2+} channels. The characteristics of the rebound-related increase in Ca^{2+} transients, in association with prolongation of duration (Fig. 2A), indicate that the rebound is induced by a cAMP-independent mechanism. However, it was reported recently that acetylcholine elicits rebound stimulation of the Ca^{2+} current that is mediated by pertussis toxin-sensitive G protein and activation of protein kinase A in atrial myocytes (Wang and Lipsius, 1995), an indication that the cAMP-mediated process might contribute to the rebound phenomenon under certain experimental conditions.

The rebound-related increase in force during the washout of carbachol was associated with a pronounced elevation of Ca^{2+} transients (Fig. 1) and it is, therefore, evident that an increase in Ca^{2+} transients plays a primary role in the rebound phenomenon. A positive inotropic effect with similar characteristics is also elicited in mammalian ventricular muscle by muscarinic receptor stimulation (e.g., Endoh et al., 1970). In isolated rat ventricular myocytes, carbachol at high concentrations (above 10 μM) elicits an increase in the amplitude of Ca^{2+} transients that is mediated by muscarinic M_1 receptors (Sharma et al., 1996), while in guinea-pig ventricular myocytes, carbachol increases the amplitude of Ca^{2+} transients and force via muscarinic M_2 receptors (Protas et al., 1998). Muscarinic M_1 and M_4 receptors are coupled to stimulation of phosphoinositide hydrolysis, while muscarinic M_2 and M_3 receptors mediate the inhibition of adenylyl cyclase (Peralta et al., 1988).

A subcellular mechanism for the increase in Ca^{2+} transients in the recovery or rebound phenomenon has been proposed, as follows. The primary increase in $[Na^+]_i$ and a subsequent increase in $[Ca^{2+}]_i$ through the operation of the Na^+–Ca^{2+} exchanger might be crucial (Korth and Kühlkamp, 1985). Muscarinic receptor stimulation elicits the acceleration of the hydrolysis of phosphoinositide with production of inositol 1,4,5-trisphosphate and diacylglycerol in cardiac myocytes (Brown et al., 1985; Poggioli et al., 1986). Stimulation of the Na^+–H^+ exchanger by protein kinase C, activated by diacylglycerol, a product of the hydrolysis of phosphoinositide, could result in an increase in $[Na^+]_i$ and an increase in myofibrillar sensitivity to Ca^{2+} ions that is due to intracellular alkalinization (Krämer et al., 1991; Gambassi et al., 1992; Terzic et al., 1992). Thus, the activation of the Na^+–H^+ exchanger is potentially a key process that could explain the regulation of Ca^{2+} signaling induced by muscarinic receptor stimulation, leading to a moderate increase in the intracellular mobilization of Ca^{2+} ions and an increase in the sensitivity of contractile proteins to Ca^{2+} ions.

Experimental evidence in support of the functional relevance of the receptor-mediated activation of the Na^+–H^+ exchanger has, however, been fragmentary and controversial and the relevance has not yet been established (for reviews, see Endoh, 1995, 1998). Furthermore, a peculiar feature of the regulation of Ca^{2+} signaling that is induced by receptor agonists that stimulate the hydrolysis of phosphoinositide in cardiac myocytes is that the increase in Ca^{2+} transients is much less pronounced than the increase induced by other inotropic interventions, such as β-adrenoceptor stimulation and elevation of $[Ca^{2+}]_o$ (Endoh and Blinks, 1988; Wang et al., 1991; Fujita and Endoh, 1996). In this context, a pronounced increase in the amplitude of Ca^{2+} transients that is observed during the rebound phenomenon (Fig. 1) appears to be dissimilar, to some extent, to the effect of receptor agonists that stimulate the hydrolysis of phosphoinositide. Nonetheless, stimulation of the Na^+–H^+ exchanger by protein kinase C and the resultant increase in $[Na^+]_i$ and intracellular alkalinization that are due to acceleration of the hydrolysis of phosphoinositide, induced by activation of muscarinic receptors, are, at the moment, the most likely candidates for mediators of recovery and the rebound positive inotropic effect that are associated with an increase in Ca^{2+} transients in atrial muscle. There is, in fact, experimental evidence that suggests a contribution of the hydrolysis of phosphoinositide

to the positive inotropic effect in atrial muscle (Eglen et al., 1988; Vites and Pappano, 1995).

4.3. Indirect inhibitory action of muscarinic receptor stimulation

The indirect inhibitory action of muscarinic receptor stimulation is elicited by antagonism of the cAMP-mediated facilitation of myocardial contractility which is induced by cardiotonic agents, such as β-adrenoceptor agonists, forskolin and phosphodiesterase inhibitors, and it is observed in both atrial and ventricular muscle. In atrial and ferret ventricular muscle, the interaction is extremely complex because of contributions of both direct and indirect inhibitory actions (Brown et al., 1980; Boyett et al., 1988; Hongo et al., 1993b).

In mammalian ventricular myocardium, in general, muscarinic receptor stimulation does not affect or barely affects the basal force but it elicits a pronounced negative inotropic effect when applied during the activation of β-adrenoceptors. This indirect inhibitory action of muscarinic receptor stimulation has been designated adrenergic–cholinergic interaction or accentuated antagonism (Hollenberg et al., 1965; Levy, 1971) and it has been postulated that this indirect inhibitory action occurs at the level of a cyclic nucleotide-mediated signaling process (Watanabe and Besch, 1975; Endoh, 1979).

Regulation of myocardial contractility via the activation of β-adrenoceptors is mediated primarily by the accumulation of cAMP and the resultant phosphorylation of functional proteins by protein kinase A (Tsien, 1977). Stimulation of β-adrenoceptors regulates myocardial contractility at different steps of cardiac excitation–contraction coupling via the phosphorylation of at least five proteins. (1) It increases the rate at which the sarcoplasmic reticulum sequesters Ca^{2+} ions as a result of the phosphorylation of phospholamban (Tada et al., 1978; Lindemann et al., 1983). (2) It increases the influx of Ca^{2+} ions through L-type Ca^{2+} channels during each action potential (Reuter and Scholz, 1977), which, in turn, increases the amount of releasable Ca^{2+} ions in the sarcoplasmic reticulum (Morad and Goldman, 1973) and facilitates the Ca^{2+}-induced release of Ca^{2+} ions (Valdivia et al., 1995). (3) It facilitates opening of Ca^{2+}-release channels, namely, the ryanodine receptors, in the sarcoplasmic reticulum, with phosphorylation of channel proteins (Takasago et al., 1991) and/or of an associated inhibitory protein, sorcin (Valdivia, 1998). (4) It decreases the responsiveness of troponin C to Ca^{2+} ions in association with the phosphorylation of troponin I (McClellan and Winegrad, 1978; Mope et al., 1980; Solaro, 1986). Finally, (5) it enhances phosphorylation of myosin-binding protein C and myosin light chain by protein kinase A, but the functional relevance of these reactions has not yet been unequivocally established.

It remains controversial whether these actions are exerted in parallel, subsequent to β-adrenoceptor stimulation and phosphodiesterase inhibition, and whether muscarinic receptor stimulation antagonizes these β-adrenoceptor-mediated changes with similar dependence on concentration of muscarinic receptor agonists and on time after administration. Rapundalo et al. (1989) showed that β-adrenoceptor stimulation and cAMP-phosphodiesterase inhibitors induce different degrees of phosphorylation of these functional proteins in intact cardiac preparations, suggesting that compartmentalized intracellular cAMP-mediated regulation might occur. It is likely that each of the various cAMP-dependent processes is modified by muscarinic receptor stimulation since a decrease in β-adrenoceptor-mediated accumulation of cAMP (Brown, 1979; Endoh, 1979; Linden et al., 1985) and the dephosphorylation of functional proteins (Watanabe et al., 1984; Lindemann and Watanabe, 1985) are crucial for the antagonism. Inhibition of the former three phenomena might contribute to attenuation of the isoproterenol-induced increase in Ca^{2+} transients. If any alterations in myofibrillar sensitivity to Ca^{2+} ions were to occur during the interaction, they would produce a dissociation of the force from the Ca^{2+} transients. It was revealed, however, that the relationship between force and Ca^{2+} transients during application of isoproterenol alone was unaffected by additional muscarinic receptor stimulation by carbachol, an indication that the myofibrillar sensitivity to Ca^{2+} ions is unaltered by muscarinic receptor stimulation as compared to the decrease in Ca^{2+} sensitivity that is induced by β-adrenoceptor stimulation alone in rabbit papillary muscle (Fig. 8). The increases in amplitude of Ca^{2+} transients and force, as well as the relaxant effect and abbreviation of both parameters, are all inhibited in parallel by muscarinic receptor stimulation (Endoh, 1990; present study). These findings in rabbit papillary muscle are not consistent with those in ferret papillary muscle (McIvor et al., 1988; Hongo et al., 1993b). The discrepancy might be attributable to the difference in the contribution of the direct inhibitory action of muscarinic receptor stimulation between the two species, as will be discussed below in greater detail.

The role of the metabolism of cyclic nucleotides in the subcellular mechanism of the indirect inhibitory action of muscarinic receptor stimulation has been studied extensively. Although it is evident that inhibition of the cAMP-mediated signal-transduction process is responsible for the indirect inhibitory action of muscarinic receptor stimulation on Ca^{2+} signaling, the site of action remains controversial. While experimental evidence has been accumulating to indicate that decreased generation of cAMP, due to inhibition of adenylyl cyclase via activation of the inhibitory G protein G_i, might play a crucial role in the indirect inhibitory action of muscarinic receptor stimulation in myocardial cells, other mechanisms, such as activation of phosphatases and activation of a nitric oxide (NO)-cGMP pathway (Balligand et al., 1995; Ungureanu-Longrois et al., 1995), have been proposed to be similarly

involved in the indirect inhibitory action. In addition, activation of cGMP-dependent protein kinase (protein kinase G) results in attenuation of I_{Ca} (Ono and Trautwein, 1991; Wahler and Dollinger, 1995; Shirayama and Pappano, 1996). By contrast, working with human atrial myocytes, Vandecasteele et al. (1998) failed to obtain any evidence for the involvement of an NO-cGMP pathway in the muscarinic receptor-mediated regulation of I_{Ca}. Because the experimental findings have been so variable, depending on animal species and/or cardiac tissue differences, it has been proposed (Méry et al., 1997) that the various discrepancies require further clarification, for example, by examination of the different steps in signal transduction as follows: (1) by characterization of the coupling process between M_2 receptors and endothelial cell NO synthase; (2) by characterization of the potential role of NO in muscarinic receptor-mediated inhibition of adenylyl cyclase; (3) by identifying the respective roles of each enzyme (adenylyl cyclase, phosphatase, NO synthase) in the overall effect of acetylcholine; (4) by identifying the targets of NO and/or cGMP that are connected with the regulation of I_{Ca}; and (5) by determining the functional relevance of the cGMP-dependent protein kinase.

There is some experimental evidence to indicate that cGMP might be involved in the cross-talk between adrenergic and cholinergic interactions. cGMP affects the rate of degradation of cAMP via modulation of the activities of phosphodiesterases. Since cGMP stimulates phosphodiesterase II but inhibits phosphodiesterase III, it attenuates or enhances the cAMP-mediated functional and electrophysiological response to β-adrenoceptor stimulation depending on whether the former (Han et al., 1996) or the latter (Ono and Trautwein, 1991; Kirstein et al., 1995) mechanism predominates in the cardiac tissue (Hove-Madsen et al., 1996). In human atrial myocytes, the basal I_{Ca} is controlled by phosphodiesterase II, and both phosphodiesterase II and phosphodiesterase III might contribute to maintain the concentrations of the cyclic nucleotide at a minimum in the absence of the stimulation of adenylyl and/or guanylyl cyclase (Rivet-Bastide et al., 1997). When I_{Ca} is activated by β-adrenoceptor stimulation in mammalian ventricular myocytes (such as those of guinea pig and rat), cGMP further enhances the current by inhibition of phosphodiesterase III. When I_{Ca} is activated by the inhibitors of phosphodiesterases, such as 3-isobutyl-1-methylxanthine (IBMX), or by cAMP, or by methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay k 8644), cGMP inhibits the current via activation of a cGMP-dependent protein kinase (Méry et al., 1991; Mubagwa et al., 1993; Sumii and Sperelakis, 1995; Shirayama and Pappano, 1996).

The role of cGMP in the regulation of Ca^{2+} transients appears to be more complicated than its role in the regulation of I_{Ca}. Hongo et al. (1993a) showed, in ferret papillary muscle loaded with aequorin, that derivatives of cGMP, such as 8-bromo-cGMP and dibutyryl-cGMP, and also sodium nitroprusside failed to mimic the inhibitory action of muscarinic receptor stimulation on the cAMP-mediated facilitation of Ca^{2+} signaling. Their results are consistent with the finding in dog ventricular myocardium that cGMP is not involved in the indirect inhibitory action of muscarinic receptor stimulation (Endoh and Shimizu, 1979; Endoh and Yamashita, 1981).

4.4. Effects of muscarinic receptor stimulation in ferret ventricular myocardium

Among mammalian ventricular muscles, ferret ventricular myocardium is exceptionally sensitive to the direct inhibitory action of the stimulation of muscarinic (Boyett et al., 1988; McIvor et al., 1988; Hongo et al., 1993a,b) and adenosine (Endoh et al., 1993; Komukai and Kurihara, 1994) receptors. This property can be ascribed to the abundant distribution of the GTP binding protein-gated muscarinic K^+ channels in ferret ventricular myocytes (Ito et al., 1995).

During the induction of the direct inhibitory action of carbachol, an apparent increase in myofibrillar sensitivity to Ca^{2+} ions, similar to that observed in atrial muscle (Fig. 3A), was elicited in ferret ventricular myocardium (Fig. 3B). It is noteworthy that the durations of aequorin signals and twitch contractions were abbreviated by carbachol during induction of the direct inhibitory action of muscarinic receptor stimulation in ferret ventricular myocardium.

The effects of muscarinic receptor stimulation during β-adrenoceptor stimulation in ferret papillary muscle are quite different from those in rabbit papillary muscle in so far as certain effects of β-adrenoceptor stimulation are not reversed by muscarinic receptor stimulation, while other effects of β-adrenoceptor stimulation are effectively inhibited (McIvor et al., 1988; Hongo et al., 1993b). In ferret papillary muscle, (1) acetylcholine increases the myofibrillar sensitivity to Ca^{2+} ions, while it antagonizes the positive inotropic effect of isoproterenol; and (2) acetylcholine does not reverse the β-adrenoceptor-mediated abbreviation of Ca^{2+} transients and contractions, while it antagonizes the increase in the amplitude of Ca^{2+} transients and the decrease of myofibrillar sensitivity to Ca^{2+} ions. These differential antagonistic actions of muscarinic receptor stimulation are considered to be due essentially to the fact that acetylcholine has a pronounced negative inotropic effect (direct inhibitory action) that is unrelated to the cAMP-mediated signaling process (Boyett et al., 1988). This observation contrasts strongly to the fact that muscarinic receptor stimulation does not, in general, have a direct inhibitory action on mammalian ventricular myocardium (Löffelholz and Pappano, 1985; Endoh, 1987). It is highly likely, therefore, that the direct inhibitory action of muscarinic receptor stimulation, which is associated with an abbreviation of Ca^{2+} transients and contractions and also with an increase in myofibrillar sensitivity to

Ca^{2+} ions, modifies the adrenergic–cholinergic interaction (indirect inhibitory action) in ferret ventricular myocardium in ways that are more complex than in the ventricular myocardium of other mammalian species (McIvor et al., 1988; Hongo et al., 1993a). This hypothesis is supported by the observation that adenosine also elicits a pronounced direct inhibitory action on ferret ventricular myocardium and antagonizes the positive inotropic effect of β-adrenoceptor stimulation. Apparently, however, adenosine does not reverse the relaxant effect of β-adrenoceptor stimulation because of the contribution of the direct inhibitory action (Endoh et al., 1993). McIvor et al. (1988) showed that acetylcholine increased myofibrillar sensitivity to Ca^{2+} ions when it antagonized the response mediated by stimulation of β-adrenoceptors in ferret papillary muscle. They ascribed these results similarly to the direct inhibitory action that is associated with an increase in myofibrillar sensitivity to Ca^{2+} ions (Fig. 3B).

5. Conclusion

The stimulation of muscarinic receptors induces a decrease and/or an increase in the amplitude of intracellular Ca^{2+} transients in mammalian cardiac muscle: the former can be classified as direct and indirect inhibitory actions depending on the involvement or non-involvement of a cAMP-mediated process. The latter is manifest as the recovery or rebound phenomenon that is observed during prolonged exposure or after washout of muscarinic receptor agonists. The direct inhibitory action, which is readily elicited in atrial muscle but is scarcely induced in most mammalian ventricular muscle, might be due primarily to activation of K^+ channels by muscarinic receptor stimulation ($I_{K,ACh}$). The ferret ventricular muscle has atrial muscle-like characteristics in that muscarinic receptor stimulation has a pronounced direct inhibitory action. The indirect inhibitory action is exerted both in atrial and ventricular myocardium. The direct and indirect inhibitory actions have different dependence on frequency: the former action is more pronounced at higher frequencies, while the latter is more pronounced at lower frequencies of contraction. The alterations in Ca^{2+} transients are essentially responsible for the negative and positive inotropic effects of muscarinic receptor stimulation. In addition, the direct inhibitory effect on atrial muscle and ferret ventricular muscle is associated with an increase in the myofibrillar sensitivity to Ca^{2+} ions. By contrast, the indirect inhibitory effect on the β-adrenoceptor-mediated response is not associated with changes in Ca^{2+} sensitivity. The alterations in Ca^{2+} signaling induced by β-adrenoceptor stimulation are reversed by muscarinic stimulation in parallel, whereas the adrenergic–cholinergic interaction in the ferret ventricular muscle is more complex because of involvement of both the direct and the indirect inhibitory action.

Acknowledgements

The author is grateful to Dr. John Blinks for his continuous encouragement and for support of the experiments performed at the Department of Pharmacology, Mayo Clinic, Rochester, MN, as well as to Dr. Youichi Kawabata for the performance of experiments with ferret papillary muscles at the Department of Pharmacology, Yamagata University School of Medicine. The author also wishes to thank Mr. Ikuo Norota for preparation of the figures.

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