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High carbachol increases the electrically induced $[Ca^{2+}]_i$ transient in the single isolated ventricular myocyte of rats

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Abstract

In order to investigate the mechanisms responsible for the inotropic effects of muscarinic acetylcholine receptor stimulation by high concentrations of muscarinic receptor agonists, we studied the effects of carbachol at 30–300 μM on the electrically induced $[Ca^{2+}]_i$ transient of rat isolated ventricular myocytes. Carbachol at this dose range increased the amplitude and duration of the electrically induced $[Ca^{2+}]_i$ transient time and dose dependently. It also increased the resting fluorescence ratio and time to 80% decline of amplitude from the peak. At 100–300 μM the increase in $[Ca^{2+}]_i$ transient was followed by a cluster of Ca^{2+} oscillations in 50–83% of the cells studied. The effects were blocked by atropine, but not pertussis toxin. Depletion of Ca^{2+} from sarcoplasmic reticulum by ryanodine, which itself reduced the amplitude of the $[Ca^{2+}]_i$ transient and increased resting fluorescence, abolished the effect of carbachol on the $[Ca^{2+}]_i$ transient without affecting its effect on resting fluorescence ratio. The caffeine-induced $[Ca^{2+}]_i$ transient was unaffected by prior addition of carbachol in a Ca^{2+} free and low Na^+ solution. Inhibition of Ca^{2+} influx by the L-type Ca^{2+} channel blocker, verapamil, which itself reduced the amplitude of the $[Ca^{2+}]_i$ transient without affecting the resting fluorescence ratio, attenuated the augmentation of the amplitude of the $[Ca^{2+}]_i$ transient elicited by carbachol. Ni^{2+}, a non-specific Ca^{2+} channel blocker and an inhibitor of Na^+-Ca^{2+} exchange, abolished the effects of carbachol on both $[Ca^{2+}]_i$ transient and resting fluorescence ratio. Low external Na^+, which increased the resting fluorescence ratio due to its inhibitory effect on Na^+-Ca^{2+} exchange, also abolished the effects of carbachol. The results indicate that the inotropic effect of muscarinic acetylcholine receptor stimulation by high concentrations of a muscarinic receptor agonist may be due to an increase in the electrically induced $[Ca^{2+}]_i$ transient in ventricular myocytes via a process which is not pertussis toxin sensitive. The increase in the electrically induced $[Ca^{2+}]_i$ transient may result from increases in Na^+-Ca^{2+} exchange and influx of Ca^{2+} via voltage-gated Ca^{2+} channels, and mobilization of Ca^{2+} from the intracellular store. The mobilization of Ca^{2+} from the intracellular store is a secondary event. The study has provided evidence for the first time that muscarinic acetylcholine receptor stimulation by high concentrations of carbachol increases Ca^{2+} influx via the Ca^{2+} channel and mobilization of Ca^{2+} from its intracellular store. The study has also demonstrated for the first time the occurrence of Ca^{2+} oscillations induced by high concentrations of carbachol.

Keywords: Muscarinic receptor; $[Ca^{2+}]_i$ transient; Electrical stimulation; Na^+-Ca^{2+} exchange; Carbachol

1. Introduction

Muscarinic acetylcholine receptor stimulation usually produces inhibitory effects on the heart (see Pappano, 1991). The inhibitory effects of muscarinic acetylcholine receptor stimulation are mediated by muscarinic M_2 receptors and are sensitive to ADP ribosylation by pertussis toxin (Endoh et al., 1985; Sorota et al., 1985). Muscarinic acetylcholine receptor stimulation by higher concentrations of muscarinic receptor agonists such as carbachol, however, produces paradoxically stimulatory effects including increases in heart rate, contractility and action potential duration (Lipsius and Gibbons, 1980; Gilmour and Zips, 1985; Berlin et al., 1987; Mubagwa et al., 1992), which may be mediated by muscarinic M_1 receptor and via a process insensitive to pertussis toxin (Tajima et al., 1987; Gallo et al., 1993). The positive inotropic effect was believed to be related to an increase in $[Ca^{2+}]_i$. The main evidence was obtained in a quiescent rat ventricular myocyte suspension, in which carbachol at 300 μM increased

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$[Ca^{2+}]_i$, which was dependent on extracellular Ca^{2+} (Korth et al., 1988). The increase in $[Ca^{2+}]_i$ may be due to an increase in Ca^{2+} influx as carbachol at 100 μM has been shown to increase a Ca^{2+} current in the guinea-pig ventricular myocyte pretreated with pertussis toxin (Gallo et al., 1993). The Ca^{2+} influx may not be mainly via the L-type Ca^{2+} channel as the increase in $[Ca^{2+}]_i$ can not be blocked by nifedipine (Korth et al., 1988). The carbachol-induced increase in $[Ca^{2+}]_i$ is abolished in the absence of external Na^+, which is known to inhibit the Na^+-Ca^{2+} exchange (Korth et al., 1988). The contribution of mobilization of Ca^{2+} from its intracellular store to the elevation of $[Ca^{2+}]_i$ in response to muscarinic acetylcholine receptor stimulation by high carbachol is not clear. In the quiescent ventricular myocyte, the carbachol-induced increase in $[Ca^{2+}]_i$ was found to remain the same in the presence of ryanodine or caffeine (Korth et al., 1988), suggesting that intracellular Ca^{2+} from the sarcoplasmic reticulum is not required for the carbachol-induced increase in $[Ca^{2+}]_i$. However, carbachol also induces inositol 1,4,5-triphosphate (IP_3) production in myocardial cells (Berg et al., 1989; Gallo et al., 1993), which may induce Ca^{2+} release from sarcoplasmic reticulum via the IP_3-sensitive binding site in sarcoplasmic reticulum.

Cardiac muscle contracts upon the arrival of an excitatory impulse that depolarizes the sarcolemma and triggers a cascade of events – Ca^{2+} influx, Ca^{2+} release from the sarcoplasmic reticulum via a Ca^{2+}-induced Ca^{2+} release mechanism, and elevation of $[Ca^{2+}]_i$, leading eventually to muscle contraction. However, measurement of $[Ca^{2+}]_i$, in the quiescent ventricular myocyte, in which such a cascade of events does not happen, is not the most suitable parameter for the study of the mechanisms of contraction of cardiac muscle in response to humoral stimulation. In support of this statement, it was found that κ-receptor (Ventura et al., 1992) and angiotensin-receptor (Kem et al., 1991) stimulation increased $[Ca^{2+}]_i$ in the quiescent ventricular myocyte, but decreased the cardiac contractility, indicating that changes in the $[Ca^{2+}]_i$ in quiescent myocytes are not directly linked to changes in the contractility of cardiac muscle. The $[Ca^{2+}]_i$ transient in the ventricular myocyte induced by electrical stimulation, which also elicits contraction, is directly related to changes in contraction (Tatsukawa et al., 1993; Janczewski and Lakatta, 1993). This is because electrical stimulation elicits muscle contraction via the cascade of events that occur on the arrival of an excitatory impulse – Ca^{2+} influx, release of Ca^{2+} from the intracellular store by a Ca^{2+}-induced Ca^{2+} release (Fabiato, 1985). Therefore, the $[Ca^{2+}]_i$ transient in the ventricular myocyte is a more suitable parameter for the study of mechanisms related to contraction of cardiac muscle.

The purpose of the present study was therefore 1. to study the effects of high concentrations of carbachol on electrically induced $[Ca^{2+}]_i$ transients in the ventricular myocyte and 2. to re-investigate the mechanisms of muscarinic acetylcholine receptor stimulation by high concentrations of carbachol on the positive inotropic effect in this preparation. The main observations were: muscarinic acetylcholine receptor stimulation by high concentrations of carbachol increased the amplitude of the electrically induced $[Ca^{2+}]_i$ transient, which was blocked by atropine, but not pertussis toxin. Interference with Na^+-Ca^{2+} exchange or Ca^{2+} mobilization from the sarcoplasmic reticulum abolished completely, while reductions in Ca^{2+} influx by verapamil attenuated, the increase in the amplitude of the electrically induced $[Ca^{2+}]_i$ transient elicited by carbachol. Carbachol did not act directly on the sarcoplasmic reticulum, suggesting that mobilization of intracellular Ca^{2+} is a secondary event. Preliminary results have been presented in abstract form (Wang et al., 1996).

2. Materials and methods

2.1. Isolation of Ca^{2+}-tolerant ventricular myocytes

Ventricular myocytes of adult Sprague Dawley rats (200–250 g) were isolated using a collagenase perfusion method described previously (Dong et al., 1993). Immediately after decapitation of the rat, the heart was rapidly removed and perfused in a retrograde manner at a constant flow of 10 ml/min with oxygenated Joklik modified Eagle's medium supplemented with 1.25 mM $CaCl_2$ and 10 mM Hepes, pH 7.4, at 37°C for 5 min. followed by another 5 min. with the same solution without Ca^{2+}. Type I collagenase was added to the medium to a concentration of 125 U/ml with 0.1% bovine serum albumin. After 35–45 min. of perfusion with the collagenase containing medium, both atria were discarded. The ventricular tissues were cut into small pieces with a pair of scissors and stirred with a glass rod in the same oxygenated collagenase solution for 5 min at 37°C. The residue was filtered through 250 μm mesh screens with 2% bovine serum albumin. More than 70% of the cells were rod-shaped and not trypan-blue permeable. The Ca^{2+} concentration of the Joklik solution was increased gradually to 1.25 mM over 30 min.

2.2. Cell culture

A minimum essential medium supplemented with final concentrations of 100 I.U./ml penicillin, 0.1 mg/ml streptomycin and 10% fetal calf serum was used. The isolated cells were seeded into 25-cm^2 tissue culture flasks containing 10 ml of the supplemented solution and maintained at 37°C in 5% CO_2-humidified air. Pertussis toxin at 1 μg/ml was added for 20–24 h.

2.3. Loading of cells with fura-2/AM

Ventricular myocytes were incubated with fura-2/AM at a concentration of 4 μM in Joklik solution supple-

mented with 1.25 mM $CaCl_2$ for 25 min. The unincorporated dye was removed by washing the cells twice with fresh incubation solution. The loaded cells were maintained at room temperature (24–26°C) for 60 min before measurement of $[Ca^{2+}]_i$ in order to allow the fura-2/AM in the cytosol to de-esterify.

2.4. Measurement of cytosolic free $[Ca^{2+}]_i$

The apparatus and fura-2 fluorescent technique have been described previously (Hohl and Li, 1991). The ventricular myocytes loaded with fura-2/AM were transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The inverted microscope was coupled with a dual excitation spectrofluorimeter (PTI). The ventricular myocytes were superfused with a Krebs bicarbonate buffer (KB buffer) containing (in mM) 115 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose with 1% dialyzed bovine serum albumin and gassed with 95% O_2-5% CO_2. Low Na^+ solution was prepared by substituting 2/3 of NaCl with an equimolar concentration of choline chloride. For the experiment on the effect of caffeine, all NaCl was replaced by an equimolar concentration of choline chloride and $CaCl_2$ was not added. A small amount of Na^+ was still present due to the use of NaOH for adjustment of pH. The single cardiac myocytes were placed on a glass coverslip to which they naturally became adherent in approximately 20 min. They were paced at 0.1 Hz by electrical field stimulation with pulses from a stimulator at 60 V for 15 ms duration. The emitted light was filtered at 510 nm. Fluorescent signals obtained at 340 nm (F340) and 380 nm (F380) were recorded in a personal computer for subsequent data processing and analysis. The ratio of F340 and F380 was used to represent $[Ca^{2+}]_i$ in the myocyte because it was considered that changes in the fluorescence ratio do accurately reflect fluctuations in cytosolic concentration occurring during a contraction-relaxation cycle (Hohl and Li, 1991). The following parameters of the $[Ca^{2+}]_i$ transient were determined: peak fluorescence ratio, resting fluorescence ratio, amplitude (peak fluorescence ratio − resting fluorescence ratio) and time to 80% decline of amplitude from the peak. There was no leakage of fura 2 during the whole experimental period of 20 min, as indicated by the fact that the fluorescence at 340 and 380 nm remained constant for 20 min.

2.5. Drugs and chemicals

Carbachol, fura-2/AM, verapamil, type I collagenase, nickel chloride, pertussis toxin, ryanodine and caffeine were purchased from Sigma Chemical Co. All chemicals were dissolved in distilled water except fura-2/AM, which was dissolved in dimethyl sulphoxide (DMSO). The concentrations of drugs used were based on previous studies-verapamil, ryanodine and caffeine (Korth et al., 1988), and Ni^{2+} (Levi et al., 1993).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the difference among multiple groups. Paired Student's *t*-test was used to determine the effects of the drugs at different time points. For the effects of caffeine on the $[Ca^{2+}]_i$ transient, unpaired Student's *t*-test was used. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1. The effects of carbachol on the electrically induced Ca^{2+} transient

Carbachol at 30–300 μM increased significantly the amplitude of the electrically induced Ca^{2+} transient dose dependently (Fig. 1A,B). Carbachol at 100–300 μM also increased the resting fluorescence ratio and time to 80% decline of amplitude from the peak (Fig. 1A,B). In addition, it triggered $[Ca^{2+}]_i$ oscillations in 3 and 5 out of 6 cells studied at 100 and 300 μM, respectively (Fig. 1A). The time course of the effect of carbachol 100 μM is shown in Fig. 2. The effect of carbachol on amplitude was obvious at 10 min after addition of the drug (Fig. 2). The peak response was reached at 15 min when a plateau appeared (Fig. 2). At 15 min both the resting fluorescence ratio and time to 80% decline of amplitude from peak

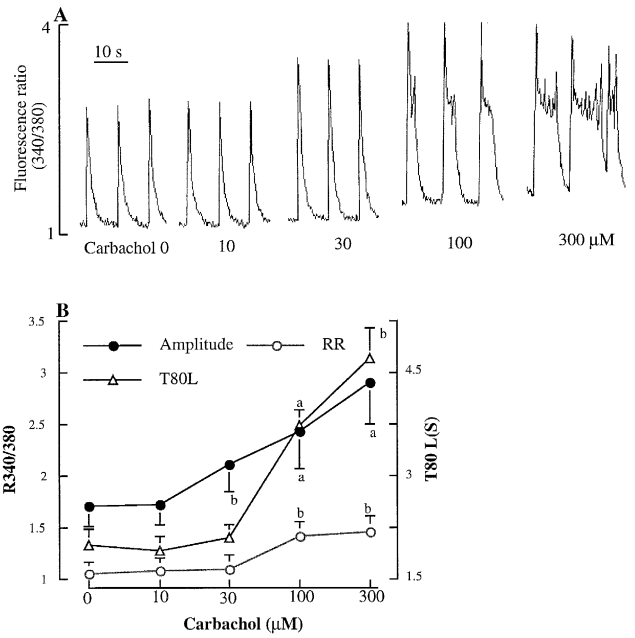

Fig. 1. Effects of carbachol (10–300 μM) on the electrically induced $[Ca^{2+}]_i$ transient in an isolated single ventricular myocyte – dose-dependent response at 15 min after drug administration. (A) Representative tracings; (B) effects on amplitude, resting fluorescence ratio and time to 80% decline in amplitude from peak (T80 L). For (B), values are means ± S.E.M; $n = 6$. [a] $P < 0.05$, [b] $P < 0.01$ vs. control.

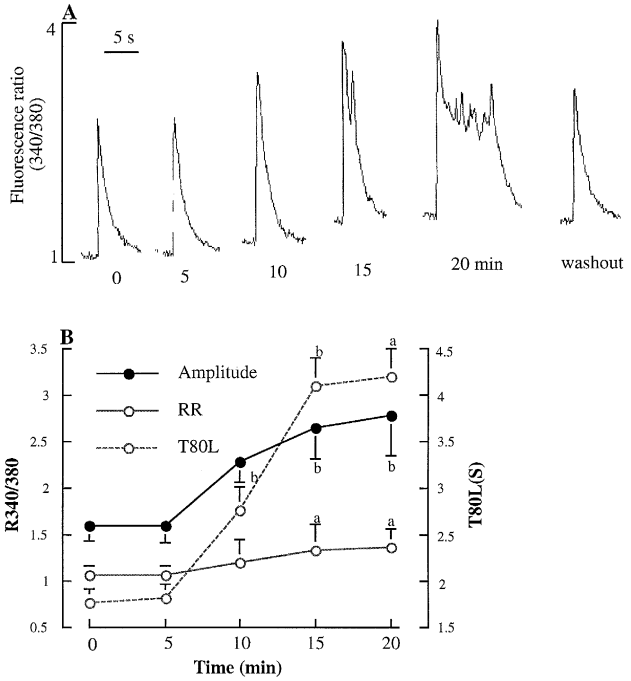

Fig. 2. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes – time-course changes. (A) Representative tracings; (B) effects on amplitude, resting fluorescence ratio and time to 80% decline in amplitude from peak (T80 L). For (B), values are means ± S.E.M; $n = 6$. [a] $P < 0.05$, [b] $P < 0.01$ vs. control.

increased significantly (Fig. 2) and $[Ca^{2+}]_i$ oscillations occurred (Fig. 2A).

3.2. The effect of carbachol on the $[Ca^{2+}]_i$ transient in ventricular myocytes pretreated with atropine and pertussis toxin

The effects of carbachol on the electrically induced Ca^{2+} transient were abolished after the addition of atropine at 1 μM for 10 min, which itself had no effect (Fig. 3A). Incubation of the ventricular myocytes with pertussis toxin at 1 μg/ml for 20–24 h failed to alter the effect of carbachol at 100 μM at all (Fig. 3B). The treatment indeed abolished the function of pertussis toxin-sensitive G-proteins in the present experimental conditions, as indicated by the fact that it completely abolished the inhibitory effects of adenosine 100 μM on the augmentation of the electrically induced Ca^{2+} transient by isoproterenol at 100 nM (data not shown), an event known to be mediated via G_i, which is sensitive to pertussis toxin (Brown et al., 1990; Ma and Green, 1992).

3.3. The effect of carbachol on the $[Ca^{2+}]_i$ transient in ventricular myocytes pretreated with ryanodine

In order to test whether the increase in the electrically induced $[Ca^{2+}]_i$ transient was related to mobilization of intracellular Ca^{2+}, the effects of carbachol were studied with pretreatment with ryanodine, which is known to deplete Ca^{2+} from its intracellular pool (Wier et al., 1985). As shown in Fig. 4A, ryanodine at 500 nM itself markedly decreased the amplitude of the electrically induced $[Ca^{2+}]_i$ transient, but increased significantly the resting fluorescence ratio, presumably due to depletion of Ca^{2+} from its intracellular pool (Wier et al., 1985). Carbachol added after ryanodine was unable to increase the amplitude of the electrically induced $[Ca^{2+}]_i$ transient, but the resting fluorescence was further elevated (Fig. 4). Neither did carbachol trigger $[Ca^{2+}]_i$ oscillation.

3.4. The effect of carbachol on the caffeine-induced $[Ca^{2+}]_i$ transient

In order to further test whether carbachol acted directly on the sarcoplasmic reticulum leading to mobilization of Ca^{2+} or not, we studied the effects of pretreatment with carbachol on the Ca^{2+} transient induced by caffeine, known to mobilize Ca^{2+} from the sarcoplasmic reticulum (Sitsapesan and Williams, 1990), in quiescent ventricular myocytes superfused with a Ca^{2+}-free and low Na^+ solution, thus removing the influence of both Ca^{2+} influx and Na^+-Ca^{2+} exchange. After superfusion of the cell with carbachol at 100 μM for 15 min, the caffeine-induced Ca^{2+} transient remained the same (Fig. 5). In a Ca^{2+}-free solution carbachol 100 μM itself had no effect on $[Ca^{2+}]_i$ in a quiescent ventricular myocyte either (data not shown).

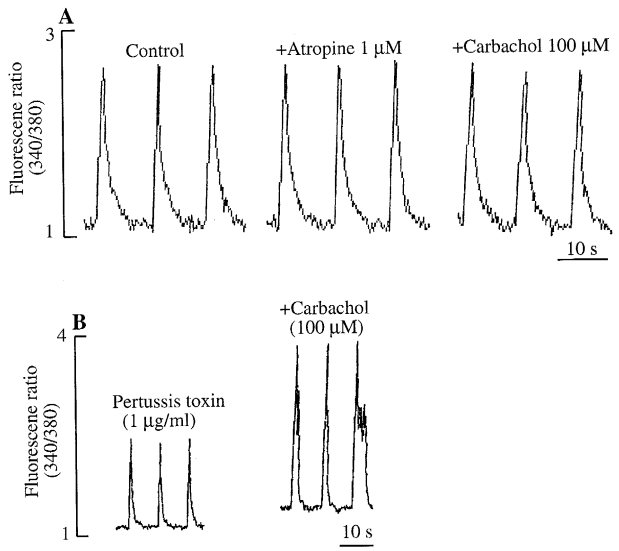

Fig. 3. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes in the presence of atropine (A) or pertussis toxin (B) – representative tracings of 5 experiments. Atropine (1 μM) was added 15 min before carbachol and pertussis toxin (1 μg/ml) was incubated with the myocytes for 20–24 h before experiment.

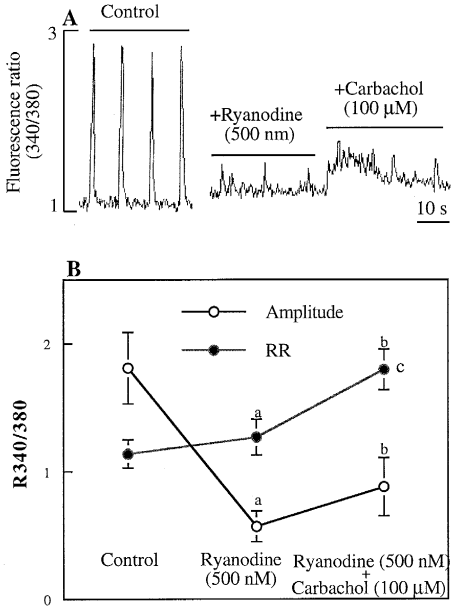

Fig. 4. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after pretreatment with ryanodine (500 nM). (A) A representative tracing; (B) effects of carbachol on the amplitude and resting fluorescence ratio (RR) of the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after pretreatment with ryanodine. Values are means ± S.E.M; $n = 5$. [a] $P < 0.05$, [b] $P < 0.01$ vs. control, [c] $P < 0.05$ vs. ryanodine only.

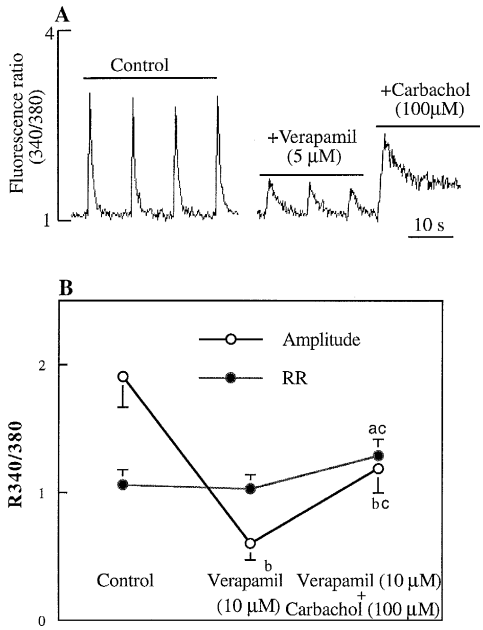

Fig. 6. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after pretreatment with verapamil (10 μM). (A) A representative tracing; (B) effects of carbachol on the amplitude and resting fluorescence ratio (RR) of the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after pretreatment with verapamil. Values are means ± S.E.M; $n = 7$. [a] $P < 0.05$, [b] $P < 0.01$ vs. control, [c] $P < 0.01$ vs. verapamil only.

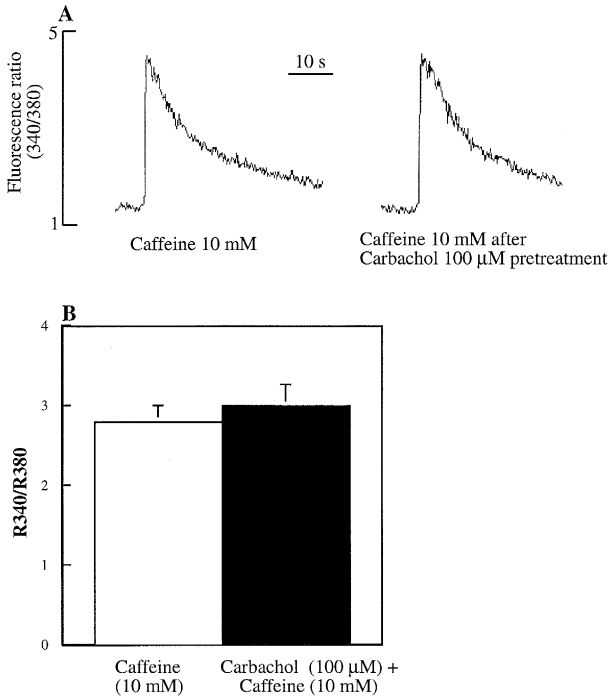

Fig. 5. Induction of $[Ca^{2+}]_i$ transients by caffeine (10 mM) in single isolated quiescent ventricular myocytes following pretreatment with carbachol (100 μM) and superfused with a Ca^{2+}-free and low $[Na^+]_o$ solution for 20 min. (A) A representative tracing; (B) amplitude of caffeine-induced $[Ca^{2+}]_i$ transients following pretreatment with carbachol and superfused with a Ca^{2+}-free and low $[Na^+]_o$ solution for 20 min. Values are means ± S.E.M; $n = 6$.

3.5. The effect of carbachol on the $[Ca^{2+}]_i$ transient in ventricular myocytes pretreated with verapamil

To determine the importance of Ca^{2+} influx via the voltage-gated Ca^{2+} channel on the augmentation of the electrically induced Ca^{2+} transient, the effects of carbachol following pretreatment with verapamil, a Ca^{2+} channel antagonist, were studied. Within 5 min after the addition of verapamil at 10 μM, the amplitude of the electrically induced Ca^{2+} transient was reduced significantly, while the resting fluorescence ratio remained unchanged. Although carbachol at 100 μM increased significantly both the amplitude of the electrically induced Ca^{2+} transient and resting fluorescence ratio in the presence or absence of verapamil, the increases in amplitude were significantly attenuated in the presence of the Ca^{2+} channel antagonist (Fig. 6). No $[Ca^{2+}]_i$ oscillation was observed.

3.6. The effect of carbachol on the $[Ca^{2+}]_i$ transient in myocardial cells pretreated with Ni^{2+}

Ni^{2+}, a non-selective Ca^{2+} channel blocker, which also inhibits Na^+-Ca^{2+} exchange (Kimura et al., 1987; Beuckelmann and Wier, 1989) was used. After the addition of Ni^{2+} at 5 mM, which itself reduced greatly the amplitude of the electrically induced Ca^{2+} transient without any effect on the resting fluorescence ratio, the muscarinic

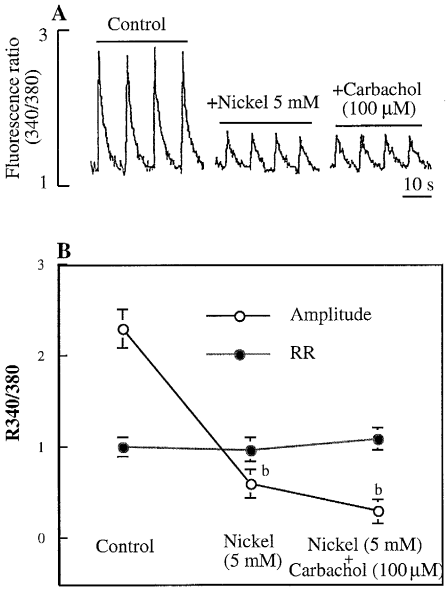

Fig. 7. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after prior superfusion with Ni^{2+} (5 mM) for 5 min. (A) A representative tracing; (B) effects of carbachol on the amplitude and resting fluorescence ratio (RR) of the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes after superfusion with Ni^{2+}. Values are means ± S.E.M; $n = 5$. [b] $P < 0.01$ vs. control.

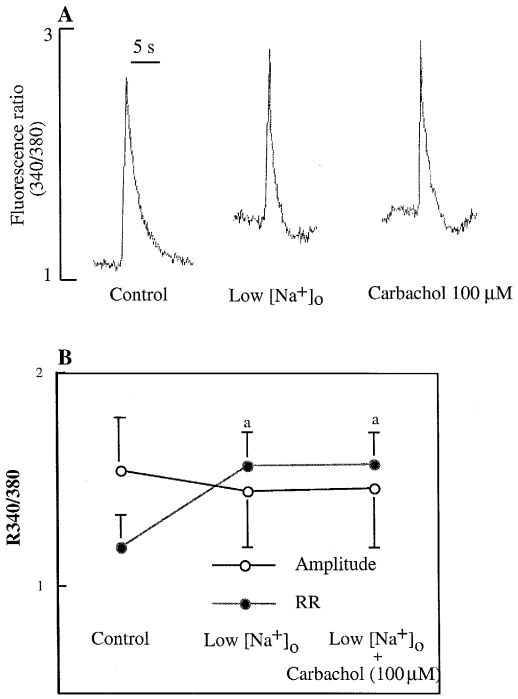

Fig. 8. Effects of carbachol (100 μM) on the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes in a low $[Na^+]_o$ solution. (A) A representative tracing; (B) Effects of carbachol on the amplitude and resting fluorescence ratio (RR) of the electrically induced $[Ca^{2+}]_i$ transient in isolated single ventricular myocytes in a low $[Na^+]_i$ solution. Values are means ± S.E.M; $n = 5$. [a] $P < 0.05$ vs. control.

receptor agonist was unable to increase the electrically induced Ca^{2+} transient any further (Fig. 7), an effect different from that of verapamil (Fig. 6). Nor did the Ca^{2+} oscillation occur (Fig. 7).

3.7. The effect of carbachol on the $[Ca^{2+}]_i$ transient in myocardial cells pretreated with low $[Na^+]_o$

Replacement of 2/3 of the sodium chloride with choline chloride, which increased the resting fluorescence ratio in agreement with its well known inhibitory action on Na^+-Ca^{2+} exchange (Barry et al., 1985; Bers, 1987; Tatsukawa et al., 1993), abolished the effects of carbachol on the electrically induced Ca^{2+} transient and $[Ca^{2+}]_i$ oscillations (Fig. 8). The resting fluorescence ratio remained high.

4. Discussion

In the present study we observed that high concentrations of carbachol increased not only the amplitude and duration of the electrically induced $[Ca^{2+}]_i$ transient, but also the resting level of cytoplasmic Ca^{2+}, indicating increases in intracellular Ca^{2+} in the ventricular myocyte in both stimulated and non-stimulated states. The observation was in agreement with the previous finding that in the quiescent cardiomyocyte, carbachol at the same dose-range increased $[Ca^{2+}]_i$ (Korth et al., 1988). That carbachol prolonged time to 80% decline of amplitude from the peak suggests that either sequestration of Ca^{2+} back to the sarcoplasmic reticulum or the extrusion of Ca^{2+} out of the cell across the sarcolemma, or both, may be attenuated. This warrants further investigation.

The effects of carbachol at high concentrations on the electrically induced $[Ca^{2+}]_i$ transient were blocked by atropine, but not pertussis toxin, which is in agreement with the previous observation in chick atria (Tajima et al., 1987). The findings of both studies indicate that the action of carbachol at this dose range is receptor-mediated via a process which does not involve pertussis toxin-sensitive G-proteins.

In the present study, the ventricular myocyte was depolarized by electrical field stimulation, which is known to lead to muscle contraction via a cascade of events that occur when an impulse generated from the S-A node arrives in the cell. It has been shown that increases in electrically induced $[Ca^{2+}]_i$ transients are directly correlated to increases in contractility (Janczewski and Lakatta, 1993). Therefore, the stimulatory effects of carbachol on the electrically induced $[Ca^{2+}]_i$ transient offer a reasonable explanation of the inotropic effect of the muscarinic receptor agonist. Unfortunately, in the present study, changes in contractility of the ventricular myocyte following treatment with carbachol were not measured and correlated

with changes in electrically induced $[Ca^{2+}]_i$ transients. Further study is needed.

In order to further investigate the mechanism of augmentation of the electrically induced $[Ca^{2+}]_i$ transient by muscarinic acetylcholine receptor stimulation, we studied the effects of muscarinic acetylcholine receptor stimulation on the electrically induced $[Ca^{2+}]_i$ transient when Ca^{2+} in the intracellular pool, Ca^{2+} influx via the L-type Ca^{2+} channels, and Na^+-Ca^{2+} exchange were interfered with. In the present study, we found that ryanodine, known to accelerate Ca^{2+} loss from the sarcoplasmic reticulum (Wier et al., 1985), as indicated by an increase in resting fluorescence ratio, when added before carbachol abolished the enhancing effect of the agonist on the electrically induced $[Ca^{2+}]_i$ transient. The observation indicates the importance of Ca^{2+} from the intracellular store in the carbachol-induced augmentation of the $[Ca^{2+}]_i$ transient. In contrast, in quiescent ventricular myocytes, the carbachol-induced increase in $[Ca^{2+}]_i$ was unaffected in the presence of either ryanodine or caffeine, suggesting that Ca^{2+} release from the sarcoplasmic reticulum was not required for the increase in $[Ca^{2+}]_i$ following muscarinic acetylcholine receptor stimulation (Korth et al., 1988). The discrepancy between the two studies is due to the different preparations used. Upon electrical stimulation, which depolarizes the sarcolemma, Ca^{2+} influx occurs via voltage-gated Ca^{2+} channels, leading to release of Ca^{2+} from the sarcoplasmic reticulum via a Ca^{2+}-induced Ca^{2+} release mechanism and eventually to contraction (Fabiato, 1985; Cleemann and Morad, 1991). Such events, and in particular the Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, do not happen in quiescent myocytes. The contribution of Ca^{2+} release from the sarcoplasmic reticulum was secondary as carbachol did not increase the $[Ca^{2+}]_i$ level in quiescent ventricular myocytes nor did it affect the caffeine-induced $[Ca^{2+}]_i$ transient in the absence of external Ca^{2+} and in the presence of low $[Na^+]_o$, which removed the influence of both Ca^{2+} influx and Na^+-Ca^{2+} exchange. The observations indicate no direct action of carbachol on the ryanodine-sensitive site in the sarcoplasmic reticulum in the ventricular myocyte. Ca^{2+} influx via voltage-gated Ca^{2+} channels has been well established to trigger Ca^{2+} release from the sarcoplasmic reticulum (Fabiato, 1985). There is also evidence suggesting that the release of Ca^{2+} from its intracellular pool may be secondary to activation of Na^+-Ca^{2+} exchange (Ganitkevich and Isenberg, 1993; Levi et al., 1993). Further studies are needed to determine the roles of these mechanisms in triggering the mobilization of intracellular Ca^{2+} upon muscarinic acetylcholine receptor stimulation in electrically stimulated myocytes.

In the present study it was found that when Ca^{2+} influx via the L-type Ca^{2+} channel was inhibited with a Ca^{2+} channel antagonist, verapamil, the effects of carbachol were greatly attenuated, although it was still able to increase the resting fluorescence ratio, suggesting Ca^{2+} influx via the L-type Ca^{2+} channel was also involved. The observation is not in agreement with the finding that the carbachol-induced increase in $[Ca^{2+}]_i$ is not affected by nifedipine in quiescent ventricular myocytes (Korth et al., 1988). The discrepancy may be also related to the different preparations used in the previous and the present studies, as discussed above.

Ni^{2+}, a non-specific Ca^{2+} channel antagonist that also inhibits Na^+-Ca^{2+} exchange (Kimura et al., 1987; Beuckelmann and Wier, 1989), significantly reduced the amplitude of the electrically induced $[Ca^{2+}]_i$ transient in a similar way as verapamil, presumably due to its inhibitory effects on Ca^{2+} influx. Unlike verapamil, Ni^{2+} added before carbachol abolished completely the augmentation of the electrically induced $[Ca^{2+}]_i$ transient by carbachol, suggesting that the Na^+-Ca^{2+} exchange may be essential for the process. In support of the suggestion, in the presence of low external Na^+, which itself increased the resting fluorescence ratio presumably due to inhibition of the Na^+-Ca^{2+} exchange (Barry et al., 1985; Bers, 1987; Tatsukawa et al., 1993), but did not affect the electrically induced $[Ca^{2+}]_i$ transient, carbachol could no longer increase the amplitude of $[Ca^{2+}]_i$ transient. Since influx of Ca^{2+} only contributed partly to the augmentation of the carbachol-induced increase in $[Ca^{2+}]_i$ transient and mobilization of Ca^{2+} from the intracellular store was a secondary event, Na^+-Ca^{2+} exchange may be important in initiating the augmentation of the electrically induced $[Ca^{2+}]_i$ transient elicited by carbachol.

It was found that carbachol further increased the resting fluorescence ratio despite the presence of ryanodine or verapamil, but not after inhibition of Na^+-Ca^{2+} exchange. The increase in the resting $[Ca^{2+}]_i$ elicited by carbachol after depletion of intracellular Ca^{2+} or blockade of Ca^{2+} influx via the Ca^{2+} channel was most likely due to an increase in Na^+-Ca^{2+} exchange because Korth et al. (1988) showed that in quiescent ventricular myocytes the carbachol-induced elevation in $[Ca^{2+}]_i$ is abolished in the absence of external Na^+, which inhibit Na^+-Ca^{2+} exchange.

In the present study, we observed for the first time that carbachol induced a Ca^{2+} oscillation in cardiac cells which occurred after a delay. It has been reported that Ca^{2+} oscillation occurs in Ca^{2+}-overloaded ventricular myocytes (Orchard et al., 1983; Wier et al., 1983). The occurrence of Ca^{2+} oscillations after a delay may be due to the fact that time is required for a sufficient amount of Ca^{2+} to accumulate to trigger the Ca^{2+} oscillations. That the carbachol-induced Ca^{2+} oscillation was abolished by pretreatment with ryanodine, verapamil or Ni^{2+} suggests that the Ca^{2+} oscillation results from Ca^{2+}-overload in cardiac cells. Changes in membrane potential may also contribute to the Ca^{2+} oscillation as high concentrations of carbachol have been shown to induce depolarization, prolong the action potential duration and increase the automaticity of the ventricular myocyte (Tajima et al., 1987; Mubagwa et al., 1992). Further studies are needed to delineate the

mechanism of the Ca^{2+} oscillation evoked by muscarinic acetylcholine receptor stimulation by high carbachol.

It is worth noting that a muscarinic receptor agonist at low and high concentrations produces inhibitory and excitatory effects via two receptor subtypes and two different pathways, respectively. The excitatory effects of high concentrations of muscarinic receptor agonist are believed to prevent the heart from excessive inhibition when the parasympathetic nervous system is overactivated (Mubagwa et al., 1992). Further studies on the pathophysiological significance are warranted.

In conclusion, the present study has provided evidence for the first time that carbachol at high concentrations augments electrically induced $[Ca^{2+}]_i$ transients via a receptor-mediated process which is not sensitive to pertussis toxin. This explains the positive inotropic action of carbachol. The present study has also demonstrated for the first time that unlike quiescent cardiomyocytes, in which the increase in $[Ca^{2+}]_i$ does not require Ca^{2+} from the intracellular pool, in active cardiomyocytes the augmentation of the electrically induced $[Ca^{2+}]_i$ transient by carbachol requires mobilization of intracellular Ca^{2+} from its store, which is a secondary event. Influx of Ca^{2+} via the L-type Ca^{2+} channel has also been demonstrated to contribute to the augmentation of the $[Ca^{2+}]_i$ transient elicited by carbachol. The Na^+-Ca^{2+} exchange may be important in initiating the augmentation of the electrically induced $[Ca^{2+}]_i$ transient by carbachol. We have also observed for the first time Ca^{2+} oscillation induced by carbachol. Further studies are needed to delineate 1. the relationship between Na^+-Ca^{2+} exchange and influx of Ca^{2+} via voltage-gated Ca^{2+} channels, and mobilization of intracellular Ca^{2+} and 2. the mechanisms of generation of Ca^{2+} oscillation upon muscarinic acetylcholine receptor stimulation in ventricular myocytes subjected to electrical stimulation.

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