Carbachol Increases Contractions and Intracellular Ca\textsuperscript{++} Transients in Guinea Pig Ventricular Myocytes\textsuperscript{1}

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Accepted for publication September 2, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

We tested hypotheses concerning the muscarinic receptor subtype and the involvement of L-type Ca current (I\textsubscript{Ca}) in the stimulation of contractions by carbachol (CCh) in single guinea pig ventricular myocytes. When superfused with Tyrode's solution (36°C, 5.4 mM [Ca\textsuperscript{2+}]) and stimulated at 0.2 Hz, CCh (EC\textsubscript{50} \approx 18 \mu M) increased the early component of isotonic contractions by acting at muscarinic receptors indistinguishable from the M\textsubscript{2} subtype because AF-DX 116 (M\textsubscript{2}-selective) was more potent than pirenzepine (M\textsubscript{1}-selective) as an antagonist of the CCh effect. Action potential duration decreased slightly and I\textsubscript{Ca} was not increased when CCh increased contractions. Carbachol increased intracellular Ca\textsuperscript{++} transients and contractions reversibly, which indicated an effect via sarcoplasmic reticulum (SR) Ca stores. Ryanodine (1–10 \mu M) blocked the early contraction component increased by CCh, another indication that CCh action depends on SR Ca stores. We previously found that CCh increased a background Na\textsuperscript{+} current by occupancy of M\textsubscript{2} receptors. We now report that these increased contractions by CCh can also originate at M\textsubscript{2} receptors and that SR Ca stores are involved in the CCh effect. Because CCh did not significantly increase I\textsubscript{Ca}, the initial increase of intracellular Na\textsuperscript{+} by CCh may eventually act through Na-Ca exchange to enhance excitation-contraction coupling.

Vagal stimulation usually evokes negative chronotropic, dromotropic and inotropic effects on the heartbeat (reviewed in Pappano, 1995; Pappano and Mubagwa, 1992) via pre- and postjunctional mACHR. Prejunctionally, the vagal transmitter ACh suppresses norepinephrine release from sympathetic adrenergic neurons. Postjunctionally, ACh-mediated signals are transduced by the G\textsubscript{i} into inhibition of I\textsubscript{Ca} via reduced synthesis and action of cAMP, activation of I\textsubscript{K(ACh)} and reduced I\textsubscript{Na}. PTX chemically inactivates G\textsubscript{i} and suppresses vagal and muscarinic inhibition (reviewed in Pappano and Mubagwa, 1992). Vagus nerve activation or muscarinic agonist also stimulates the heartbeat (reviewed in Pappano, 1991, 1995). Examples include the reflex-induced phase of sinus tachycardia in humans (Prystowsky and Zipes, 1985) and the positive inotropic effects in rabbit atria (Endoh and Blinks, 1984), canine (Gilmour and Zipes, 1985) and sheep (Iacono and Vassalle, 1989) Purkinje fibers and guinea pig ventricle (Korth and Kühlkamp, 1985; Yang et al., 1996). Muscarinic agonists increase contraction force in embryonic chick ventricle (Protas, 1990) and in atria from embryonic (Protas, 1992) and hatched chicks (Webb and Pappano, 1995). These results are interesting for two reasons. First, they are initiated by ACh at mACHR and are catecholamine-independent.

Second, muscarinic stimulation of the heart can occur in the absence of PTX. Indeed, PTX treatment may only serve to reveal the stimulant effects more clearly because G\textsubscript{i}-transduced inhibition is lacking (reviewed in Pappano, 1991). However, there are reports of stimulant actions of CCh in guinea pig (Gallo, et al., 1993) and rat (Sharma et al., 1996) ventricular myocytes that apparently require PTX pretreatment.

The proposed mechanism(s) for muscarinic stimulation of the heart are varied. Intracellular Na\textsuperscript{+} increased in guinea pig ventricle (Korth and Kühlkamp, 1985; Yang et al., 1996) and in sheep cardiac Purkinje fibers (Iacono and Vassalle, 1989) in the presence of either ACh or CCh. Increased Na\textsuperscript{+} entry via TTX-resistant channels was suggested for ventricular muscle (Korth and Kühlkamp, 1985) and verified in myocytes (Matsumoto and Pappano, 1989) of guinea pig.

ABBREVIATIONS: ACh, acetylcholine; PTX, pertussis toxin; CCh, carbachol; Gi, inhibitory guanine nucleotide binding protein; AF-DX 116, (11)[2-[diethylamino)methyl]-1- piperidinyl][acetyl]-5,11-dihydro-6H-pyrido[2,3-6]1,4][benzodiazepine-6-jone; mACHR, muscarinic receptor; SR, sarcoplasmic reticulum; InsP\textsubscript{3}, inositol 1,4,5-triphosphate; I\textsubscript{Ca}, L-type calcium current; I\textsubscript{Na-Ca}, sodium-calcium exchange current; HEPES, N-(2-hydroxyethyl)piperazine-N-(2- ethanesulfonic acid); cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; I\textsubscript{K(ACh)}, acetylcholine-induced potassium current; fura-2AM, fura-2-acetoxymethyl ester; EC\textsubscript{50}, effective concentration for 50% maximum effect; APD\textsubscript{50}, action potential duration at 50% amplitude; PKC, protein kinase C; TTX, tetrodotoxin.
inhibition of the Na/K pump was proposed for the muscarinic agonist stimulant effect in sheep Purkinje fibers (Iacono and Vassalle, 1989). Altered Na-Ca exchange secondary to increased Na$^+$ entry has experimental support that could link it to increased contractions (Korth et al., 1988). Alternatively, an increased $I_{\text{Ca}}$ by CCh was advanced as an explanation of the stimulation of guinea pig ventricular myocytes treated with PTX (Gallo et al., 1993). This hypothesis is assumed to underlie the increased intracellular Ca$^{++}$ transient by CCh in rat ventricular myocytes treated with PTX (Sharma et al., 1996). Increased release of Ca$^{++}$ secondary to augmentation of InsP$_3$ synthesis by muscarinic agonist also is a possible explanation (Pappano, 1991). Activation of pirenzepine-sensitive (M$_2$) receptors reportedly initiates muscarinic stimulation of $I_{\text{Ca}}$ (Gallo et al., 1993) and intracellular Ca$^{++}$ transients (Sharma et al., 1996). In contrast, the CCh-induced background Na current is initiated at M$_2$ mACHR (Matsumoto and Pappano, 1991) as is the positive inotropic effect in human ventricular muscle (Du et al., 1995).

The divergent results and mechanisms for muscarinic stimulation could arise from differences in preparations. Our experiments describe a guinea pig ventricular myocyte model in which muscarinic agonist-induced stimulation of cell contractions is reproducibly obtained and which replicates that found in guinea pig papillary muscle. We evaluated the hypothesis that CCh increases contractions by augmenting $I_{\text{Ca}}$ (Gallo et al., 1993) and also tested a hypothesis concerning the nature of the mACHR that initiates the increased contractions and ascertains the effect of CCh on intracellular Ca$^{++}$ transients. We chose ventricular myocytes because they illustrate the importance of mACHR signaling in a tissue that is usually considered not to have direct actions either to choline esters or to vagus nerve stimulation. A preliminary account of a portion of this work has been presented as an abstract (Protas et al., 1997).

**Methods**

**Cell isolation.** Single ventricular myocytes were isolated enzymatically from the hearts of male and female guinea pigs (250–350 g) anesthetized with sodium pentobarbital (30 mg/kg i.p.) and anti-coagulated with heparin (400 IU i.p.). The heart was retrogradely perfused with Tyrode’s solution for 5 min at a rate of 8 to 10 ml/min through an aortic cannula in a Langendorff apparatus. The composition of Tyrode’s solution was (in mM): NaCl, 135; KCl, 5.4; CaCl$_2$, 1.8; MgCl$_2$, 1.0; NaH$_2$PO$_4$, 0.33; HEPES, 10; glucose, 27.5; pH was adjusted to 7.4 with NaOH. Next, Ca-free Tyrode’s solution was perfused for 45 to 60 s until the heart stopped beating. Subsequently, the heart was perfused with Tyrode’s solution having 30–40 µM Ca and containing 36 mg collagenase (Boehringer Mannheim, Indianapolis, IN, type A) and 3.6 mg protease (Sigma, St. Louis, MO, type IV) in 50 ml. This solution was recirculated for 10 min, after which the enzymes were washed out by perfusion with 50 ml of Recovery solution. Recovery solution contained (in mM): K aspartate, 130; K$_2$ATP, 5; HEPES, 5; glucose, 20; pH was adjusted to 7.4 with KOH. Afterward, the ventricles were cut off, the cells dispersed in Recovery solution and kept at 4°C for at least an hour. An aliquot of cell suspension was placed in a recording chamber (500 µl volume) mounted on the stage of an inverted microscope. After 10 min, it was superfused with Tyrode’s solution (2 ml/min); the glucose concentration was reduced to 10 mM for experiments. Temperature was 36 ± 0.5°C unless otherwise indicated.

**Electrophysiology.** In some experiments, external stimuli were applied to the myocyte from a programmable stimulator (WPI Electronics, Sarasota, FL) equipped with an isolation unit. The stimulating electrode was a glass capillary microelectrode filled with Tyrode’s solution; the tip was ~50 µm. The electrode was placed within 20 to 100 µm of the myocyte; the anode was a platinum wire immersed in the bath.

An EPC 7 patch clamp amplifier (List Electronics, Darmstadt, Germany) was used to deliver either voltage clamp or current clamp pulses in whole cell mode. All voltage commands and current data acquisition were controlled by an IBM-compatible computer equipped with pClamp software (version 5.5, Axon Instruments, Burlingame, CA) and a Labmaster TL-1 interface (Axon Instruments). Electrodes were prepared from glass capillary tubes (inner diameter, 1.1 mm; outer diameter, 1.3 mm) and filled with a pipette solution whose composition was (in mM): K aspartate, 120; KCl, 30; Na$_2$ATP, 4; MgCl$_2$, 1.0; HEPES, 5; pH = 7.2. The resistance was 2 to 4 MΩ. The pipette was connected to the amplifier by a Ag-AgCl wire, and the tip was gently pushed against the cell surface. Negative pressure was applied to the pipette interior until a gigahm seal was formed. After the electrode capacitance was compensated electronically in the cell-attached mode, the cell membrane was ruptured by additional negative pressure. In some experiments where action potentials were initiated by current passed through the patch electrode, nystatin (50 µg/ml) was added to the pipette solution to obtain electrical access to the cell interior. The stock solution of nystatin (200 mg/ml) was prepared in methanol, protected from light and used to prepare fresh pipette solutions at the time of experiments.

**Cell contraction.** Contractions of single myocytes were elicited by either external or internal stimuli. With the cell shortening along its long axis, displacement of one or both ends of the cell edge is an indicator of the extent of cell contraction. A video-edge detection system (Crescent Electronics, Sandy, UT) was used to track the motion of cell edge. A microscope-magnified (400×) cell image was observed continuously on a high-resolution black-and-white TV monitor via a sequential scanning video camera attached to a sideport of the microscope. The camera position was rotated so that the video monitor raster lines were parallel with the long axis of the cell. The video dimension analyzer monitored a selected raster line for light intensity differences between the end of the myocyte and the surrounding field. The temporal resolution of this detector was 16.7 ms and motion of as little as 0.25 µm could be detected. The signal from the detector was sent to a strip chart recorder and to a VCR for storage and off-line analysis. The extent of shortening of each cell was measured and was expressed either as micrometer of displacement or as percent cell length.

**Intracellular calcium transients.** A suspension of myocytes was incubated in 2 ml Tyrode’s solution (36°C) containing 2 to 3 µM fura-2 AM ester for 5 to 10 min. The incubation solution was de-esterified and the cells washed in Tyrode’s solution for up to 30 min to remove extracellular dye and to allow time for its intracellular de-esterification. The epifluorescence system (PTI Deltascan) had two monochromators that passed excitation wavelengths of 340 and 380 nm reflected onto the myocyte through a dichroic mirror (410 nm). Emitted fluorescence passed through the mirror to a 510 nm bandpass filter and then to the photomultiplier. Cytoplasmic Ca$^{++}$ was reported as the 340/380 ratio rather than the concentration of free Ca$^{++}$ because the calibration may be complicated by dye compartmentation and incomplete de-esterification. Cell contractions and the 340/380 were recorded alternately under steady-state conditions.

**Statistical analysis.** Data are expressed as mean ± S.E.M. Significance between means was calculated by the Student’s $t$ test (paired or independent as appropriate).

**Results**

Effects of extracellular calcium [Ca$^{++}$]$_{o}$ and stimulation frequency. We used combinations of two frequencies of extracellular stimulation (1 Hz and 0.2 Hz) and two
[Ca\(^{++}\)]_o (1.8 mM and 5.4 mM) to find the conditions needed to detect a stimulant effect of CCh in single ventricular myocytes. The CCh concentration used in these experiments, 30 \(\mu\)M, was very close to the EC\(_{50}\) found for the positive inotropic effect of CCh in multicellular heart preparations.

The average increase in shortening produced by CCh at 1.0 Hz was \(6 \pm 0.6\% (n = 6)\) and \(3 \pm 2.9\% (n = 5)\) of control shortening for myocytes bathed in normal (1.8 mM) and in high (5.4 mM) [Ca\(^{++}\)]_o, respectively. The difference between control shortening and shortening in the presence of CCh was not significant for either group (P = .147 and .274, respectively). Myocytes stimulated at a frequency of 0.2 Hz and superfused with 1.8 mM [Ca\(^{++}\)]_o demonstrated a greater response to CCh (16 \(\pm\) 6.7% of control shortening; n = 6) but the difference was not significant (P = .099). The greatest CCh-induced increase in shortening was observed in myocytes stimulated at 0.2 Hz and bathed in a modified Tyrode’s solution containing 5.4 mM [Ca\(^{++}\)]_o, respectively. The shortening in the presence of CCh was 9.2 \(\pm\) 1.45 \(\mu\)m (6.8 \(\pm\) 0.84% cell length), the difference being highly significant (P = .005). The additional increase in shortening evoked by CCh was calculated to be \(2.4 \pm 0.61\% (1.7 \pm 0.35\% \text{ cell length})\) or \(39 \pm 7.7\%\) of control shortening (n = 8).

An illustration from an experiment conducted under these conditions (0.2 Hz; 5.4 mM [Ca\(^{++}\)]_o) is shown in figure 1. Cell contraction amplitude increased within 30 s after addition of 100 \(\mu\)M CCh and maximum effect occurred at \(\sim\)3 min. The lower panel shows that individual isotonic contractions display two components of shortening. The increase of myocyte shortening by CCh is registered primarily on the early and more rapid contraction phase whose amplitude doubled from an initial value of 2.3 (a) to 4.7 \(\mu\)m (b) in the presence of CCh. Myocyte shortening returned to 2.2 \(\mu\mM\) after washout of CCh (c).

**Stimulus frequency-cell shortening relation and its importance for CCh effect.** At the beginning of all experiments with extracellular stimulation, we checked the influence of stimulation frequency on the amplitude of myocyte shortening. Stimuli at 0.2 Hz and 1 Hz were applied to the cell and the ratio \(\Delta L_{o.2Hz}/\Delta L_{o.1Hz}\) was calculated where \(\Delta L_{o.2Hz}\) and \(\Delta L_{o.1Hz}\) are the cell shortenings at 0.2 Hz and 1 Hz, respectively. In more than half the experiments, the change from higher to lower stimulation frequency was accompanied by a decrease in contraction amplitude which is typical for guinea pig ventricle. The magnitude of the decrease, however, was variable. In figure 2, the effect of 100 \(\mu\)M CCh is presented as function of \(\Delta L_{o.2Hz}/\Delta L_{o.1Hz}\) ratio. The results show that there is a correlation between the ratio and the response to CCh; the greater the ratio (the smaller the decrease of amplitude with lowering of stimulation rate), the smaller the response to CCh. In 4 of 12 myocytes, the contraction amplitude was the same at 0.2 and 1 Hz stimulus frequencies; in those myocytes application of CCh was without effect (fig. 2). We considered the myocytes with a flat frequency-shortening dependence to have maximally filled SR Ca\(^{++}\) stores and rejected experiments in which \(\Delta L_{o.2Hz}/\Delta L_{o.1Hz}\) index was \(>0.8\). All results obtained with extracellular stimulation, including those described above (fig. 1), were selected by this criterion.

**Temperature.** We carried out experiments at 23°C with other conditions at which body temperature provided positive results, namely, a stimulus frequency of 0.2 Hz and 5.4 mM [Ca\(^{++}\)]_o. At 23°C, control shortening (10.9% cell length) was nearly twice as great as at 36°C. We used 100 \(\mu\)M CCh, however, even at this concentration, the effect of CCh was much smaller than at body temperature. In two experiments, I
![Fig. 1.](image1.jpg)  
An example of the increased contraction amplitude produced by CCh (100 \(\mu\)M) in a myocyte superfused with 5.4 mM Ca\(^{++}\)-Tyrode’s solution at 36°C and stimulated at 0.2 Hz. (A) Recording of contractions at slow chart speed. The time of exposure to CCh is shown by the thin line above the trace; time calibration is 2 min. (B) Single contractions recorded at fast chart speed taken at the times indicated by a, b and c in panel A. Contraction amplitude doubled in CCh whose effect is completely reversed by washout. Calibration for shortening in B applies to both panels.

![Fig. 2.](image2.jpg)  
Dependence of CCh-induced increase in cell contractions on the stimulus frequency-shortening relation. (A) Decrease in amplitude of cell contraction as stimulus frequency is reduced from 1 Hz to 0.2 Hz. (B) Ordinate, response to 100 \(\mu\)M CCh as % of control shortening at 0.2 Hz; abscissa, ratio of shortening at 0.2 Hz to that at 1.0 Hz obtained before addition of CCh. The linear regression is fit by y = \(-150 (\pm 31)x + 140 (\pm 22)\) with r = 0.84.
CCh did not change the cell contraction; in the remaining four, the increase in shortening did not exceed 0.5% cell length (0.55 μm). The average shortening in CCh was $11 \pm 1.6\%$ of cell length and did not significantly differ from control shortening ($P = .34$).

Thus, consistent and significant CCh-induced increases in ventricular myocyte shortening can be achieved at 36°C, 5.4 mM [Ca$^{2+}$]o in bath solution and a stimulus frequency of 0.2 Hz. Experiments described in the next sections were performed under these conditions.

**Concentration dependence and sensitivity to muscarinic antagonists.** External stimulated cells were exposed to CCh which was added by a discrete or cumulative method. In discrete addition, CCh was used only once or twice (for example, 3 μM and then 300 μM, 20–30 min after the first portion was washed out) on one cell. In the experiments with discrete addition, the maximal response occurred at 100 μM CCh and amounted to a 68 ± 18.5% increase above control shortening (+2.3 ± 0.36% of cell length; $n = 7$); the EC$_{50}$ of CCh was 17.4 μM.

In cumulative addition, CCh was added in increasing concentrations until the maximal response was reached. When expressed as percent of maximum response, the CCh EC$_{50}$ was 18.1 μM (see fig. 3). The maximal response to CCh was a 104 ± 29% increase above control (+5 ± 1.1% of cell length; $n = 10$). The close similarity between results of discrete and cumulative addition of CCh allowed us to use cumulative addition in experiments with antagonists.

Atropine, 3 μM, completely blocked the stimulant effect of 30 μM CCh on myocyte contractions (three experiments, not shown). To specify the mACHr subtype responsible for this effect we used two muscarinic antagonists, pirenzepine, which is selective for the M$_1$ receptor, and AF-DX 116, which has greater affinity for the M$_2$ receptor. Each antagonist produced a rightward shift in the cumulative concentration-effect curve for CCh (fig. 3). The EC$_{50}$ values for CCh were 129 μM and 78 μM in the presence of 1 μM pirenzepine or 30 nM AF-DX 116, respectively. The apparent antagonist dissociation constants ($K_D$) were calculated from the Schild equation: $[(D)/[D_o] - 1] = [A]/K_D$, where $[D]$ and $[D_o]$ are equieffective agonist drug concentrations in the presence and absence of antagonist, respectively, and $[A]$ is the antagonist concentration. The apparent $K_D$ values are 1.6 × 10$^{-7}$ M for pirenzepine and 9.1 × 10$^{-8}$ M for AF-DX 116. Thus, the M$_2$ selective drug, AF-DX 116, is the more potent antagonist of the CCh effect.

**Action potentials.** The effect of CCh on excitation-contraction coupling was examined by simultaneous recording of action potentials and contractions under current clamp conditions. The bath contained 5.4 mM [Ca$^{2+}$]o in modified Tyrode’s solution (36°C); action potentials were initiated by currents delivered through the patch electrode at a frequency of 0.2 Hz. The electrode filling solution included 50 μg/ml nystatin; electrical access was achieved by perforation of the patch rather than by rupture. An example is shown in figure 4A. The resting potential was −67 mV before, in the presence of, and after washout of 100 μM CCh. There was a small decrease in APD$_{50}$ which was 170 ms in the absence and 142 ms in the presence of CCh. At the same time, myocyte contractions increased by 26% from 8.8% of cell length in the absence of CCh to 11.1% of cell length in its presence (fig. 4A).

After washout of CCh, myocyte contractions diminished to 8.0% of cell length; APD$_{50}$ increased to 165 ms during this time.

The summary of CCh effects on APD$_{50}$ and contractions recorded simultaneously with the perforated patch technique is given in figure 4B. There was no significant change in the resting potential which averaged −72 ± 1.3 mV in control (n = 9), −72 ± 1.4 mV in CCh (n = 9; $P = .55$) and −71 ± 1.3 mV after washout (n = 6). The APD$_{50}$ in the presence of CCh was slightly but significantly smaller than in control (154 ± 12 ms, control; 132 ± 10 ms, CCh; $P = .016$). The APD$_{50}$ recovered partially 5 min after CCh was washed out (145 ± 11 ms, P = .097) in six of the nine original cells. The values for remaining cells were not taken into account because access was lost in 1 to 3 min after washout. Myocyte shortening increased significantly by 20% from an initial value of 6.7 ± 0.5% cell length to 8.1 ± 0.7% in 100 μM CCh ($P = .01$). On washout of CCh, cell shortening decreased to 4.6 ± 0.8% of cell length (six cells).

In another set of experiments, the effects of CCh on APD$_{50}$ and contractions were measured simultaneously in eight cells with the patch rupture technique. There was no significant change in the resting potential (−69 ± 0.8 mV, control; −69 ± 1.3 mV, CCh; $P = .41$) or the APD$_{50}$ (121 ± 12.6 ms, control; 114 ± 12.8 ms, CCh; $P = .11$). Myocyte shortening increased significantly by 37% from an initial value of 5.4 ± 1.34 μm to 7.4 ± 1.84 μm in 100 μM CCh ($P = .01$). On washout of CCh, cell shortening decreased to 4.4 ± 0.95 μm in seven of the eight original cells. The recording in the remaining cell was lost inadvertently because the myocyte was dislodged. The results obtained for the CCh effect on contractions with the patch rupture technique were much the same as those from the perforated patch technique.

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**Fig. 3.** Influence of the muscarinic receptor antagonists pirenzepine (1 μM, □) and AF-DX 116 (30 nM, ▲) on the stimulant effect of CCh. The control concentration-dependence relation (∗) is taken from the data for cumulative addition and the EC$_{50}$ is 18.1 μM. From the shift in the EC$_{50}$ for CCh at each antagonist concentration, apparent dissociation constants ($K_D$) taken from the Schild equation (see text) are 1.6 × 10$^{-7}$ M (pirenzepine) and 9.1 × 10$^{-8}$ M (AF-DX 116).
reflected the common tendency of shortening to run down in the course of the experiment.

When $I_{Ca}$ is evoked by voltage steps from the holding potential of $-40$ mV, the changes in $I_{Ca}$ and in shortening produced by CCh do not extend beyond the possible limits of spontaneous changes of these parameters under the conditions we used. On the other hand, CCh increased contractions evoked by action potentials (see above). A significant difference between conditions for experiments with action potentials and for those with $I_{Ca}$ described above was the membrane potential between the depolarizing stimuli (about $-70$ mV for AP and $-40$ mV for $I_{Ca}$). In another series of experiments, cells were held at a holding potential of $-80$ mV between stimuli. The stimulation procedure was repeated every 5 s and included a 1-s ramp depolarization to $-30$ mV that inactivated $I_{Na}$ and the T-type Ca$^{2+}$ current and a step depolarization to $+10$ mV (100 ms) to evoke $I_{Ca}$.

Seven cells were chosen for these experiments with a mean index $0.37 Hz/1 Hz$ of $0.55 \pm 0.06$. As shown in figure 5A, CCh (100 $\mu$M) reversibly increased contraction triggered by $I_{Ca}$ without any increase in the current itself. The stimulant-induced effect of CCh on contractions was well demonstrated in five of seven cells; $I_{Ca}$ was decreased or slightly increased. In the two remaining cells, cell shortening was not affected by CCh. When data were averaged for all seven cells, the mean shortening in the presence of CCh ($3.3 \pm 0.6%$ cell length) was significantly greater than in control ($2.5 \pm 0.68%$ cell length, $P = .045$) and at 5 min after CCh was washed out ($2.5 \pm 0.45%$ cell length, $P = .012$) (Fig. 5B). At the same time, the mean $I_{Ca}$ in CCh ($-2253 \pm 426$ pA) was smaller than the control value and the value after washout ($-2640 \pm 550$ pA and $-2353 \pm 529$ pA, respectively; $P = .035$ and 0.288, respectively). The real decrease in $I_{Ca}$ produced by CCh was probably somewhat smaller than the difference between values obtained for control and in the presence of CCh because of spontaneous rundown of $I_{Ca}$.

In a separate series of experiments (six cells, index $0.37 Hz/1 Hz = 0.58 \pm 0.03$) with the same protocol, 20 mM Cs$^+$ was added to Tyrode’s solution to block potassium currents. As in the experiments without Cs$^+$, the shortening in the presence of $100 \mu$M CCh ($5.8 \pm 1.3%$ cell length) was greater than in control ($5.0 \pm 1.11%$ cell length, $P = .038$) and greater than the shortening after CCh was washed out ($3.3 \pm 0.86%$ cell length, $P = .04$). $I_{Ca}$ in the presence of CCh was smaller than in control ($-1979 \pm 152$ pA and $-2232 \pm 147$ pA, respectively; $P = .011$). After washout of CCh, $I_{Ca}$ decreased further ($-1847 \pm 130$ pA, $P = .012$), which indicates that the main reason for the decline in $I_{Ca}$ values in this series of experiments was spontaneous rundown.

The effects of ryanodine on $I_{Ca}$ and contraction were studied with the voltage clamp protocol as in figure 5. As shown in figure 6, CCh evoked a phasic contraction in control (CTR). At 3 min after 10 $\mu$M ryanodine was added to the bath solution, $I_{Ca}$ was unchanged but myocyte contraction was eliminated. Addition of 100 $\mu$M CCh did not affect myocyte shortening in the presence of ryanodine; $I_{Ca}$ was unchanged. Washout of both ryanodine and CCh did not restore contractions (data not shown). In this series of experiments, ryanodine eliminated contractions in 2.7 $\pm 0.26$ min ($n = 12$). As in the experiments of figure 5, $I_{Ca}$ appeared to run down during the course of experiments and was unaffected by CCh. $I_{Ca}$ (pA, mean $\pm$ S.E.M.) was $-1606 \pm 217$ (control), $-1443 \pm$
Effect of CCh on intracellular Ca\(^{2+}\) transients and contractions.

The lack of I\(_{Ca}\) stimulation by CCh in otherwise untreated myocytes pointed toward altered SR Ca\(^{2+}\) handling and/or myofilament Ca sensitivity as mechanisms for the positive inotropic action. To evaluate the first possibility, myocytes were incubated in Fura-2 AM ester (see “Methods”). Myocyte contractions were evoked by extracellular stimuli in the same manner as described previously (see fig.1) and an increase of cell shortening recorded on increasing stimulus frequency from 0.2 to 1.0 Hz (data not shown).

The intracellular Ca\(^{2+}\) transient was recorded as the 340/380 ratio whereas the myocyte was stimulated continuously at 0.2 Hz. The results of nine experiments showed that raising [Ca\(^{2+}\)]\(_o\) from 1.8 to 5.4 mM increased the 340/380 ratio by 22\(\pm\)4% (P < .002) and cell contraction by 130\(\pm\)34% (P < .001). In 5.4 mM Tyrode’s solution with Ca\(^{2+}\) added, CCh produced increases of the 340/380 ratio and cell shortening as shown in fig. 7. The data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test.

Effect of CCh on intracellular Ca\(^{2+}\) transients and contractions. The intracellular Ca\(^{2+}\) transient was recorded as the 340/380 ratio whereas the myocyte was stimulated continuously at 0.2 Hz. The results of a typical experiment are shown in fig. 7. The amplitudes of the 340/380 ratio (intracellular Ca\(^{2+}\) transient) and cell contraction were 0.80 and 3\(\mu\)m in Tyrode’s solution with 1.8 mM [Ca\(^{2+}\)]\(_o\). Raising [Ca\(^{2+}\)]\(_o\) to 5.4 mM was accompanied by increases of the 340/380 ratio and cell shortening to 0.86 and 4\(\mu\)m, respectively. Addition of 100\(\mu\)M CCh in the presence of 5.4 mM [Ca\(^{2+}\)]\(_o\) increased the 340/380 ratio by 13% to 0.97 and cell contraction by 42% to 5.7\(\mu\)m, respectively. Washout of CCh restored both variables to values seen initially in 5.4 mM [Ca\(^{2+}\)]\(_o\) (fig. 7).

Increases of intracellular Ca\(^{2+}\) transient and cell contraction by 100 \(\mu\)M CCh. The upper row shows individual contractions evoked by extracellular stimuli (0.2 Hz) in Tyrode’s solution containing 1.8 mM Ca\(^{2+}\) (CTR), at 8 min after raising [Ca\(^{2+}\)]\(_o\) to 5.4 mM, at 3 min after adding 100 \(\mu\)M CCh on top of the 5.4 mM Ca-Tyrode’s solution and 6 min after washout (W.O.) of CCh when the cell was superfused again with 5.4 mM Ca-Tyrode’s solution. The time and displacement calibrations apply to all contraction records. The lower row shows Ca\(^{2+}\) transients recorded from the same myocyte with a 340/380 ratio of Fura-2 fluorescence as the index of intracellular Ca\(^{2+}\). Pairs of intracellular Ca\(^{2+}\) transients are shown in control (CTR), at 7 min in 5.4 mM Ca-Tyrode’s solution, at 2.5 min in 5.4 mM Ca-Tyrode’s with 100 \(\mu\)M CCh and at 5.5 min after washout (W.O.). The cell contractions and intracellular Ca transients were recorded alternately.
[Ca\textsuperscript{2+}]_o, CCh further increased the 340/380 ratio by 13 ± 2.5% (P = .003) and myocyte shortening by 33 ± 4.8% (P = .001). Carbachol increased the ratio, signaling an increased intracellular Ca\textsuperscript{2+} transient, and cell shortening in each of the nine myocytes.

**Discussion**

Carbachol increased the extent of myocyte shortening significantly when the experimental conditions replicated those in the original description of the phenomenon in multicellular ventricular preparations of reserpine-treated guinea pigs (Korth and Kühlkamp, 1985). The half-maximal CCh concentration (EC\textsubscript{50}) of 18 μM in our experiments is close to the value of 32 μM in guinea pig ventricle (Korth and Kühlkamp, 1987). One additional qualification is the occurrence of a positive staircase when increasing stimulus frequency. With this proviso, acutely dissociated single myocytes from guinea pig ventricle are a reliable system to evaluate the mechanism(s) for the stimulant effect of choline ester muscarinic agonists on ventricular myocyte contractions.

Pharmacological experiments (fig. 3) indicate that the mAChR initiating the contraction effect of CCh is indistinguishable with the presently available data from the M\textsubscript{2} subtype because AF-DX 116 (M\textsubscript{2}-selective) is a more potent antagonist than pirenzepine (M\textsubscript{1}-selective). The apparent K\textsubscript{d} for pirenzepine (1.6 × 10\textsuperscript{-7} M) is ~18 times greater than that of AF-DX 116 (9.1 × 10\textsuperscript{-9} M). The CCh-induced background Na\textsuperscript{+} current, thought to underlie muscarinic stimulation of ventricular contractions, is also characterized as M\textsubscript{2} because AF-DX 116 was ~6 times more potent than pirenzepine as an antagonist (Matsumoto and Pappano, 1991). In human ventricle, ACh evoked a positive inotropic effect attributed to M\textsubscript{2} mAChR (Du et al., 1995). Expression of M\textsubscript{2} mRNA in Xenopus oocytes yielded mAChR with greater affinity for AF-DX 116 than for pirenzepine (Fukuda et al., 1987). The possibility that stimulation of ventricular myocyte contractions by CCh might also involve M\textsubscript{1} mAChR must also be considered. The detection of M\textsubscript{1} mRNA in guinea pig (Gallo et al., 1993) and rat ventricular myocytes (Sharma et al., 1996) has been linked to stimulant effects of CCh (see below). Although we favor the view that the M\textsubscript{2} mAChR more likely initiates the stimulant effect of CCh, we cannot exclude the possibility of some M\textsubscript{1} mAChR participation. Experiments done with selective agonists would help solve this matter.

Carbachol did not change the resting potential when it increased cell shortening; the APD sometimes decreased significantly (see also Arreola et al., 1994; Yang et al., 1996). The latter effect was observed when the perforated patch technique was used to minimize diffusional loss of intracellular constituents. Muscarinic agonists activate K\textsubscript{AChR} channels in ventricular myocytes (McMorn et al., 1993); however, some membrane hyperpolarization >1 mV (the limit of detection) would be expected because the average membrane potential was 16 mV less negative than the K\textsuperscript{+} equilibrium potential (~88 mV). An increase of outward I\textsubscript{Na-K} at plateau potentials (Protas et al., 1997) could explain the reduced APD by CCh detected by us in single myocytes and by others in guinea pig papillary muscle (Arreola et al., 1994; Yang et al., 1996). Alternatively, CCh might induce a Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current (I\textsubscript{Cl(Ca)}) and reduce APD. When oocytes were transfected with mRNA for M\textsubscript{2}, ACh induced a monovalent current (Na\textsuperscript{+},K\textsuperscript{+}), whereas in cells transfected with M\textsubscript{1} mRNA, ACh induced I\textsubscript{Cl(Ca)} (Fukuda et al., 1987). We favor the participation of I\textsubscript{Na-K} because CCh acts through M\textsubscript{2} mAChR to increase cell contractions. If CCh also activated I\textsubscript{Cl(Ca)} when it augmented contractions, this agonist would be interacting simultaneously with both M\textsubscript{2} and M\textsubscript{1} mAChR.

The L-type Ca\textsuperscript{2+} current is an important trigger for the release of Ca\textsuperscript{2+} from the SR (McDonald et al., 1994). In CCh, I\textsubscript{Ca} was not significantly greater, yet myocyte shortening increased (fig. 5). This result confirms the lack of effect of CCh or ACh on the Ca\textsubscript{2+}-dependent action potential in K\textsuperscript{+}-depolarized guinea pig ventricle (Korth and Kühlkamp, 1985) and in rabbit ventricle (Inui et al., 1982) during the positive inotropic effect of these choline esters. It is unlikely that the signal transduction pathway underlying the increased cell shortening by CCh in guinea pig ventricle involved an increase of I\textsubscript{Ca}. The mechanism for the stimulant effect of CCh on guinea pig ventricular myocyte contractions must reside in other steps of E-C coupling.

Intracellular Ca\textsuperscript{2+} transients increased in the presence of CCh. This effect paralleled the stimulation of cell contractions by CCh and was reversible (fig. 7). This novel action for the choline ester muscarinic agonists agrees with their positive inotropic effect (Korth and Kühlkamp, 1985; Yang et al., 1996). Ryanodine (10 μM) suppressed the fast component of the isotonic contraction (fig. 6) and CCh did not restore this contraction phase in the presence of ryanodine. Our results are consistent with the hypothesis that the increased contractions by CCh depend on CR Ca release. In isolated guinea pig ventricular myocytes, ryanodine (up to 10 μM) selectively suppressed the fast component of isotonic contractions, an indication that the contraction depended on Ca release from the SR (Shepherd et al., 1990). In unstimulated rat ventricular myocytes, CCh increased intracellular Ca\textsuperscript{2+} by a mechanism involving Na-Ca exchange but independent of SR Ca release, because it was not opposed by 1 μM ryanodine which blocked caffeine-induced Ca release (Korth et al., 1988). In electrically paced myocytes, we find that the size of the intracellular Ca\textsuperscript{2+} transient increases with CCh at constant stimulation rate. Previous attempts to record intracellular Ca\textsuperscript{2+} transients provided hints that these could be augmented by ACh. In rabbit atrial muscle, vagal stimulation produced an atropine-sensitive rebound positive inotropic effect accompanied by an increase of the aerotonin light signal (Endoh and Blinkers, 1984). In rabbit SA node, intracellular Ca\textsuperscript{2+} transients detected with Indo-1 were modestly increased when ACh reduced spontaneous beat frequency (Lee et al., 1987).

Carbachol, in the presence of PTX, reportedly acts through M\textsubscript{2} mAChR to increase the amplitude of intracellular Ca\textsuperscript{2+} transients in paced rat ventricular myocytes (Sharma et al., 1996). This effect, attributed to calcium-induced calcium release in rat ventricular myocytes, was assumed to arise from a larger I\textsubscript{Ca} because CCh increased I\textsubscript{Ca} in guinea pig ventricular myocytes treated with PTX to suppress muscarinic inhibition of this current (Galbo et al., 1993). The stimulant effect of CCh on I\textsubscript{Ca} was prevented by pirenzepine (Galbo et al., 1993) and attributed to activation of M\textsubscript{1} receptors whose mRNA was detected in myocytes (Galbo et al., 1993; Sharma et al., 1996). Although our results in guinea pig ventricular myocytes also report an increased Ca\textsuperscript{2+} release transient, the effect is initiated at M\textsubscript{2} mAChR in the absence of PTX...
and occurs without an increased $I_{\text{Na}}$. On the other hand, the divergent results may be caused by the different conditions used. On the other hand, our results for stimulation of contractions replicate those found in multicellular preparations of guinea pig ventricle where the importance of intracellular Na$^+$ for regulation of contractions has been emphasized (Korth and Kuhlkamp, 1985; Matsumoto and Pappano, 1989).

The similarity of the frequency-shortening relation in single myocytes (fig. 2) to the frequency-force relation in multicellular ventricular preparations from guinea pigs (Gibbons and Zigmun, 1992) is an important qualification for the reliability of the isolated myocyte preparation. It may also provide a clue for the mechanism of CCh action. For example, increasing the frequency of voltage clamp pulses ($-80$ to 0 mV for 100 ms) from 0.5 to 3 Hz augmented the extent of shortening of guinea pig ventricular myocytes (Harrison and Boyett, 1995). The increased contractions during the positive staircase are initiated by elevation of intracellular Na$^+$ which, through the Na-Ca exchange mechanism, increases the Ca content of the SR. This allows increased contractions in the presence of an unchanged or reduced $I_{\text{Na}}$. Carbachol elevates intracellular Na$^+$ (Korth and Kuhlkamp, 1985; Yang et al., 1996) by increasing Na$^+$ entry via TTX-resistant channels (Matsumoto and Pappano, 1989, 1991). Therefore, if CCh acted like increased frequency to raise intracellular Na$^+$, the effects of CCh and stimulus frequency on intracellular Na$^+$ would be additive. A similar view applies to sheep Purkinje fibers, although the mechanism of muscarinic action underlying the increased intracellular Na$^+$ inhibition of the Na/K pump (Iacono and Vassalle, 1989).

At room temperature Na-Ca exchange contributes to SR Ca loading but does not trigger SR Ca release by action potentials (Sipido et al., 1995). That Na-Ca exchange might trigger SR Ca release was first proposed by Leblanc and Hume (1990) and was supported by the observation that contractions could be triggered by Na-Ca exchange at 35°C (Levi et al., 1994; Vornanen et al., 1994; Wasserstrom and Vites, 1996) but not at 22°C (Vornanen et al., 1994). Our experiments were not designed to evaluate the loading versus triggering functions of Na-Ca exchange. In preliminary studies, we find that CCh increased the magnitude of caffeine-induced $I_{\text{Na-Ca}}$, an indication that SR Ca$^{2+}$ stores are increased by CCh (Protas et al., 1997). Another bit of evidence for the Na-Ca exchanger as a link between increased intracellular Na$^+$ and increased contraction during the positive staircase is voltage dependence (see above). The positive staircase was absent when voltage jumps to 0 mV began from a holding potential of $-40$ mV because the cells did not gain intracellular Na$^+$ under this condition (Harrison and Boyett, 1995). We find that CCh has no effect on contractions when the membrane holding potential is kept continuously at $-40$ mV between voltage jumps to $+10$ mV.

An increase of myofilament sensitivity, if it occurred, would provide an additional mechanism for muscarinic agonist stimulation of cell contractions. Our experiments did not address this issue. The proportionately greater increase of contractions than of Ca$^{2+}$ transients by CCh might indicate that myofilament Ca$^{2+}$ sensitivity increased. However, complexities (compartmentation, incomplete de-esterification, nonlinear relation between fluorescence ratio and Ca$^{2+}$) arising from the use of Fura 2 ester preclude any conclusions regarding this possibility. Carbachol reportedly increased myofilament Ca$^{2+}$ sensitivity in guinea pig papillary muscle (Yang et al., 1996). The mechanism for this effect is not known but the increase of [Na$^+$]i is PKC-independent.

Divergent results have been reported for regulation of myofilament Ca$^{2+}$ sensitivity by cGMP and PKC, each of which has been identified as an intracellular messenger for muscarinic agents (reviewed in Pappano, 1995). Myofilament Ca$^{2+}$ sensitivity diminished in the presence of 8-bromo-cGMP whereas the intracellular Ca$^{2+}$ transient was unchanged (Shah et al., 1994). In acutely dissociated guinea pig ventricular myocytes, CCh (10 μM) increased cGMP by 100% and cell shortening by 18%. Methylene blue suppressed the cGMP effect but not the increased contractions caused by CCh (Stein et al., 1993). This pattern of results also militates against a role of cGMP in the positive inotropic action of CCh under the conditions of our experiments.

Activation of PKC by phorbol ester elicited an increased myofilament Ca$^{2+}$ sensitivity in permeabilized rat ventricular myocytes (reviewed in Terzic et al., 1993). However, phorbol esters have negative inotropic effects in many cardiac tissues and also reduce intracellular Ca$^{2+}$ transients (Terzic et al., 1993). In chick atrial muscle (Webb and Pappano, 1995) and in guinea pig papillary muscle (Arreola et al., 1994), phorbol ester had a small negative inotropic effect and suppressed the positive inotropic action of CCh. In rat ventricular myocytes, phorbol ester stimulated Na-H exchange by activation of PKC (Korth et al., 1988). However, CCh did not mimic this action of phorbol ester, which indicates that PKC activation was not a component of the reaction mechanism whereby CCh stimulated ventricular contractions. Experiments with staurosporine confirmed that CCh did not regulate Na-H exchange (Yang et al., 1996). Carbachol also increased InsP$_3$ in cardiac muscle (reviewed in McDonald et al., 1994; Pappano, 1991). Although this intracellular messenger can release Ca$^{2+}$ from the SR, it has no effect on myofilament Ca sensitivity (Nosek et al., 1986; Vites and Pappano, 1990).

Our results provide evidence for the suitability of a single myocyte model that replicates the features of cells in guinea pig papillary muscle. The increased contractions by CCh appears to be initiated at M2 mAChR, the same mAChR subtype that mediates muscarinic inhibition. The increase of Ca$^{2+}$ release transients and of contractions by CCh without a concomitant increase of $I_{\text{Ca}}$ indicates that the effect of CCh on contractions resides in other steps connected to E-C coupling, perhaps the Na-Ca exchanger and myofilament Ca$^{2+}$ sensitivity.

Acknowledgments

We thank Yu-e Zhang for expert technical assistance, Dr. Xu Chang for editorial advice and Rene Bumbera for secretarial help.

References


McDonald TF, Pelzer S, Trautwein W and Pelzer DJ (1994) Regulation and modulation of the cardiac sarcoplasmic reticulum during action potentials in guinea-pig ventricular myocytes. J Physiol (Lond) 489:1422–1433.


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