The choline esters acetylcholine (ACh) and carbamylcholine (Cch) can increase contraction force in the mammalian heart (reviewed in Levy and Pappano, 1994). Stimulation of contractions by ACh or Cch has been detected in atrial muscle (Webb and Pappano, 1985), Purkinje cells (Gilmour and Zipes, 1985), and ventricular muscle (Korth and Kühlkamp, 1985; Yang et al., 1996). This phenomenon does not require antecedent inhibition although examples of “rebound” contraction stimulation by ACh or Cch has been detected in atrial muscle (Webb and Pappano, 1985; Yang et al., 1996). This phenomenon does not require antecedent inhibition although examples of “rebound” contraction stimulation by ACh and Cch are reported in sinoatrial node (McMorn et al., 1993) and ventricle (Endoh et al., 1970). The choline esters act at muscarinic receptors (mAChR) to stimulate contractile force because atropine antagonizes their effect.

The mAChR subtype that initiates ventricular muscle contraction stimulation is disputed (Sharma et al., 1997). Experiments in guinea pig ventricular myocytes (Protas et al., 1998) and human heart ventricular muscle (Du et al., 1995) implicate the M2 mAChR because the effect of CCh or ACh is more susceptible to block by (11)([2-[diethylamino)methyl]-1-piperidinyl][acetyl]-5,11-dihydro-6H-Ypyrido [2,3- 6][1,4]benzodiazipine-6-one (AF-DX 116), an M2-selective agonist, than by pirenzepine, an M1-selective antagonist. The M1 mAChR reportedly is involved in rat ventricular myocytes because pirenzepine was more potent than methoctramine (M2-selective) as an antagonist of Cch-induced increases in intracellular Ca2+ transients (Sharma et al., 1996). Participation of M1 mAChR in stimulation of L-type Ca2+ [ICa(L)] and of the synthesis of inositol monophosphate also has been presented (Gallo et al., 1993). These results form the basis of a mechanism by which agonist occupancy of M1 mAChR increases [ICa(L)] and, thereby, intracellular Ca2+ transients (Sharma et al., 1996). Although mRNA and cell surface expression indicate the abundance of M2 mAChR in heart (Sharma et al., 1996), the M1 mAChR is also detected, particularly in ventricle (Gallo et al., 1993; Sharma et al., 1996).

In this study, we tested the hypotheses for muscarinic stimulation of ventricular myocytes with 3-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium (McN-A-343) on the favorable assumption that this drug is an M1-selective agonist (Goyal and Rattan, 1978; Hammer and Giachetti, 1982; Watson et al., 1983). McN-A-343 is a “functionally selective” M1 agonist (reviewed in Eglen et al., 1996), although agonist/antagonist effects at M2 mAChR have been reported (Christopoulos and Mitchelson, 1997). Does McN-A-343 increase [ICa(L)] in ventricular myocytes in the absence or presence of elevated intracellular cyclic AMP (cAMP)? Does...
McN-A-343 increase the extent of shortening in electrically stimulated myocytes? We compared the actions of McN-A-343 with those of the nonselective mAChR agonist Cch and with the M₂-selective agonist oxtremorine (Oxo). A preliminary account of these findings has been reported in abstract form (Shen et al., 1999).

Materials and Methods

Preparation of Single Myocytes. Ventricular cells were obtained from the hearts of anesthetized guinea pigs (250–450 g) by an enzymatic dissociation procedure as described previously (Protas et al., 1998). After dissociation, the cells were kept in recovery solution (150 mM K aspartate, 5 mM K₃ATP, 5 mM HEPES, and 20 mM dextrose, pH 7.4, adjusted with KOH) for at least 1 h before conducting experiments.

Electrophysiologic Experiments. Cells were superfused with Tyrode’s solution at room temperature (22–24°C) because at this temperature I_{Ca,L} was more stable and the low basal current allowed easier detection of the effects of stimulating drugs. The Tyrode’s solution composition was 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 0.33 mM NaH₂PO₄, 10 mM HEPES, and 10 mM dextrose. CsCl (10 mM) was added to the Tyrode’s solution to block potassium currents.

The whole-cell, patch-clamp technique was used for the experiments. Electrodes had resistances of 2 to 4 MΩ when filled with Cs⁺-rich pipette solution: 135 mM aspartic acid, 10 mM NaCl, 5 mM MgATP, 10 mM HEPES, 10 mM EGTA, pH 7.3, adjusted with CsOH, whose final concentration was 130 to 140 mM. Voltage-clamp protocols were generated by pClamp software (version 5.5; Axon Instruments, Foster City, CA). Cell capacitance was obtained immediately after patch rupture by integration of the current generated during a 20-ms hyperpolarization of 5 mV from a holding potential of ~−40 mV. A specific membrane capacitance of 1 nF/cm² was assumed. The voltage-clamp protocol consisted of a voltage jump from −80 mV (holding potential) to ~−40 mV for 350 ms to inactivate the fast Na⁺ and T-type Ca²⁺ currents followed by a second depolarizing jump to +10 mV for 300 ms to elicit the L-type Ca²⁺ current [I_{Ca,L}] and a repolarizing step back to −80 mV. The frequency of stimulation was 0.1 Hz. I_{Ca,L} amplitude was taken as the difference between the peak inward current at +10 mV and zero membrane current. Drug-containing solutions were applied to the bath (0.5-ml volume) by a gravity-fed system at a rate of 2 ml/min.

Contraction Experiments. Cell contraction experiments were carried out at 35°C; the cells were superfused with Tyrode’s solution without added CsCl. Our previous experience indicated that stimulation of contractions by carbachol is evident at 35°C but not at 22°C (Protas et al., 1998). Carbachol or McN-A-343 was applied by a solenoid-controlled rapid superfusion device (Saeki et al., 1997) via a thin, polyethylene tube positioned within 100 μm of the cell to be tested. Exposure time to either Cch or McN-A-343 was 2 to 3 min followed by washout (WO). At the beginning of each experiment, the ratio of contraction amplitudes at 0.2 and 1.0 Hz (Δ 0.2/Δ1.0) was obtained. We selected those cells with a ratio ≥0.8 to test the contraction effect of McN-A-343 or Cch because of our previous experience with guinea pig ventricular myocytes, which have a positive frequency-shortening relation (Protas et al., 1998).

Contractions of single myocytes were elicited by external stimuli applied through a broken-tip (~50-μm tip diameter) microelectrode and were detected by a video edge-detector device. The cell image was projected on a high-resolution video monitor through a sequential-scanning video camera attached to the microscope. The camera was rotated to keep the video detector raster lines parallel with the long axis of the cell. The video dimension analyzer monitored a selected raster line for differences in light intensity between the cell end and the surrounding field. The time constant and apparent spatial resolution of the video analysis system response at 400× magnification were 16.7 ms and 0.15 μm, respectively. The signals were sent to a chart recorder and to a videocassette recorder for storage and analysis.

Data Analysis. The amplitudes of I_{Ca,L} and cell contractions were expressed as means ± S.E. Student’s paired t test was used to evaluate the statistical significance of the difference between means of the results. P ≤ .05 is taken as statistically significant.

Results

Basal L-Type Calcium Current. Basal I_{Ca,L} was measured in experiments using a Cs⁺-rich pipette solution and 10 mM CsCl-containing bath solution to block potassium currents.

In eight cells tested with 0.1 mM McN-A-343, I_{Ca,L} was not changed after 5 to 8 min of exposure to the drug. The average I_{Ca,L} (pA) in these eight cells was 682 ± 145 (control), 635 ± 113 in McN-A-343, and 507 ± 88 after 8 to 10 min of WO. As reported previously (Protas et al., 1998), 0.1 mM Cch had no significant effect on basal I_{Ca,L} (n = 5). The results of these experiments indicate that I_{Ca,L} (pA) simply ran down from 734 ± 103 (control) to 671 ± 100 (Cch) and then to 585 ± 112 in WO. Oxo (0.1 mM) also was tested in two cells; no change of I_{Ca,L} was detected.

Isoproterenol (ISO)-Stimulated L-Type Calcium Current. The effects of McN-A-343 (M₁-selective), Cch (nonselective), and Oxo (M₂-selective) were examined on ISO-stimulated I_{Ca,L} to ascertain whether M₁- and M₂-selective agonists have similar inhibitory actions on the current that triggers contractions.

The records shown in Fig. 1 are illustrative of the results in 12 of 14 experiments. ISO (3 nM) increased I_{Ca,L} from 0.9 to 1.4 nA. In the continued presence of ISO, addition of 0.1 mM McN-A-343 did not change I_{Ca,L}, whereas the subsequent addition of 0.1 mM Cch decreased this current to ~1.0 nA (80% reduction). The Cch effect was completely reversed by 1 μM atropine, indicating that Cch acted through mAChR. Removal of ISO caused I_{Ca,L} to return toward the initial level. It is noted that the slight initial increase of I_{Ca,L} after the addition of Cch and the “rebound” increase of I_{Ca,L} when atropine was added occurred only in this experiment. Whether the effect of Cch arose from the inositol (1,4,5)-trisphosphate-dependent mechanism (Gallo et al., 1993) or from an effect of accumulated cGMP that inhibited cAMP hydrolysis (Shirayama and Pappano, 1996) is not known.

In 2 of 14 experiments in this series, McN-A-343 exerted an atypical action that is illustrated in the records of Figs. 2 and 3 taken from one of the cells. ISO (3 nM), as expected, increased I_{Ca,L} to 990 pA (Fig. 2A-b). Addition of McN-A-343 (Fig. 2A-c) reduced this current to 790 pA, and subsequent addition of Cch reduced I_{Ca,L} to 550 pA (Fig. 2A-d). In the presence of ISO, McN-A-343, and Cch, 1 μM atropine reversed the inhibition seen in the presence of McN-A-343 plus Cch (Fig. 2A-e); removal of atropine left I_{Ca,L} at the level to which ISO had elevated it (Fig. 2A-f). A later test of Cch alone (Fig. 2A-g) indicated that Cch inhibited I_{Ca,L} and that this effect was muscarinic in nature (Fig. 2A-h). Upon removal of atropine and Cch, I_{Ca,L} returned to the value seen in ISO alone (Fig. 2A-i). The experiment continues in the records of Fig. 3 with the cell superfused with 3 nM ISO (Fig. 3A-j). A second test with McN-A-343 (Fig. 3A-k) again resulted in diminished I_{Ca,L}, but this action was not muscarinic because atropine did not affect it (Fig. 3A-l), yet WO restored the...
L-type Ca\(^{2+}\) current to the sustained increase produced by ISO (Fig. 3A-m). Addition of Oxo (0.1 mM; Fig. 3A-n) reduced \(I_{\text{Ca}(L)}\) through an atropine-sensitive mechanism (Fig. 3A-o); atropine brought the current to the level seen in ISO alone (Fig. 3A-p). Addition of 1 mM propranolol reduced \(I_{\text{Ca}(L)}\) to the initial level (Fig. A-r) seen in the control portion of the experiment; this confirms the reliability of the recordings in the continued presence of ISO with the several additions of agonists and antagonist during the experimental period of slightly more than 2 h.

The results from the data collected in these 14 cells with ISO-stimulated \(I_{\text{Ca}(L)}\) (Fig. 3A-m). Addition of Oxo (0.1 mM; Fig. 3A-n) reduced \(I_{\text{Ca}(L)}\) through an atropine-sensitive mechanism (Fig. 3A-o); atropine brought the current to the level seen in ISO alone (Fig. 3A-p). Addition of 1 mM propranolol reduced \(I_{\text{Ca}(L)}\) to the initial level (Fig. A-r) seen in the control portion of the experiment; this confirms the reliability of the recordings in the continued presence of ISO with the several additions of agonists and antagonist during the experimental period of slightly more than 2 h.

The results from the data collected in these 14 cells with ISO-stimulated \(I_{\text{Ca}(L)}\) are shown in Fig. 4. ISO (3–10 nM) increased \(I_{\text{Ca}(L)}\) by 81% from the control value of 699 ± 58 pA (\(p < .001\)). McN-A-343 had no effect on \(I_{\text{Ca}(L)}\) that exceeded rundown of this current (\(p = .16\)). In a subset of these (\(n = 6\)), Cch significantly reduced ISO-stimulated \(I_{\text{Ca}(L)}\) (\(p < .05\)), an effect that was atropine-sensitive. These data indicate not only that the action of the nonselective agonist Cch was muscarinic but also that McN-A-343 did not interfere with Cch action.

The effect of Oxo, a relatively selective M\(_2\) mAChR agonist, was compared with that of Cch on ISO-stimulated \(I_{\text{Ca}(L)}\). In eight cells, 3 to 10 nM ISO increased \(I_{\text{Ca}(L)}\) from 623 ± 80 to 1178 ± 111 pA (\(p < .05\)), and 0.1 mM Cch reduced this current to 728 ± 105 pA (\(p < .05\)). When the changes from each cell were averaged, ISO increased \(I_{\text{Ca}(L)}\) by 102 ± 18.8% and Cch reduced the effect of ISO by 87 ± 6.7%. In four other cells (Fig. 5), 3 to 10 nM ISO increased \(I_{\text{Ca}(L)}\) from 726 ± 129 to 1381 ± 145 pA (\(p < .05\)), and it declined to 1093 ± 145 pA (\(p < .05\)) in 0.1 mM Oxo. From the individual data, ISO
increased $I_{\text{Ca(L)}}$ by 102 ± 29.9% and Oxo suppressed 49 ± 9.0% of the ISO effect. Atropine (1 μM) fully reversed this effect of Oxo. These results indicate that activation of M2 mAChR by Oxo plays an important role in the inhibition of ISO-stimulated $I_{\text{Ca(L)}}$ by mAChR. From our experiments at equimolar concentrations of muscarinic agonist, the inhibition of ISO-stimulated $I_{\text{Ca(L)}}$ by Oxo is less than that by Cch (49 versus 87%, respectively). This difference can be explained by two possibilities, namely, that Cch is a more potent activator of the M2 mAChR or that activation of other mAChR subtypes such as M1 by Cch somehow facilitates the inhibition. However, the evidence from experiments with McN-A-343 and Cch show that activation of the M1 mAChR alone could not inhibit ISO-stimulated $I_{\text{Ca(L)}}$, nor did it affect the response to Cch.

$I_{\text{Ca(L)}}$, Stimulated by Intrapipette cAMP. Cch increased $I_{\text{Ca(L)}}$ in guinea pig ventricular myocytes dialyzed with cAMP and a low concentration of the M1-selective antagonist; pirenzepine opposed this effect (Gallo et al., 1993). This result supported the conclusion that the stimulant effect of Cch was initiated at M1 mAChR. We examined this hypothesis by testing the effects of McN-A-343 or Cch on cAMP-stimulated $I_{\text{Ca(L)}}$. The patch electrode contained 300 μM cAMP in the pipette solution. Cells were subjected to this condition from the time of patch rupture to stimulate $I_{\text{Ca(L)}}$. Stimulation of $I_{\text{Ca(L)}}$ by 300 mM cAMP was maximal inasmuch as we confirmed in three cells that superfusion of 10 nM ISO could not further increase $I_{\text{Ca(L)}}$. In a total of 12 cells dialyzed with a pipette solution containing 300 μM cAMP, the $I_{\text{Ca(L)}}$ density was 20.6 ± 1.62 mA/cm², which is about three times larger than that observed in 30 cells without cAMP in the pipette solution (6.7 ± 3.01 μA/cm²).

The effect of McN-A-343 on cAMP-stimulated $I_{\text{Ca(L)}}$ was tested in 8 of 12 cells in this series (Fig. 6). Representative results with McN-A-343 or Cch are shown in Fig. 6A. Neither drug changed $I_{\text{Ca(L)}}$, which simply ran down during the time
of the experiment. The average $I_{Ca(L)}$ in these cells was $2650 \pm 353$ pA at control (5 min after membrane rupture), $2188 \pm 368$ pA at 6 min with 0.1 mM McN-A-343, and $2022 \pm 373$ pA after 8-min WO (Fig. 6B). In the remaining four cells tested with Cch, the average $I_{Ca(L)}$ was $2668 \pm 246$ pA at control, $2319 \pm 204$ pA at 6 min with Cch, and $1843 \pm 305$ pA after 8-min WO (Fig. 6B). These results indicate that neither McN-A-343 nor Cch had any additional effect on $I_{Ca(L)}$ when it is maximally activated by a high concentration of intrapipette cAMP.

Fig. 5. The effect of the M$_2$-selective agonist Oxo on ISO-stimulated $I_{Ca(L)}$. In four cells, 3 to 10 nM ISO increased $I_{Ca(L)}$ from $726 \pm 129$ to $1381 \pm 145$ pA (90% increase) after 4 to 6 min. At 6 min after addition, 0.1 mM Oxo reduced $I_{Ca(L)}$ to $1093 \pm 145$ pA (44% decrease); this effect was fully reversed by treatment with 1 $\mu$M atropine for 4 to 6 min. WO of atropine and Oxo for 8 to 10 min showed no significant change in the stimulant effect of ISO. Asterisks indicate a significantly different current ($p < .05$) from the immediately preceding $I_{Ca(L)}$. Number of cells tested are shown in parentheses.

Fig. 6. Lack of effect of McN-A-343 or Cch on cAMP-stimulated $I_{Ca(L)}$. The pipette solution contained 300 $\mu$M cAMP that stimulates $I_{Ca(L)}$ maximally because addition of 10 nM ISO ($n = 3$ cells) did not further increase $I_{Ca(L)}$. A, neither McN-A-343 (upper left) nor Cch (upper right) had any effect that varied from the spontaneous rundown of $I_{Ca(L)}$. The control (CTR) records were taken at 5 min after patch rupture. The traces in either McN-A-343 (McN) or Cch (Cch) are at 6 min in drug-containing solution, and WO records are at 6 min after drug removal. B, summary of experiments with McN-A-343 ($n = 8$) and Cch ($n = 4$). These results indicate that McN-A-343 and Cch have no effect on $I_{Ca(L)}$ when this current is saturated by a high concentration of intrapipette cAMP.

Cell Contractions. Contractions of single myocytes were elicited by external stimuli at 35°C; the Tyrode’s solution bathing the cells contained 1.8 mM Ca$^{2+}$. At the beginning of each experiment, the ratio of contraction amplitude at 0.2 Hz and 1.0 Hz ($\Delta 0.2/\Delta 1.0$ Hz) was calculated. Our previous experience with guinea pig ventricular myocytes showed that they had a positive frequency-shortening relation as expected and that a stimulant effect of Cch could be detected if the ratio ($\Delta 0.2/\Delta 1.0$ Hz) was $\leq 0.8$ (Protas et al., 1998). For these reasons, we chose those cells with a ratio $\leq 0.8$ to test the effects of McN-A-343 or Cch on contractions. McN-A-343 did not change the cell contractions in most of the cells tested, either at 0.2 Hz (16 unchanged, 5 decreased, and 1 increased) or at 1.0 Hz (15 unchanged and 1 decreased). However, we confirmed that Cch increased cell contractions in 10 of 15 cells at 0.2 Hz (5 showed no change) and in 8 of 10 cells at 1.0 Hz (1 unchanged and 1 decreased). In some cells, both McN-A-343 and Cch were tested in random sequence. An example of the typical results obtained is given in Fig. 7; cell shortening was 5.7 $\mu$m before drug addition (Fig. 7A-a). Addition of 0.1 mM McN-A-343 did not change the extent of contraction (Fig. 7A-b), but Cch increased cell contraction from 5.7 to 7.9 $\mu$m reversibly (Fig. 7A-d). As shown in Fig. 7B, McN-A-343 decreased cell contractions by only $0.17 \pm 0.11$ $\mu$m at 0.2 Hz ($n = 22$) and by $0.06 \pm 0.16$ $\mu$m ($n = 16$) at 1.0 Hz. McN-A-343 had no significant effect on cell contractions when the rundown of contractions in the WO period was taken into account. In contrast, Cch increased cell contrac-
triggers contractions. In a few instances (see Figs. 2 and 3), A-343 does not regulate ICa(L) in guinea pig ventricular myocytes. However, atropine did not antagonize this effect; there- fore, it is not muscarinic in nature. We conclude that McN-A-343 is an agonist at such receptors. Our experiments report two principal findings: namely, that McN-A-343 had no effect either on ICa(L) in the absence or presence of cAMP (ISO, intrapipette cAMP) or on contractions in guinea pig ventricular myocytes. When Cch or Oxo inhibited ICa(L), McN-A-343 had no effect on this current that triggers contractions. In a few instances (see Figs. 2 and 3), ISO-stimulated ICa(L) diminished in the presence of McN-A-343. However, atropine did not antagonize this effect; therefore, it is not muscarinic in nature. We conclude that McN-A-343 does not regulate ICa(L) in guinea pig ventricular myocytes. Does McN-A-343 affect contractions by acting at a site distal to the L-type Ca2+ channel? Attempts to detect such an effect yielded negative results. Our evidence is inconsistent with the hypotheses that agonist occupancy of M1 mAChR accounts for M1 mAChR and the Heart. The M2 subtype is the principal mAChR in heart muscle cells. In rat heart, the M1 subtype accounts for ~3% of total mAChR (Watson et al., 1983). Recent experiments document the presence of mRNA for M1 mAChR in guinea pig and rat heart (Galloy et al., 1993; Sharma et al., 1996). M1 mAChR expression on the surface of guinea pig and rat ventricular myocytes has contributed to the hypothesis that the M1 mAChR initiates the novel stimulant effect of muscarinic agonist in mammalian ventricle (Galloy et al., 1993; Sharma et al., 1996). The presence of two cell surface mAChR subtypes initiating opposite effects is not unprecedented. Muscarine causes depolarization (M1) after hyperpolarization (M2) in rat parasympathetic ganglion cells (Allen and Burnstock, 1990).

In the human heart, pirenzepine-sensitive M2 mAChR are autoreceptors on postganglionic parasympathetic nerves (Pitschner and Wellstein, 1988; Brodde et al., 1998). In cat atrial myocytes, the M2 mAChR participated in the ACh-induced potentiation of a glibenclamide-sensitive K+ current (Wang and Lipsius, 1995). In the same preparation, M2 mAChR activation by ACh caused an inward Na+ current, as we reported in guinea pig ventricular myocytes (Matsumoto and Pappano, 1991). Thus, M1 and M2 mAChR can function on different tissues within the heart or on the same cell. Other variations include having M1 mAChR on postganglionic cholinergic nerves and on cardiac myocytes in the chicken heart (Jeck et al., 1998) and M2 mAChR on postganglionic cholinergic nerves and on cardiac muscle cells in rat and guinea pig hearts (Jeck et al., 1988; Bognar et al., 1990).

**Fig. 7.** Cch but not McN-A-343 increases isotonic shortening in ventricular myocytes. A, individual contraction traces (a–e) taken from the continuous record below it of a myocyte stimulated at 1 Hz and superfused with Tyrode’s solution (35°C). At 0.1 mM, McN-A-343 did not change the extent of cell contractions (b), but Cch (d) increased cell contraction reversibly. B, on average, when compared with control, McN-A-343 had no significant effect on cell contractions when the rundown of contractions is considered. However, Cch increased cell contractions significantly at 0.2 Hz (n=15; p<.004) and at 1.0 Hz (n=10; p<.03).
where ACh acts as a negative inotropic agent. McN-A-343 did not induce a Na\(^+\) current in ventricular myocytes but prevented Cch from doing so at M\(_2\) mACHR (Matsumoto and Pappano, 1991). Similarly, McN-A-343 actions on rabbit atrium were attributed to M\(_2\) mACHR antagonism (Lambrecht et al., 1993; Christopoulos and Mitchelson, 1997).

**Limitations.** Our results (Matsumoto and Pappano, 1991; Protas et al., 1998; present report) indicate negligible participation of M\(_1\) mACHR in the stimulant effect of muscarinic agonist in guinea pig ventricular myocytes. How can one explain the discrepancies inasmuch as pirenzepine prevented stimulation of I\(_{Ca,L}\) in guinea pig ventricular myocytes (Gallo et al., 1993) and was more potent than methoctramine (M\(_2\)-selective) as an antagonist of increased intracellular Ca\(^{2+}\) transients by Cch in rat ventricular myocytes (Sharma et al., 1996)?

We did not detect an increase of I\(_{Ca,L}\) by Cch, Oxo, or McN-A-343 in the presence of saturating concentrations of intrapipette cAMP. Others have obtained the same result (reviewed in Méry et al., 1997), contrary to the reported increase of the L-type Ca\(^{2+}\) current by Cch via M\(_1\) receptor activation under this condition (Gallo et al., 1993). Although increases of I\(_{Ca,L}\) are easier to detect at 22–24°C than at 35°C, the temperature used by Gallo et al. (1993), the activity of M\(_1\) mACHR may be highly temperature dependent. Also, we did not treat cells with pertussis toxin. We found that pertussis toxin treatment is not essential to detect the Cch-induced Na\(^+\) current (Matsumoto and Pappano, 1991), the stimulation of Na/Ca-exchange current (Saeki et al., 1997), and the increase of intracellular Ca\(^{2+}\) transients and cell contractions (Saeki et al., 1997; Protas et al., 1998). Conceivably, we could miss the effects reported by others because pertussis toxin treatment either allows (Gallo et al., 1993) or increases the likelihood (Sharma et al., 1996) of M\(_1\)-mediated stimulation. With respect to the possibility that the products of phosphoinositide metabolism could be involved in muscarinic stimulation of I\(_{Ca,L}\), only a partial answer can be given.

We find that inositol (1,4,5)-triphosphate (1–300 μM) had no effect on I\(_{Ca,L}\) in guinea pig ventricular myocytes (see also Shuba et al., 1990; Saeki et al., 1999). Activation of protein kinase C (PKC) by diacylglycerol, the other product of phospholipase C stimulation, might have promoted L-type Ca channel phosphorylation in the experiments of Gallo et al. (1993). Although such an effect has been reported for L-type Ca channels expressed in Xenopus oocytes (Bourinet et al., 1992), cardiac PKC requires Ca\(^{2+}\), which was buffered by EGTA in the pipette solution. If so, the free myoplasmic Ca\(^{2+}\) needed to activate PKC would have to be less than that required to permit contraction in our experiments.

Could species differences explain the discrepancy? The M\(_1\)-mediated increase of intracellular Ca\(^{2+}\) transients by Cch occurred in rat ventricular myocytes (Sharma et al., 1996) that differ from guinea pig ventricular myocytes in electrophysiology and excitation-contraction coupling (reviewed in Bers, 1991). Such differences also may extend to neurotransmitter regulation of I\(_{Ca,L}\). However, ACh did not increase I\(_{Ca,L}\) in rat ventricular myocytes (McMorn et al., 1993). When ACh was washed out of the bath solution, a rebound stimulation of contractions and an increase in the amplitude of the late depolarization phase of the rat ventricular action potential occurred (McMorn et al., 1993). This action potential phase is generated by the Na/Ca-exchange current in rat ventricular myocytes rather than I\(_{Ca,L}\) (reviewed in Noble, 1995).

In general, muscarinic agonists are ineffective against basal I\(_{Ca,L}\) in ventricle (reviewed in Pappano, 1995); however, there are reports to the contrary. In frog and rat ventricular myocytes, atropine increased I\(_{Ca,L}\) in the presence and, occasionally, in the absence of ISO (Hanh et al., 1993). If mACHR are constitutively active in guinea pig ventricular myocytes, we could not detect an effect of muscarinic agonists at 22–24°C.

The findings with the M\(_1\)-selective agonist McN-A-343 together with our previous observations with selective antagonists are consistent with the hypothesis that the stimulant effect of muscarinic agonists in guinea pig ventricular myocytes is initiated at M\(_1\) mACHR rather than at M\(_2\) mACHR. The lack of effect of McN-A-343 does not exclude the presence of M\(_1\) mACHR because low-receptor reserve or inefficient receptor-effector coupling could account for negligible effect even though M\(_1\) mACHR are present (Eglen et al., 1996).

**References**


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