Signaling Mechanisms Underlying Muscarinic Receptor-mediated Increase in Contraction Rate in Cultured Heart Cells*

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Henry M. Colecraft‡, Joanne P. Egamino, Virendra K. Sharma, and Shey-Shing Sheu§

From the Department of Pharmacology and Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14622

We have investigated the mechanisms by which stimulation of cardiac muscarinic receptors result in paradoxical stimulatory effects on cardiac function, using cultured neonatal rat ventricular myocytes as a model system. Application of low concentrations of carbachol (CCh) (EC50 = 35 nM) produced an atropine-sensitive decrease in spontaneous contraction rate, while, in cells pretreated with pertussis toxin, higher concentrations of CCh (EC50 = 26 μM) elicited an atropine-sensitive increase in contraction rate. Oxtremorine, an m2 muscarinic acetylcholine receptor (mAChR) agonist, mimicked the negative but not the positive chronotropic response to CCh. Reverse transcription followed by polymerase chain reaction carried out on mRNA obtained from single cells indicated that ventricular myocytes express mRNA for the m1, m2, and, possibly, m4 mAChRs. The presence of m1 and m2 mAChR protein on the surface membranes of the cultured ventricular myocytes was confirmed by immunofluorescence. The CCh-induced positive chronotropic response was significantly inhibited by fluorescein-tagged antisense oligonucleotides directed against the m2, but not the m3 and m4, mAChR subtypes. The response was also inhibited by antisense oligonucleotides against Gαq protein. Finally, inhibition of CCh-induced phosphoinositide hydrolysis with 500 μM neomycin or 5 μM U73122 completely abolished the CCh-induced positive chronotropic response. These results are consistent with the stimulatory effects of mAChR activation on the rate of contractions in cultured ventricular myocytes being mediated through the m1 mAChR coupled through Gq to phospholipase C-induced phosphoinositide hydrolysis.

Stimulation of postsynaptic muscarinic acetylcholine receptors (mAChRs)† on cardiac cells is able to produce dual effects on the heartbeat. Classically, stimulation of the vagus nerve or the application of low concentrations of muscarinic cholinergic agonists produces characteristic negative chronotropic and inotropic effects on cardiac tissue and cells (1). Paradoxically, under appropriate conditions, activation of cardiac mAChRs elicits stimulatory effects on the rate of beating and contractile force of the heart. These latter effects often require higher concentrations of agonist and in some cardiac cell types are only seen after pretreatment with pertussis toxin (PTx) (2–4).

It has been widely believed, based on both radioligand binding techniques as well as Northern blot analyses, that cardiac cells express only the m2 mAChR subtype on their surface membranes (5–8). However, more recent findings suggest limits in the sensitivity of these techniques in detecting low levels of protein and mRNA (6). For example, it has been demonstrated that mRNA for mAChR subtypes that were missed by Northern blot studies were detected by use of reverse transcription followed by polymerase chain reaction (RT-PCR) in neuroblastoma cells as well as in guinea pig ventricular myocytes (7, 9). Previous work in this laboratory, employing the techniques of single-cell RT-PCR on cardiac myocytes isolated from adult rat ventricle has demonstrated the existence of m1 mAChR mRNA in addition to m2 mRNA (10, 11). Furthermore, we have demonstrated that the activation of m3 mAChRs leads to an increase in muscle shortening and Ca2+ transients. However, the signal transduction pathway mediating this stimulatory response is still unknown. Moreover, there is no report on the role of m1 mAChRs in the regulation of heart rate.

In the present studies, we have taken advantage of the ability of cultured neonatal rat ventricular myocytes to contract spontaneously and have utilized them as a cardiac cell model system to investigate the role of m1 mAChRs in the regulation of rate of contraction. By applying a number of complementary approaches, including single-cell intracellular Ca2+ concentration ([(Ca2+]i) measurements, single-cell RT-PCR, immunocytochemistry and antisense technology, four specific questions were addressed. 1) Do cultured neonatal rat ventricular myocytes exhibit dual responses to mAChR stimulation in a manner similar to that observed in other cardiac cell preparations? 2) How many subtypes of mAChRs are expressed in cultured neonatal rat ventricular myocytes? 3) Which mAChR subtype mediates the stimulatory effects of muscarinic agonists on the rate of contraction? 4) What second messenger system is involved in generating this response?

A preliminary report of this work has appeared in abstract form (12).

EXPERIMENTAL PROCEDURES

Culture of Ventricular Myocytes—Cultured neonatal rat ventricular myocytes were prepared using modifications of a previously published procedure (13). Briefly, 1–3-day-old Sprague-Dawley rats were anesthetized with ether and decapitated, and their hearts isoelectrically excised. The bottom half of the hearts were cut, rinsed in magnesium- and calcium-free phosphate-buffered saline (PBS), and finely minced with a pair of scissors. Single cells were obtained by brief alternating periods of enzymatic digestion with 0.125% trypsin and mechanical disaggregation at room temperature. The cells dispersed in the supernatant from the first two digestions were discarded. The supernatant from the following digestions were collected.

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‡ Advanced predoctoral fellow of the Pharmaceutical Research and Manufacturers of America Foundation during the tenure of this work.
§ To whom all correspondence and reprint requests should be addressed. Tel.: 716-275-3381; Fax: 716-244-9283; E-mail: sheus@pharmacol.rochester.edu.
† The abbreviations used are: mAChR, muscarinic acetylcholine receptor; PTx, pertussis toxin; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PSS, physiological saline solution; IP, inositol phosphate; PLC, phospholipase C; PI, phosphoinositide; GPBS, PBS containing normal goat serum.

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Immunofluorescence Detection of mAChR Subtypes—Subtypes of mAChR protein expressed on the surface membranes of cultured neonatal rat ventricular myocytes were probed by immunofluorescence as described previously (10). Briefly, cultured ventricular myocytes were fixed with 3% paraformaldehyde for 10 min and subsequently permeabilized with 0.5% Triton X-100. mChRs were blocked by preincubating the fixed cells for 30 min with PBS containing 10% normal goat serum (GPBS). Cells were then exposed to GPBS, containing either no primary antibody or affinity-purified rabbit polyclonal antibodies specific for the m1, m2, and m4 mAChRs (diluted 1:200; 0.5 µg/ml final concentration) for 48 h at 4 °C. These antibodies have been characterized by immunoprecipitation and immunocytochemistry of high yield. Briefly, 20–30 ml of cloned mChRs as well as receptors expressed in their native tissues (20). At the end of the incubation period, excess primary antibody was removed with washing with GPBS and the cells were exposed to a fluorescein-labeled goat anti-rabbit secondary antibody, used at 1:500 dilution, for 45 min at room temperature. Following the removal of excess secondary antibody by washing with PBS, the cells were mounted on glass slides in Mowiol 4-88 (Calbiochem). Images were acquired through the use of a confocal microscope.

Functional Inhibition of Gene Expression with Antisense Oligonucleotides: Design and Fluorescein Labeling of Oligonucleotides—Antisense oligonucleotides targeted to mRNA for the m1, m2, and m4 mAChRs as well as to Gq and Gα protein were designed based on their published sequences. Phosphorothioated, 20-mer oligonucleotides synthesized using phosphoramidite chemistry and purified by gel filtration were obtained from the Midland Certified Reagent Co. (Midland, TX). The antisense oligonucleotides were further modified by the addition of a 3’-amino linker arm to allow their labeling with fluorescein isothiocyanate (Molecular Probes). The oligonucleotides were each designed to contain comparable amounts of pyrimidines and purines. The uniqueness of the sequences targeted by each of the oligonucleotides used in this study was determined by comparing the targeted sequence against sequences found in GenBank and other databases using BLAST (21). The antisense oligonucleotide sequences used and the positions in the published mRNA sequences (17, 18, 22, 23) to which they are targeted are as follows.

The modified oligonucleotides were labeled with fluorescein using established methods (24). Briefly, 0.1 µmol of oligonucleotide was incubated with 0.6 µmol of fluorescein isothiocyanate for 18 h in 1 ml of sodium carbonate buffer (pH 9). The reactions were terminated using 100 mM ammonium chloride, pH 7.3. The labeled fluorescein-tagged oligonucleotide was separated from free fluorescein isothiocyanate by spin column chromatography using Sephadex G-25 equilibrated with deionized water. The labeled oligonucleotides were sterilized by filtration through a 0.2-µm membrane filter and quantitated by measuring the absorbance at 260 nm (A260). One A260 unit was taken as being equivalent to 30 µg of oligonucleotide.

m1: 5’-TGGATTGTTGACGTGCAGCAAGAGCC-3’ (sense, 801–818)
m2: 5’-GGATTGTTGTGCAGCTTATGCATC-3’ (antisense, 1380–1361)
m4: 5’-CCATTGCAGGCGGCTGAAATGTTGTT-3’ (22–21)

The treatment of cells with Oligonucleotides—Antisense oligonucleotides were dispensed from a stock concentration of 100 µM stored at –20 °C. For transfection of cells, 200–500 nM of fluorescein-tagged oligonucleotides and 8 µg/ml Lipofectin (Life Technologies, Inc.) were then each diluted in 100 µl of serum-free Dulbecco’s modified Eagle’s medium warmed to 37 °C. The two solutions were then combined and incubated at room temperature for 20 min. For each transfection, 0.5 ml of serum-free medium was added to each tube containing the Lipofectin-oligonucleotide complexes and the resulting mixture overlaid onto the cultured cells. Unless otherwise stated, the cells were incubated with the Lipofectin-DNA complexes for 18 h at 37 °C in a CO2 incubator, after which time this solution was removed and replaced with serum-containing normal growth medium.

Nuclear Localization of Oligonucleotides—To identify cells with nuclear translocation of fluorescein-tagged oligonucleotides, they were viewed by fluorescence microscopy. Cells were placed on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence. Selected cells were excited with 490 nm wavelength light through a 40× oil immersion objective. The emitted signal at 510 nm was focused onto the photomultiplier tube. The signals in response to the two excitation wavelengths were digitized and stored in a microcomputer. All experiments were conducted at 37°C with continuous perfusion of cells with HEPES buffer containing no drugs (control recordings) or the indicated concentrations of drugs in a final constant bath volume of 1 ml.

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the fluorescent microscopic pictures to determine the intracellular localization of oligonucleotides. Immunofluorescence Detection of mAChR Subtypes in Antisense-treated Cells—Cultured neonatal rat ventricular myocytes, treated with antisense oligonucleotides directed against m1, m2, mAChR mRNA, were probed by immunocytochemistry using m1 and m2 mAChRs subtype-specific antibodies and the ABC Elite kit from Vector Laboratories. Briefly, cultured ventricular myocytes were treated with 200 nM antisense oligonucleotides and 8 µg/ml Lipofectamin in serum-free culture medium, and maintained at 37 °C in a 5% CO2 incubator for 18 h. At this time, the existing medium was removed and replaced with normal serum-supplemented culture medium. Twenty-four to 48 h later, cells were fixed with a 50% acetone:50% methanol solution for 5 min and subsequently permeabilized and blocked with PBS containing 5% goat serum, 0.3% Triton X-100, and 3% bovine serum albumin. Cells were then exposed to affinity-purified rabbit polyclonal antibodies specific for the m1 and m2 mAChR (R&D Ab, used at 1:1000 dilution) in GPBS for 24 h at 4 °C. Excess primary antibody was removed by repeated washing with GPBS and the cells were exposed to a biotinylated goat anti-rabbit secondary antibody, used at 1:100, for 1 h at room temperature. Following the removal of excess secondary antibody, the cells were incubated with avidin DH and biotinylated horseradish peroxidase H, premixed according to manufacturer’s instructions. After washing, cells were stained by incubation for 5 min with a peroxidase substrate solution containing an equal volume of 0.02% hydrogen peroxide and 0.1% diaminobenzidine tetrahydrochloride made in 0.1 M Tris buffer, pH 7.2. Following washing and air drying, the cells were placed on coverslips with Mowiol (Calbiochem) and viewed first using fluorescence microscopy to identify nuclear fluorescein staining, and then under the light microscope to examine peroxidase staining.

Measurements of Phosphoinositide Hydrolysis—Insitol phosphate (IP1) accumulation was assessed as a measure of phospholipase C (PLC)-mediated phosphoinositide (PI) hydrolysis using modifications of previously described procedures (25). Briefly, ventricular myocytes were cultured in 12-well tissue culture dishes for 5 days, at which time the cells were incubated with normal growth medium containing 2 µCi/ml [3H]myoinositol (Amersham Pharmacia Biotech) for another 36 h. The cells were rinsed twice with a physiological saline solution (PSS) which contained (mM): NaCl 118, KCl 4.7, CaCl2 3, MgSO4 1.2, KH2PO4 1.2, glucose 10, EDTA 0.5, and HEPES 20 (pH 7.4). The cells were then incubated with 10 nM LiCl in PSS for 30 min at 37 °C, followed by incubation with the indicated concentrations of drugs for another 30 min at 37 °C. The reactions were terminated by removing the PSS bathing the cells, followed by the addition of 0.6 ml of ice-cold methanol to each well. The cells were scraped from the wells and transferred to centrifuge tubes, to each of which was added 0.3 ml of chloroform and 0.25 ml of water. The tubes were vortexed vigorously to break up the cells, after which 0.3 ml each of chloroform and water was added. The tubes were again vortexed and centrifuged at 1500 × g for 10 min, after which 0.7 ml of the upper phase was withdrawn and added to 2.3 ml of water in a conical tube. Approximately 175 mg of Dowex AG1 × 8 anion exchange resin (Bio-Rad) was added to each tube and vortexed. The gel was allowed to settle and the supernatant poured off. The resin was then washed four times with 3 ml of water. IP1 was extracted by the addition of 0.5 ml of 200 mM ammonium formate/100 mM formic acid to each tube. After vortexing and allowing to settle, 0.4 ml of the supernatant was removed and placed into a 20-ml scintillation vial. This procedure was repeated, and 0.5 ml of supernatant was removed and combined with the other extract. Ten ml of scintillation fluid was added to each combined extract and counted. To account for differences in cell number between different wells, the counts obtained for each extract were divided by the total radioactivity present in the respective lipid fraction.

RESULTS

Dual Effects of mAChR Activation on the Rate of Spontaneous Contractions—In cardiac cells, each contraction is dependant on a transient elevation of [Ca2+]. Thus, we recorded the frequency of fura-2 reported Ca2+ transients in spontaneously beating cultured neonatal rat ventricular myocytes as a measure of the contraction rate. Fig. 1A shows the effect of 500 nM CCh in the perfusion solution on the frequency of spontaneous Ca2+ transients. At this concentration, CCh immediately and completely abolished the spontaneous contractions as evidenced by the lack of Ca2+-transients. Washout of CCh resulted in the reappearance of Ca2+ transients, demonstrating the reversible nature of this inhibitory response. In most cases, the reversal in rate was accompanied by a decrease in the diastolic Ca2+ concentration. This negative chronotropic response to CCh was observed at all concentrations (>10 nM) tested; however, at higher CCh concentrations (>1 µM) there was often an attenuation of the response in the continued presence of agonist. In experiments in which the CCh-induced decrease in rate was measured, only the response in the first 2 min after the addition of drug was taken into account for analyses. During this time no appreciable desensitization or rebound of the response occurred.

In a number of cardiac cell types, the stimulatory effects of mAChR stimulation are only seen after pretreatment with PTXs. To determine whether CCh could also have stimulatory effects on the rate of spontaneous contractions in cultured neonatal rat ventricular myocytes, cells were treated with 100 ng/ml PTXs for 18–24 h to inactivate the Gi/Go protein and thus abolish the CCh-induced negative chronotropic response. Under these conditions, exposure of cells to higher concentrations of CCh resulted in a concentration-dependent, reversible in-
increase in the rate of spontaneous contractions (Fig. 1B). The increase in rate was often accompanied by an increase in the intracellular Ca\(^{2+}\) concentration. The concentration-response curves for the CCh-induced negative and positive chronotropic responses are shown in Fig. 1C. A comparison of the curves for the two responses demonstrates that CCh is more efficacious in eliciting the inhibitory response (\(EC_{50} = 35 \text{nM}\)) as compared with the stimulatory response (\(EC_{50} = 26 \text{\mu M}\)). The maximal inhibitory response on the rate of spontaneous contractions was a complete abolition of the heartbeat (100% inhibition) and was achieved with concentrations of CCh \(\geq 500 \text{nM}\). For the positive chronotropic response, the threshold concentration for the observation of a response was 5 \(\mu \text{M}\) CCh and the maximum response was obtained with 300 \(\mu \text{M}\) CCh. At this concentration, CCh increased the rate of spontaneous contractions from a control value of 59 ± 6 beats/min (\(n = 12\)) to a value of 119 ± 8 beats/min (\(n = 12\)). After two minutes of wash, the rate of spontaneous contractions returned toward control rates with a value of 73 ± 7 beats/min, thus demonstrating the reversible nature of the positive chronotropic response.

To determine whether the dual and opposite effects of CCh on the rate of spontaneous contractions are both mediated through cell surface mACHRs, the non-selective muscarinic antagonist, atropine, was used to inhibit the observed responses. In the presence of 100 nM atropine, the inhibitory effect of 500 nM CCh on the rate of spontaneous contractions was completely abolished (Fig. 2A). Similarly, atropine inhibited the CCh-induced positive chronotropic response. In cells treated with 200 ng/ml PTx for 24 h, 50 \(\mu \text{M}\) CCh increased the rate of spontaneous contractions by 34 ± 3 beats/min (\(n = 5\)). In the presence of 100 nM atropine, however, 50 \(\mu \text{M}\) CCh produced a change in the rate of spontaneous contractions of −3 ± 3 beats/min, indicating a complete block of the positive chronotropic response (Fig. 2B). Thus, both the CCh-induced negative and positive chronotropic effects are mediated through cell surface mACHRs.

One hallmark of the dual effects of muscarinic activation on the heartbeat obtained from previous studies on various cardiac tissue preparations is that the two responses can be differentiated by different muscarinic agonists. It has been reported that while CCh and acetylcholine are able to elicit both the inhibitory and stimulatory effects, the \(m_2\) mAChR-selective agonist, oxotremorine, is able to elicit only the inhibitory response (4, 26). Therefore, we determined whether oxotremorine could similarly differentiate between the negative and positive chronotropic effects of mAChR activation in the cultured ventricular myocytes. In three separate experiments, addition of 1 \(\mu \text{M}\) oxotremorine to the superfusion buffer completely abolished spontaneous contractions in an atropine-sensitive manner (Fig. 3A). In cells pretreated with 100 ng/ml PTx, however, oxotremorine was unable to mimic the CCh-induced positive chronotropic response at any concentration tested (Fig. 3B; \(n = 4\)). These results establish that oxotremorine differentiates between the dual effects of mAChR stimulation on the heartbeat in a manner similar to that found in other cardiac cell preparations.

Expression of mAChR Subtypes in Cultured Ventricular Myocytes—We investigated the possible expression of multiple mAChR subtypes in the cultured heart cells using the highly sensitive and specific technique of RT-PCR. A potential problem with these studies arose from the fact that, as is typical with primary cultures, the cultured neonatal rat ventricular myocyte preparation was always found to be contaminated by a population of non-myocyte cells most of which are fibroblasts. This contamination was evident even after the use of effective procedures such as preplating to limit the initial amount of fibroblasts, and exposure to \(\gamma\)-irradiation to inhibit their proliferation thereafter (14). These methods resulted in cultures...
which identifiably contained about 80–90% ventricular myocytes after 2 weeks in culture. Nevertheless, due to the extreme sensitivity of the RT-PCR procedure, it was recognized that even this low level of contaminating cells could potentially be the source of a confounding artifact by contributing to the mAChR subtypes identified. For this reason, it was necessary to use a method capable of identifying mAChR subtypes that are expressed in the ventricular myocytes to the exclusion of those expressed in the contaminating fibroblasts. This was achieved in two different ways. First, single-cell RT-PCR was carried out on mRNA derived by sucking up the contents of single ventricular myocytes into glass micropipettes. Using this technique, we consistently obtained amplification products corresponding to the m1 and m2 mAChRs (Fig. 4A). In two experiments, we obtained an amplification product corresponding to the m3 mAChR, however, this observation could not be reproduced consistently. We did not observe products corresponding to the m4 or m5 mAChRs in any experiment. The identity of the m1 mAChR, however, was confirmed by Southern blot (Fig. 4B) and by sequencing the PCR products (data not shown). Controls for the single-cell RT-PCR reactions were provided by experiments in which reverse transcriptase was omitted during the reverse transcription step (Fig. 4A). In a second approach, immunofluorescence was used to confirm the results obtained from the single-cell RT-PCR experiments. Fig. 5 shows confocal images obtained from immunofluorescence studies when cells were incubated with either no primary antibody (A), or primary antibodies against the m1 and m2 mAChRs (B and C, respectively). Staining of the cell membrane was seen for both m1 and m2 mAChR antibodies. Thus, the results obtained by immunocytochemistry are consistent with the single-cell RT-PCR data, and together these provide strong evidence for the existence of multiple mAChR subtypes on the surface membranes of cultured neonatal rat ventricular myocytes.

Functional Inhibition of mAChR Subtypes with Antisense Oligonucleotides—To determine which mAChR subtype mediates the CCh-induced positive chronotropic response in cultured neonatal rat ventricular myocytes, antisense oligonucleotides were used to specifically and selectively reduce the expression of the different mAChR subtypes. Based on the results obtained from the single-cell RT-PCR experiments, fluorescein-tagged antisense oligonucleotides targeted against mRNA for the m1, m2, and m3 mAChRs were designed and tested for their ability to inhibit the CCh-induced positive chronotropic response in cultured ventricular myocytes. The cells were treated with 200–500 nM fluorescein-tagged oligonucleotide in the presence of Lipofectin and functional assays carried out 48–72 h later. Measurements were only made in cells that demonstrably exhibited nuclear fluorescence at this time. In cells not treated with oligonucleotide, 300 μM CCh resulted in an increase in rate of 48 ± 9 beats/min (n = 3) (Fig. 6). This response was not inhibited in cells treated with antisense oligonucleotides directed against the m2 or m3 mAChRs, which responded to 300 μM CCh with increases in rate of 49 ± 15 beats/min (n = 4) and 49 ± 12 beats/min (n = 3), respectively. Treatment of cells with antisense oligonucleotides targeted against the m1 mAChR, however, resulted in a significant reduction in the CCh-induced increase in single-cell contraction rate. In these cells, 300 μM CCh resulted in an increase in rate of only 16 ± 2 beats/min (n = 5) (Fig. 6). These results indicate that the CCh-induced positive chronotropic response observed in cultured neonatal rat ventricular myocytes is mediated through the m1 mAChR. A number of control experiments were performed to demonstrate the specificity of the antisense methods applied here. Immunocytochemistry was performed, using m1- and m3-specific primary antibodies and the ABC Elite kit from Vector Labs, on cells treated with m1 antisense oligonucleotides to monitor changes in protein expression. Fig. 7 (A and C) illustrates peroxidase staining for control cells treated with either m2 or m1 mAChR primary antibody, respectively. In cells treated with antisense oligonucleotides to m1 mAChR protein, a significant decrease in m1 receptor staining was observed (Fig. 7D), whereas no change in m2 receptor staining was observed (Fig. 7B). In functional experiments, the anti-m2 mAChR oligonucleotides were able to inhibit the CCh-induced negative chronotropic response in contrast to their lack of effect on the positive chronotropic response (data not shown). Additionally, the anti-m1 mAChR oligonucleotide was found to be ineffective in inhibiting isoproterenol-mediated increase in contraction rate, even though this latter response could be inhibited by an antisense oligonucleotide directed against Gα protein mRNA (data not shown).

 Previous studies have demonstrated that agonist binding to the m1 mAChR leads to the activation of Gq protein (27). We, therefore, tested the hypothesis that the Gqα protein was responsible for transducing the mAChR-mediated increase in single-cell contraction rate in response to CCh using an antisense approach. Antisense oligonucleotides directed against the Gqα protein were also effective in significantly inhibiting the CCh-induced positive chronotropic response. In cells treated with anti-Gqα oligonucleotides, 300 μM CCh induced an increase in the rate of spontaneous contractions of 18 ± 5 bpm (n = 7). This result suggests the involvement of Gqα protein in mediating the CCh-induced positive chronotropic response (Fig. 6). To demonstrate that inhibition of the Gqα-mediated pathway by antisense oligonucleotides does not interfere with isoproterenol-mediated activation of Gqα or carbachol-mediated activation of Gqα protein pathways, spontaneously beating cells treated with Gqα protein-specific oligonucleotides were perfused with either 1 μM isoproterenol or 500 nM carbachol. Table 1 demonstrates that blockade of the Gqα-mediated pathway has no effect on either the ability of isoproterenol to produce an increase in spontaneous beat rate via Gqα activation, or the ability of carbachol to decrease the spontaneous beat rate via Gqα activation.

Role of the PI Hydrolysis Pathway in the CCh-induced Posi-
tive Chronotropic Response—Studies with cloned mAChRs have shown that the m₃ mAChR preferentially couples to PLC-mediated PI hydrolysis when transfected into cells. Also, it has been demonstrated that stimulation of mAChRs can induce PI turnover in cardiac cells (28). We therefore studied the possible involvement of this signal transduction pathway in the CCh-induced positive chronotropic response in cultured ventricular myocytes. In 6-day-old cultures, application of CCh resulted in a concentration-dependent increase in the accumulation of IP (Fig. 8A). The half-maximal concentration of CCh required for this response was 17 μM, while a maximum response of a 3-fold increase in IP was observed with 300 μM CCh. The increase in IP accumulation induced by 150 μM CCh was inhibited by 1 μM atropine, indicating its mediation through cell surface mAChRs (Table II). To determine whether a cause and effect relationship exists between CCh-induced PI hydrolysis and the positive chronotropic response, we used two putative inhibitors of PLC-mediated PI hydrolysis, neomycin and U73122. Pretreatment of cells with 500 μM neomycin or 5 μM U73122 resulted in a complete blockade of the positive chronotropic response induced by 300 μM CCh (Fig. 8B). The effect was recovered upon washout of neomycin or U73122 in all cases. At these concentrations, both neomycin and U73122 were shown to inhibit the increase in IP accumulation induced by 150 μM CCh (Table II).

In contrast, 500 μM neomycin or 5 μM U73122 did not inhibit either the CCh-induced negative chronotropic response, or the isoproterenol-induced positive chronotropic response, in cultured ventricular myocytes (Table I).

FIG. 5. Detection of mAChR protein on cultured ventricular myocytes by immunofluorescence. Confocal microscopy images obtained from immunofluorescence experiments where cells were incubated with no primary antibody (A), anti-m₁ mAChR antibody (B), or anti-m₂ mAChR antibody (C).

FIG. 6. Effects of mAChR antisense oligomers on CCh-induced increase in contraction rate. Bar graph showing the effect of fluorescein-tagged antisense oligonucleotides directed against mRNA for the m₁, m₂, and m₄ mAChRs, and G₉α protein on the increase in contraction rate induced by 300 μM CCh in PTx-treated cultured neonatal rat ventricular myocytes. Values represent means ± S.E. with the number of experiments shown in parentheses (*, p < 0.05 when compared with control using Student’s unpaired t test).

DISCUSSION

Dual Effects of CCh on the Heartbeat—The phenomenon in which activation of cardiac mAChRs produces dual and opposite effects on the heartbeat has been described in almost all cardiac cell types in virtually every species in which this question has been addressed. For example, muscarinic agonists have been reported to have both negative and positive inotropic effects in guinea pig and rat atria (3), canine and guinea pig ventricular muscle (2, 26), as well as in canine Purkinje fibers (29). In addition, negative and positive chronotropic responses to mAChR stimulation have been observed in chick sinoatrial node (30) and canine Purkinje fibers (31). Although the negative inotropic and chronotropic effects of mAChR receptor activation are the best well known and considered physiologically relevant, the remarkable conservation of stimulatory responses to muscarinic agonists in all cardiac cell types and across species argues strongly for a physiological role for this phenomenon. What this physiological role may be is not altogether clear at present, although it has been suggested that a stimulatory component of mAChR activation on the heartbeat may act in an autoregulatory capacity to prevent excessive, and thus detrimental, inhibition of the heartbeat by the vagus nerve (4). In this regard, it is interesting to note that the phenomenon whereby an agonist produces dual and opposite effects on the heartbeat is not unique to the mAChR system but is in fact prevalent among other receptor systems that are able to influence the heartbeat. For example, both inhibitory and stimulatory effects on the heart have been reported to occur with the stimulation of cardiac adenosine receptors (32), P₂₄-purinoceptors (33), and α-adrenergic receptors (34). Given this apparent ubiquity, it is reasonable to speculate that the phenomenon of agonists having dual effects on the heartbeat is a common mechanism employed by cardiac cells to regulate or fine-tune the effects of various agonists on the chronotropic and inotropic state of the heart.

A consistent finding relating to the stimulatory effects of mAChR activation on the force and frequency of the heartbeat is that these responses are insensitive to PTx and invariably require much higher concentrations of agonist than do the inhibitory responses (4). Two competing hypotheses have been invoked to explain these observations (35). First, the two responses could be mediated through a single mAChR, which couples with different efficiency to two different G proteins.
m2 mAChR subtype, other than m2, coupled to a PTx-insensitive G protein. In this work, cultured neonatal rat ventricular myocytes—

Table I

Effect of inhibitors of PI hydrolysis and Gα antisense oligonucleotides on carbachol-mediated inhibition of beat rate and isoproterenol mediated stimulation of beat rate

Cultured neonatal rat ventricular myocytes were pretreated with 500 μM neomycin sulfate (Neo), 5 μM U73122, or 200 nM Gα antisense oligonucleotides in the presence of 8 μg/ml lipofectin, and the effects of 500 nM carbachol (CCh) and 1 μM isoproterenol (Iso) on beat rate were monitored. 1 μM atropine = Atr; 1 μM propranolol = Prop. Values represent means ± S.E. with n representing the number of experiments performed. *, p < 0.05 when compared to control using Student’s unpaired t test.

| Condition            | Rate  | n  |
|----------------------|-------|----|
| Control              | 30 ± 2| 25 |
| CCh                  | 5 ± 2 | 8  |
| CCh + Atr            | 31 ± 4| 4  |
| CCh + Neo            | 0 ± 0 | 3  |
| CCh + U73122         | 2 ± 2 | 3  |
| Iso                  | 51 ± 5| 9  |
| Iso + Prop           | 23 ± 5| 4  |
| Iso + Neo            | 50 ± 12| 3  |
| Iso + U73122         | 60 ± 15| 3  |
| Gα antisense         | 33 ± 6| 9  |
| Gα antisense + CCh   | 0 ± 0 | 4  |
| Gα antisense + Iso   | 76 ± 11| 3  |

Alternatively, the two responses could be mediated through two different mAChR subtypes. In the latter model, the inhibitory response to cholinergic agonists is proposed to be mediated through the m2 mAChR coupled to a Gι/Go protein while the stimulatory response is mediated through a less abundant m1-specific antibody.

Expression of mAChR Subtypes in Cultured Ventricular Myocytes—It has been a long held view that cardiac cells express only the m2 mAChR on their surface membranes (27). This view was supported by Northern blot experiments in which it was shown that mRNA for the m2 mAChR, but not other mAChR subtypes, could be detected in rat atrial preparations (5). This result is difficult to reconcile with the fact that the atrial preparations used for these studies presumably contained cell types, other than cardiac cells, such as smooth muscle cells, fibroblasts, and nerve cells, some of which have
been found to possess mAChR subtypes other than m2 (6, 27). Thus, the detection of mRNA for only the m2 mAChR in this preparation using Northern blot analysis suggests a limit to the sensitivity of this technique in detecting small amounts of mRNA. This possibility is supported by recent experiments in which mAChR subtypes that were missed by Northern blot studies were detected by use of RT-PCR in guinea pig ventricular myocytes (7). In addition, work in this laboratory has demonstrated, through the use of single-cell RT-PCR, the existence of m1 and m2 mRNA in acutely isolated ventricular myocytes from adult rat heart (10).

In this work, using the highly sensitive technique of RT-PCR, we found that cultured neonatal rat ventricular myocytes express mRNA for the m1, m2, and, possibly, m4 mAChRs. Furthermore, the presence of m1 and m2 mAChR protein on the surface membranes of the cultured ventricular myocytes were confirmed by immunofluorescence. The finding of mRNA for the m1 and m2 mAChRs in these cardiac cells is in agreement with recent work in which both these subtypes were found in adult guinea pig and adult rat ventricular myocytes (7, 10). In our experiments we were also able to occasionally detect mRNA for the m1 mAChR. The inconsistent nature of this observation could reflect a lower abundance of this mRNA when compared with those for the m1 and m2 mAChRs. Clearly, further work is needed to establish whether the cultured neonatal rat ventricular myocytes do indeed express the m1 mAChR. It is noteworthy, however, that in embryonic chick ventricle a mAChR subtype termed chick m4, which has high homology to the mammalian m2 mAChR mRNA, has been found to be expressed (36).

Use of Antisense Oligonucleotides to Assess mAChR Subtypes Linked to Negative and Positive Chronotropy—The identification of multiple mAChR subtypes in cultured neonatal rat ventricular myocytes provides a rational basis for the hypothesis that the CCh-induced negative and positive chronotropic responses are mediated through different mAChR subtypes. The absolute determination of this question, however, requires the use of agents that are able to selectively and specifically inhibit the function of different mAChR subtypes. The use of “selective” antagonists to mAChR subtypes in this role has yielded conflicting results. Although data obtained using selective mAChR subtype antagonists generally agree that the inhibitory effects of mAChR stimulation on the heartbeat are mediated through the m2 mAChR, the situation is not so clear for the stimulatory response. Thus, it has been reported that “low affinity” mAChR responses in cardiac cells are mediated through m1 mAChRs (7, 10, 31), m2 mAChRs (3, 35), m3 mAChRs (37), or through receptors that are neither m1 nor m2 (38).

In this study we have used antisense oligonucleotides to selectively inhibit expression of specific mAChR subtypes in cultured rat ventricular myocytes. These agents have found widespread use as specific inhibitors of gene expression in a variety of in vitro systems as well as in vivo (39). A number of factors have been identified as being important in determining the efficacy of antisense oligonucleotides in inhibiting gene expression. These include the stability of the oligonucleotide, its intracellular concentration, and its intracellular distribution. Phosphorothioate oligonucleotides were used in this study since this modification has been found to render the oligonucleotides nuclease-resistant, thus enhancing their stability, without interfering with their hybridization properties (40). To assess uptake and intracellular distribution, oligonucleotides were tagged with fluorescein and observed within cells by fluorescence microscopy. To allow for mAChRs already expressed on the cell surface at the time of transfection to turnover, functional assays were performed 48–72 h after transfection. At this time, however, only 30% or less of the cells exhibited a nuclear localization of oligonucleotide. Thus, a crucial advantage of the fluorescence-based method described here is that it allowed for the determination of the efficacy of antisense treatment only on the single cells that exhibited a nuclear localization of oligonucleotide.

Fluorescein-tagged oligonucleotide antisense to mRNA for the m1, but not the m2 or m4 mAChRs inhibited the positive chronotropic response elicited by 300 μM CCh. Importantly, 1) cells treated with anti-m1 mAChR oligonucleotides displayed decreased staining with m1-specific antibodies while showing no change in m2 staining, 2) the anti-m1 mAChR oligonucleotide had no effect on isoproterenol-induced increase in contraction rate, and 3) the anti-m2 mAChR oligonucleotide was able to inhibit the CCh-induced negative chronotropic response. These results demonstrate the specificity of the antisense treatment and indicate that the CCh-induced negative and positive chronotropic responses are mediated through m2 and m1 mAChRs, respectively. We suggest that the stimulatory effects of mAChR activation on the force and frequency of the heartbeat observed in other cardiac cell preparations could be similarly mediated through m1 mAChRs. Recent data in agreement with this conclusion are provided by experiments conducted on adult guinea pig and rat ventricular myocytes in which activation of pharmacologically identified m1 mAChRs were associated with an increase in the L-type Ca2+ current and an increase in the magnitude of cytosolic Ca2+ transients, respectively (7, 10).

Involvement of Gα Activation and PI Hydrolysis in the CCh-Induced Positive Chronotropic Response—In other cell systems, m1 mAChRs are known to couple to and activate Gα protein (27). Involvement of Gα protein in the CCh-induced increase in spontaneous beat rate in cultured neonatal rat ventricular myocytes was investigated through the use of Gα-specific antisense oligonucleotides. Inhibition of Gα protein expression resulted in a significant decrease in the ability of CCh to stimulate spontaneous beat rate, suggesting the involvement of this protein in the stimulatory pathway. In contrast, blockade of the Gα-mediated pathway in cultured ventricular myocytes had no effect on the ability of those cells to activate Gα protein in response to isoproterenol treatment or to activate Gα protein in response to low concentrations of CCh.

It has long been suggested that the products of PI hydrolysis may be the second messengers responsible for mediating the stimulatory effects of muscarinic agonists on the heartbeat. This suggestion is based on a number of factors. 1) The concentrations of muscarinic agonist required to elicit PI turnover (ÊC50 = 17 μM) are high and comparable to those required to elicit positive inotropic and chronotropic responses (ÊC50 = 26 μM), 2) similar to mAChR-mediated stimulatory effects on the heartbeat, the stimulation of PI hydrolysis by muscarinic agonists is insensitive to PTx, and 3) other receptor systems that couple to PI hydrolysis in the heart also generally have stimulatory effects on the rate and force of the heartbeat. Despite this obvious correlation between mAChR agonist-induced PI turnover and stimulatory effects on the heartbeat, there is scant evidence establishing a cause and effect relationship between the two events. Here, we attempted to determine whether such a relationship existed by observing the effects of blockade of PI hydrolysis on the CCh-induced positive chronotropic response. At high concentrations, neomycin blocks PI turnover by binding to phosphatidylinositol 4,5-bisphosphate and preventing its hydrolysis by PLC (41). Consistent with this, 500 μM neomycin blocked CCh-induced PI turnover by over 80% in the cultured ventricular myocytes. More interest-
ingly, neomycin also completely blocked the CCh-induced positive chronotropic response in these cells, suggesting the direct involvement of a product of the PI hydrolysis pathway in generating this response. Consistent with this notion, another putative inhibitor of PLC-mediated PI hydrolysis, U73122 (42), blocked both the positive chronotropic response, and PI turnover induced by 300 μM CCh. One concern relating to these results relates to the specificity of neomycin and U73122 in inhibiting PLC-mediated PI hydrolysis. Conceivably, these agents could inhibit other second messenger pathways that might be important in mediating the stimulatory effects of mACHR activation on the heartbeat (41, 42). However, in the presence of 500 μM neomycin sulfate or 5 μM U73122, these drugs had no effect on the ability of 500 nM CCh or 1 μM isoproterenol to produce a decrease or increase in spontaneous beating rate, respectively.

Finally, what are the possible mechanisms by which m1 mACHR activation can elicit a positive chronotropic response? Stimulation of m1 mACHR has been found to inhibit different types of K+ channels through a pathway involving PLC activation and direct tyrosine phosphorylation (43, 44). An inhibition of K+ channels could presumably depolarize resting membrane potential sufficiently to increase automaticity and produce a positive chronotropic effect. Consistent with this idea, our recent preliminary results indicate that 300 μM CCh significantly prolongs the action potential duration and depolarizes the membrane potential in PTx-pretreated neonatal ventricular myocytes. The signaling mechanisms for these effects are currently under investigation.

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