J Pharmacol Sci **103**, 75 – 82 (2007)

Journal of Pharmacological Sciences

Full Paper

Muscarinic Receptor Subtypes Mediating Positive and Negative Inotropy in the Developing Chick Ventricle

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Received July 5, 2006; Accepted November 27, 2006

Abstract. The inotropic response to muscarinic receptor stimulation of isolated chick ventricular myocardium was examined at various developmental stages, and the receptor subtype involved was pharmacologically characterized. In embryonic chick ventricles, carbachol (CCh) produced positive inotropy at micromolar concentrations. In hatched chick ventricles, CCh produced negative inotropy at nanomolar concentrations. Neither positive nor negative inotropy was observed in the 19 – 21-day-old embryos. Both positive and negative inotropy were also observed with acetylcholine and oxotremoline-M. The CCh-induced positive inotropy in 7 – 9-day-old embryonic ventricles and the negative inotropy in 1 – 3-day-old hatched chick ventricles were antagonized by muscarinic receptor antagonists; pA_2 values for the positive and negative responses of pirenzepine were 7.5 and 7.2, those of AF-DX116 (11-[(2-[(diethylamino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4] benzodiazepine-6-one) were 6.8 and 6.9, those of 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) were 9.0 and 8.5, and those of himbacine were 7.0 and 8.0, respectively. CCh had no effect on action potential configuration. In conclusion, the positive inotropy is most likely mediated by muscarinic M_1 receptors and the negative inotropy is mostly likely mediated by muscarinic M_4 receptors.

Keywords: carbachol, chick, ventricle, muscarinic receptor, contractile force

Introduction

Acetylcholine (ACh) is a transmitter present in many species throughout the animal kingdom and is involved in a wide variety of physiological responses. In mammalian hearts, ACh is present as the parasympathetic neurotransmitter and generally mediates negative chronotropy and inotropy through its action on muscarinic receptors. However, its effect on the heart is known to vary depending on the animal species, developmental stage, and pathophysiological conditions.

The mammalian muscarinic receptor family belongs to the superfamily of G-protein-coupled receptors and consists of five members, M_1, M_2, M_3, M_4, and M_5 (1). The odd-numbered muscarinic receptor subtypes M_1, M_3, and M_5 are coupled to $G_{q/11}$-protein that activates the phospholipase C-diacylglycerol-inositol phosphate system (1). The even-numbered muscarinic receptors, M_2 and M_4, are coupled to $G_{i/o}$ and can activate potassium channels and/or inhibit adenylate cyclase when the enzyme is activated by some other stimuli (1). Five cDNAs, m1, m2, m3, m4, and m5, have been cloned that correspond to the five muscarinic receptors M_1, M_2, M_3, M_4, and M_5, respectively (1). In the mammalian heart, the predominant subtype is M_2 (1).

The chicken heart has long been used for electrophysiological and pharmacological studies on cardiac ontogeny and regulation (2 – 7). Concerning autonomic regulation, anabolic and catabolic enzymes for norepinephrine and ACh as well as the receptors for these primary autonomic transmitters are present in the chick from the first quarter of embryonic life (8, 9). Earlier studies with electrical stimulation established that autonomic efferents are capable of releasing ACh from cholinergic fibers in the 12-day-old embryo,

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Published online in J-STAGE: January 13, 2007
doi: 10.1254/jphs.FPJ06013X

while sympathetic release of norepinephrine becomes possible in the 21-day-old embryo (10). Concerning the muscarinic regulation of ventricular contractility, both positive inotropy in the embryo and negative inotropy in the hatched chick have been reported (11, 12). However, the precise receptor subtype involved has not yet been clarified. Pharmacological (13 – 15) and radioligand binding (15) studies showed that the muscarinic receptors in the chick ventricle have affinity to pirenzepine, which suggests the presence of muscarinic M_1 receptors. However, the affinity to pirenzepine was lower than that of typical mammalian muscarinic M_1 receptors which may indicate co-existence of muscarinic M_1 and some other receptor subtypes. cDNAs corresponding to chicken muscarinic receptor subtypes M_2, M_3, and M_4 have been cloned and were designated as cm2 (16), cm3 (17), and cm4 (18), respectively.

The present study was performed with the following goals in mind: 1) to provide a systematic description of the developmental changes in muscarinic receptor-mediated inotropy, and 2) to clarify the muscarinic receptor subtypes involved in the positive and negative inotropy.

Materials and Methods

Animal and cardiac tissue preparation

Fertilized chicken eggs were incubated at 37°C, and the ventricles were removed at various stages of development. Right ventricular free wall was rapidly isolated and placed horizontally in an organ bath. The organ bath contained physiological salt solution of the following composition: 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, and 11.1 mM glucose. The solution was gassed with 95% CO_2 – 5% O_2 and maintained at 36°C – 37°C (pH 7.4). To eliminate adrenergic influence, all experiments were performed in the presence of 0.3 μM propranolol, which had no direct effect on contractile force and action potential parameters.

Measurement of contractile force

The stages examined were 7 – 9-day-old embryos, 11 – 13-day-old embryos, 15 – 17-day-old embryos, 19 – 21-day-old embryos, and 1 – 3-day-old chicks. One end was pinned down and the other end was hooked to the needle of a force-displacement transducer (TB-612T; Nihon Kohden, Tokyo) connected to a minipolygraph (Nihon Kohden, EF-601GS) and developed tension was recorded isometrically. All preparations were driven by a pair of platinum plate electrodes (field stimulation) with rectangular current pulses (1 Hz, 3 ms, 1.5 × threshold voltage) generated from an electronic stimulator (DPS-07; Dia Medical System, Kunitachi). The resting tension applied to each preparation was adjusted so that the muscle was stretched to the peak of its length-tension curve as previously described (19 – 21).

Measurement of cardiac action potential

The stages examined were 7 – 9-day-old embryos and 1 – 3-day-old chicks. The right ventricular free wall was used. They were driven by external electrical stimulation using bipolar platinum electrodes and rectangular current pulses (5-ms duration, about 1.2-fold threshold strength) at a constant frequency (1 Hz) generated by an electronic stimulator (Nihon Kohden, SEN-3101). Conventional microelectrodes experiments were performed as previously described (21 – 23). The amplifier (Nihon Kohden, MEZ-8201) was monitored through a dual-beam cathode ray oscilloscope (CS-1040; KENWOOD, Hachioji) and fed into an AD converter (Analogue Pro; Canopus, Kobe) attached to a computer (PC-9801FA; NEC, Tokyo) for analyses. Drugs were added after 60 min, after which the action potentials were well maintained. All drug solutions were prepared immediately before the start of the experiments.

Statistical analyses

Agonists were applied cumulatively in the absence or presence of antagonists and the increase or decrease in contractile force by each addition was expressed as a percentage of the maximum increase by the agonist. The EC_{50} values (concentration of agonists that caused half-maximum response) were determined by least-squares nonlinear regression analysis of each concentration-response curve. The sigmoidal concentration-response curve fitting was carried out using GraphPad Prism™ (Version 3.00; GraphPad Software, Inc., San Diego, CA, USA) assuming a Hill slope of 1. Antagonists were applied 30 min before agonist. Apparent pA_2 values for antagonists were calculated assuming competitive antagonism according to the method of Van Rossum (24) from the equation: $pA_2 = pA_X + \log (X - 1)$, in which X represents the factor of the shift of the concentration-response curves to the right at the level of EC_{50} and pA_X, negative logarithm of the concentration of antagonist that caused this shift. Data were expressed as the mean ± S.E.M. from 6 – 12 experiments.

To evaluate statistical significance of differences between means, we used one way or two way ANOVA followed by Fisher's least significance difference test or Dunnett's test for multiple comparisons, the paired *t*-test, or unpaired *t*-test with Welch's correction if necessary. A *P* value less than 0.05 were considered significant.

Drugs and chemicals

The drugs used were acetylcholine chloride (ACh) (Daiichi Seiyaku, Tokyo); carbamylcholine chloride (carbachol: CCh), himbacine, oxotremorine-M, pirenzepine dihydrochloride, and propranolol hydrochloride (Sigma, St. Louis, MO, USA); atropine sulfate and McN-A-343 (4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltri-methylammonium chloride) (Wako Pure Chemical Industries, Osaka); hexamethonium chloride (Tokyo Chemical Industry Co., Ltd., Tokyo); AF-DX116 (11-[(2-[(diethylamino)methyl]-1-piperidinyl) acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4] benzodiazepine-6-one) (Nissan Chemical Industries, Tokyo); and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP, Funakoshi, Tokyo). All other drugs and chemicals were commercial products of the highest available quality.

Results

CCh produced concentration-dependent positive inotropy in the ventricle from 7 – 9-day embryos (7-9E), 11 – 13-day embryos (11-13E), and 15 – 17-day embryos (15-17E). The maximum level of contractile

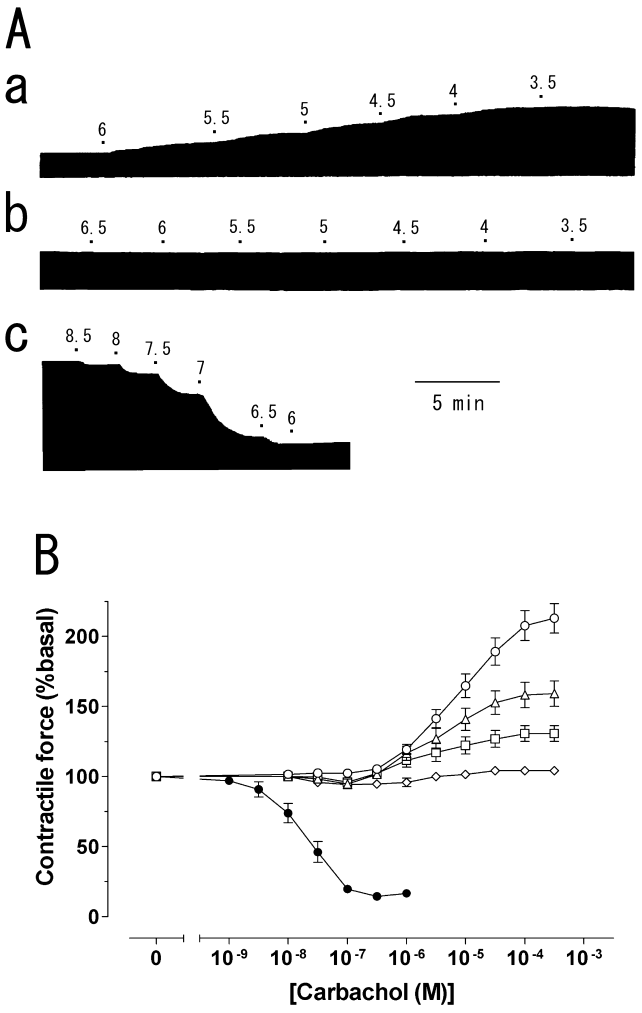

Fig. 1. Inotropic responses to CCh of developing chick ventricle. A: Typical traces obtained in the 8-day-old embryo (a), 20-day-old embryo (b), and 2-day-old hatched chick (c). Dots and numbers above the traces indicate the addition of CCh and the negative logarithm of the molar concentration. B: Concentration-response curves for ventricular preparations from 7 – 9-day-old embryos (open circles), 11 – 13-day-old embryos (open triangles), 15 – 17-day-old embryos (open squares), 19 – 21-day-old embryos (open diamonds), and 1 – 3-day-old hatched chicks (filled circles). Contractile force under each concentration was expressed as a percentage of the basal value. Symbols with vertical bars indicate the mean ± S.E.M. of 5 – 6 preparations for each group.

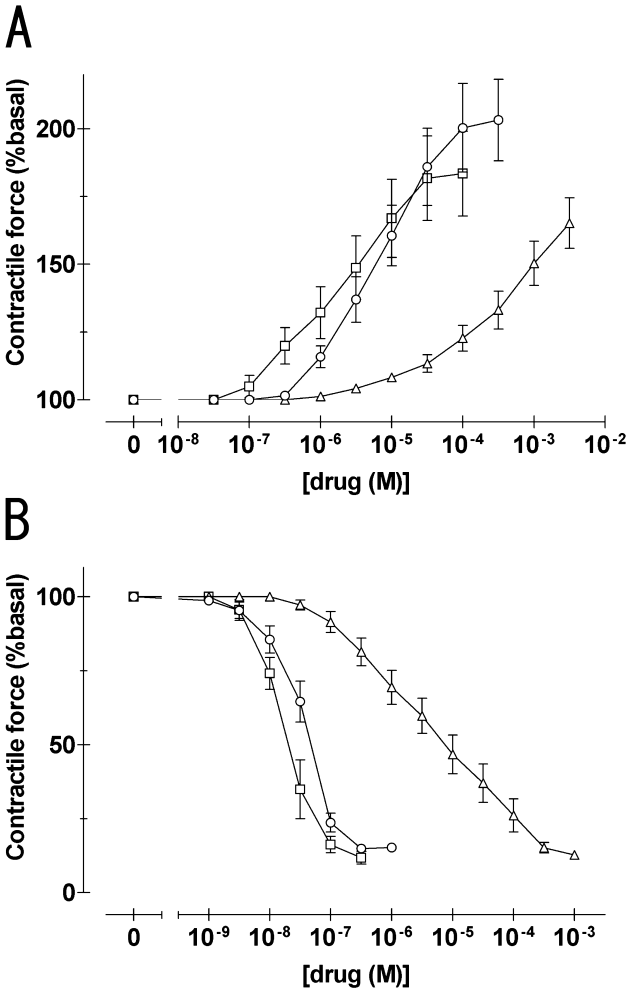

Fig. 2. Inotropic effect of muscarinic receptor agonists. A: Concentration-response relationship for the positive inotropic responses to CCh (open circles), ACh (open triangles), and oxotremorine-M (open squares) in 7 – 9-day-old embryos. B: Concentration-response relationship for the negative inotropic responses to CCh (open circles), ACh (open triangles), and oxotremorine-M (open squares) in 1 – 3-day-old hatched chicks. Contractile force under each concentration was expressed as a percentage of the basal value. Data points with bars indicate the mean ± S.E.M. from 6 experiments.

force produced by CCh in the 7-9E, 11-13E, and 15-17E was 212.9 ± 10.4% (n = 12), 159.2 ± 9.0% (n = 6), and 130.7 ± 5.7% (n = 6) of the basal contractile force, respectively; and the $-\log EC_{50}$ value was 5.2 ± 0.1 (n = 12), 5.5 ± 0.2 (n = 6), and 5.7 ± 0.3 (n = 6), respectively (Fig. 1). Inotropic response to CCh was totally absent or only slight toward either direction in the 19 – 21-day embryos (19-21E) (Fig. 1). In the 1 – 3-day-old hatched chicks (1-3H), CCh produced concentration-dependent negative inotropy. The contractile force reached minimum at 0.3 μM CCh, which was 14.9 ± 1.0% (n = 12) of the basal contractile force (Fig. 1). The $-\log EC_{50}$ value was 7.4 ± 0.1 (n = 12, Fig. 3B).

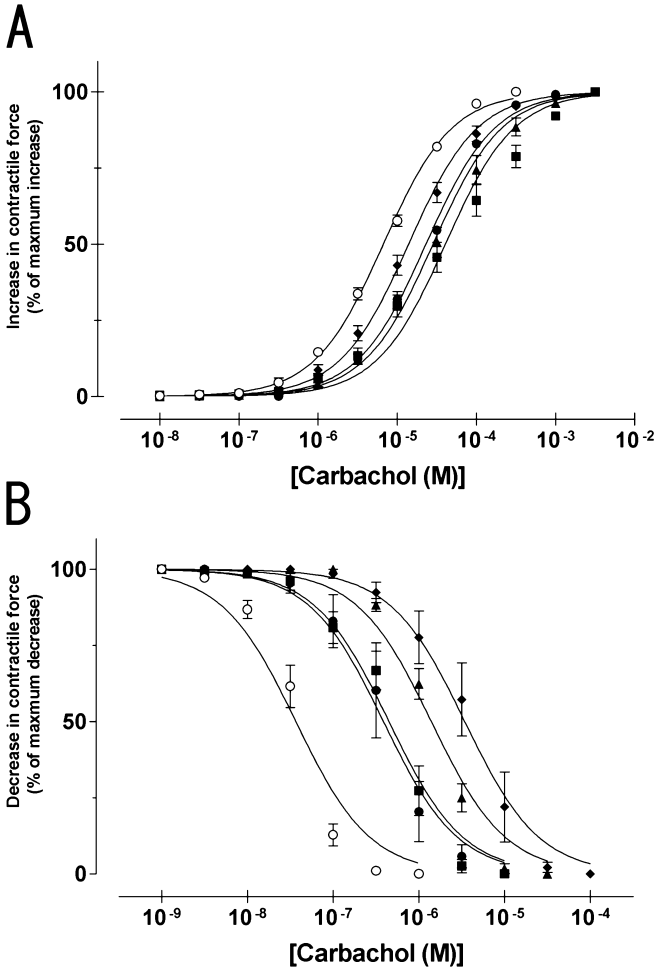

Fig. 3. Effect of muscarinic antagonists on the inotropic responses to CCh. A: Concentration-response curves for the positive inotropic effect in 7 – 9-day-old embryos in the absence of antagonist (open circles), in the presence of 60 nM pirenzepine (filled circles), 0.5 μM AF-DX116 (filled triangles), 5 nM 4-DAMP (filled squares), and 0.1 μM himbacine (filled diamonds). B: Concentration-response curves for the negative inotropic effect in 1 – 3-day-old hatched chicks in the absence of antagonist (open circles) and in the presence of 0.6 μM pirenzepine (filled circles), 5 μM AF-DX116 (filled triangles), 50 nM 4-DAMP (filled squares), and 1 μM himbacine (filled diamonds). Contractile force under each concentration is expressed as a percentage of the maximum increase or decrease induced by CCh. Data points with bars indicate the mean ± S.E.M. from 6 – 16 experiments.

In 7 – 9-day-old embryonic chicken ventricles (embryo), the physiological parasympathetic transmitter ACh and a non-choline ester muscarinic receptor agonist, oxotremorine-M, produced concentration-dependent positive inotropy (Fig. 2A). The positive inotropy by ACh did not reach maximum (n = 6) (Fig. 2A) at the concentration range tested. The maximum level of contractile force produced by oxotremorine-M was 183.4 ± 15.6% of the basal contractile force, and the $-\log EC_{50}$ value was 5.7 ± 0.2 (n = 6) (Fig. 2A). The M_1-receptor-selective agonist McN-A-343 slightly but significantly increased contractile force, which reached 114.9 ± 5.5% of the basal contractile force at 100 μM (n = 3) (data not shown). The CCh-induced positive inotropy was completely abolished by 1 μM atropine, but was not affected by hexamethonium; the contractile force after the addition of 100 μM CCh and that after the addition of 100 μM CCh in the presence of 100 μM hexamethonium was 200.3 ± 16.3% (n = 6) and 198.9 ± 12.1% (n = 6) of the basal contractile force, respectively.

The concentration-response curve for the CCh-induced positive inotropy in the embryo was shifted to the right by 60 nM pirenzepine (n = 6), 0.5 μM AF-DX116 (n = 8), 5 nM 4-DAMP (n = 9), and 0.1 μM himbacine (n = 16) in a competitive manner, with the pD_2 values being 4.64 ± 0.01, 4.55 ± 0.03, 4.39 ± 0.03, and 4.86 ± 0.02, respectively (Fig. 3A). The apparent pA_2 values of these muscarinic receptor antagonists are summarized in Table 1.

In ventricles from 1 – 3-day-old hatched chicken, ACh and oxotremorine-M produced concentration-dependent negative inotropy. The minimum contractile force after the addition of these drugs was 12.7 ± 1.2% (n = 6) and 11.8 ± 2.1% (n = 6) of the basal contractile

Table 1. Effect of muscarinic antagonists on CCh-induced inotropy

Antagonist	pA_2 values	
	7 to 9 days embryonic	1 to 3 days hatched
Pirenzepine	7.8	7.2
AF-DX116	6.9	6.9
4-DAMP	9.1	8.4
Himbacine	7.3	8.0

pA_2 values for antagonist on the CCh-induced positive inotropy in ventricles from 7 – 9-day-old chick embryo and negative inotropy in ventricles from 1 – 3-day-old hatched chick calculated from the data presented in Fig. 3.

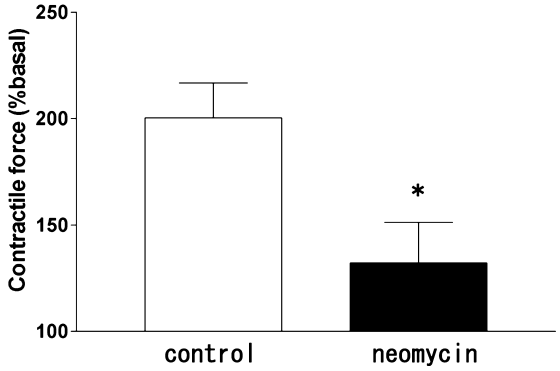

Fig. 4. Effect of neomycin on the inotropic responses to CCh in 7 – 9-day-old embryo. Increase in force of contraction by 100 μM CCh in the presence or absence of 100 μM neomycin were expressed as a percentage of the basal force of contraction. Symbols with vertical bars indicate the mean ± S.E.M. from 6 experiments for each group. * indicates significant difference from the control group, $P<0.05$.

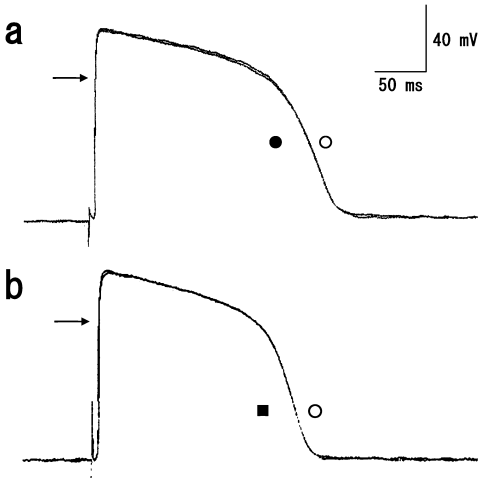

Fig. 5. Effect of CCh on ventricular action potential. Typical action potential recordings from 7 – 9-day embryo (a) and at 1 – 3-day-old hatched chicks (b) obtained in the absence of CCh (open circles), in the presence of 1 μM CCh (filled circle), and in the presence of 100 μM CCh (filled square). Arrows indicate zero mV level.

force, respectively, and the $-\log EC_{50}$ value was 5.5 ± 0.1 (n = 6) and 7.8 ± 0.1 (n = 6), respectively (Fig. 2B). The CCh-induced negative inotropy was completely abolished by 1 μM atropine, but was not affected by hexamethonium; the contractile force after the addition of 1 μM CCh and that after the addition of 1 μM CCh and 100 μM hexamethonium were 16.7 ± 1.1% (n = 6) and 18.6 ± 1.8% (n = 6) of the basal contractile force, respectively. The concentration-response curve for the CCh-induced negative inotropy in the hatched chick heart was shifted to the right by 0.6 μM pirenzepine, 5 μM AF-DX116, 50 nM 4-DAMP, and 1 μM himbacine in a competitive manner, with the pD_2 values being 6.41 ± 0.07, 5.86 ± 0.03, 6.35 ± 0.05, and 5.47 ± 0.06, respectively (Fig. 3B). The apparent pA_2 values of these muscarinic receptor antagonists are summarized in Table 1.

In ventricles from 7 – 9-day-old embryonic chick, positive inotropy by CCh at 100 μM was inhibited by 100 μM neomycin, which inhibits phospholipase C (25) (Fig. 4). The maximum level of contractile force produced by 100 μM CCh in absence and presence of neomycin was 200.3 ± 16.3% (n = 6) and 132.1 ± 19.1% (n = 4) of the basal contractile force, respectively.

In 7 – 9-day-old embryonic (n = 6) and 1 – 3-day-old hatched (n = 16) chicken ventricles, CCh produced no change in the action potential duration (Fig. 5). The action potential parameters are summarized in Table 2.

Discussion

Muscarinic agonists induced positive inotropy in embryonic and negative inotropy in hatched chick ventricle. Acetylcholine was the weakest of the agonists used in this study, which may be explained by the

Table 2. Effect of CCh on action potential

	7 – 9-day-old embryo		1 to 3 days after hatching	
	Control	CCh, 100 μM	Control	CCh, 1 μM
RP (mV)	−79.8 ± 0.5	−80.1 ± 0.5	−82.6 ± 0.4	−82.8 ± 0.6
OS (mV)	40.5 ± 0.9	40.9 ± 0.7	36.7 ± 0.9	36.5 ± 0.9
APD_{90} (ms)	202.1 ± 3.6	201.9 ± 5.6	182.6 ± 3.6	180.6 ± 2.7
APD_{50} (ms)	174.7 ± 3.8	174.5 ± 5.4	161.4 ± 3.8	159.1 ± 4.4
APD_{20} (ms)	130.8 ± 6.8	131.6 ± 4.8	118.3 ± 3.6	118.1 ± 1.7

Right ventricular myocardial tissues from 7 – 9-day-old embryo and from chicks at 1 to 3 days after hatching were stimulated at 1 Hz, and action potentials were measured with glass microelectrodes in the absence (control) or presence of CCh. RP and OS indicate resting potential and Overshoot, respectively. APD_{90}, APD_{50}, and APD_{20} indicate action potential duration at 20%, 50%, and 90% repolarization, respectively. Values are the mean ± S.E.M. from 5 – 8 experiments.

existence of choline esterase (8, 9). Indeed, physostigmine at 1 μM induced negative inotropy in the ventricle from 1 – 3-day-old hatched chicks (data not shown). The lack of or reduced inotropy in 19 – 21-day-old embryos is not specific for muscarinic receptor stimulation; decrease in pD_2 value for β-adrenergic agonists (26) and lack of response to histamine (27) are also observed during this period. It appears that the heart is in a "low sensitivity state" during this short period just before hatching, which may have to be considered separately from the gradual developmental changes taking place throughout the embryonic to post hatching periods. This period coincides with the onset of functional sympathetic innervation to the chick heart (10). Sympathetic innervation of the myocardium has been shown to produce reductions in its inotropic sensitivity to autonomic transmitters (28 – 30). Negative results have been reported for the causal relationship between sympathetic innervation and decreased sensitivity to β-adrenergic stimulation in the chick embryo heart (26).

In the mammalian heart, the predominant subtype of muscarinic receptors is muscarinic M_2 (1). Muscarinic receptor stimulation of the ventricular myocardium produces a decrease in contractile force only when the cAMP pathway has been pre-stimulated due to activation of adenylate cyclase (1). In some cases, however, positive inotropy by high concentrations of muscarinic receptor agonists has been reported in mammalian atrial and ventricular myocardial preparations (1). It appears to be mediated by muscarinic M_1 receptor, stimulation of phospholipase C, and enhancement of I_{Ca} (1). The muscarinic receptor-mediated positive inotropy in the chick ventricle was antagonized by pirenzepine, AF-DX116, 4-DAMP, and himbacine with approximate pA_2 values of 7.8, 6.9, 9.1, and 7.3, respectively. These are within the reported range of negative logarithm affinity constants or pK_i values of the four drugs for human muscarinic M_1 receptor (1). According to the review by Dhein (1), the $-\log K_i$ value range for the M_1 receptor is 7.8 – 8.5 for pirenzepine, 6.4 – 6.9 for AF-DX 116, 8.6 – 9.2 for 4-DAMP, and 6.9 – 7.4 for himbacine. Moreover Mac-N-343 at 100 μM, a selective M_1 agonist, produced positive inotropy (data not shown). These results indicate that muscarinic M_1 receptors mediate the positive inotropy in the chick embryonic heart. This conclusion is also supported by the observation that stimulation of muscarinic receptors in the chick ventricle results in increase in a ^{3}H-inositol phosphate production (31, 32) and activation of phospholipase C (33), which are both inhibited by phospholipase C inhibitor neomycin (Fig. 4). The positive inotropy was not accompanied by changes in the action potential duration (Fig. 5 and Table 2).

Muscarinic receptors are known to be present not only in cardiomyocytes but also in other cell types in the myocardium. In isolated mouse atria, a biphasic response to ACh was observed: a transient negative inotropy was followed by positive inotropy that were both inhibited with atropine (34). The positive inotropy in this case was revealed to be mediated by prostaglandins released from the endocardial endothelium (35). In some cases, muscarinic receptor agonist-induced positive inotropy are mediated by norepinephrine released from sympathetic nerve terminals in the myocardium (1). The CCh-induced positive inotropy in the developing chick ventricle was insensitive to indomethacin, propranolol, and phentolamine which rules out the involvement of endothelium-derived prostaglandins or norepinephrine released from sympathetic nerve endings (data not shown).

In mammalian atria, muscarinic receptor stimulation is known to produce negative inotropy through inhibition of adenylate cyclase and activation of the G-protein coupled potassium channel (1). In contrast, in mammalian ventricle, muscarinic receptor stimulation usually results in negative inotropy only under elevated adenylate cyclase activity. Direct negative inotropy by muscarinic receptor stimulation of ventricular myocardium has been reported in animal species such as the ferret, rat, dog, and frog (1), but the subtypes of the muscarinic receptor involved in the inotropy as well as its mechanisms remain to be investigated. The muscarinic receptor-mediated negative inotropy in chick ventricle was inhibited by pirenzepine, AF-DX116, 4-DAMP, and himbacine with pA_2 values of 7.2, 6.9, 8.4, and 8.0, respectively. These are close to the reported negative logarithm of affinity constants or pK_B values for the mammalian muscarinic M_4 receptor of the four drugs (1). According to the review by Dhein (1), the $-\log K_i$ value range for the M_4 receptor is 7.1 – 8.1 for pirenzepine, 6.6 – 7.0 for AF-DX 116, 8.4 – 9.4 for 4-DAMP, and 8.0 – 8.8 for himbacine. In hatched chick ventricle, CCh produced no change in ventricular action potential configuration, which rules out the augmentation of repolarizing potassium currents (Fig. 5 and Table 2). This is different from the case in atrial (23) and ferret papillary muscles (1) where ACh produced membrane hyperpolarization and a decrease in action potential duration. In hatched chick ventricle, SQ22,526 at 100 μM, an adenylate cyclase inhibitor, produced negative inotropy. Further addition of CCh did not produce negative inotropy (data not shown). Moreover, intracellular cAMP levels are elevated in hatched chick ventricle compared with embryonic chick ventricle (36). These results showed that the muscarinic receptor-

mediated direct negative inotropy in the hatched chick ventricle may rather be through inhibition of intrinsically pre-activated adenylate cyclase. Negative inotropy by SQ22,536 was not observed in the mouse ventricle, suggesting that this phenomenon is characteristic of the chick ventricle (data not shown). The ACh-induced negative inotropy in the 2 – 3 days after hatched chick ventricle was reported to be inhibited by pretreatment with pertusiss toxin (37).

In conclusion, the overall inotropy in response to muscarinic receptor stimulation shifted from positive to negative in the developing chick ventricular myocardium. The positive inotropy is most likely mediated by muscarinic M_1 receptors and the negative inotropy is most likely mediated by muscarinic M_4 receptors. To our knowledge, this is the first systematic characterization of functional muscarinic receptor subtypes in the chick ventricle.

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