

M₃ Muscarinic Receptors Mediate Positive Inotropic Responses in Mouse Atria: A Study with Muscarinic Receptor Knockout Mice

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ABSTRACT

The potential functional roles of M₃ muscarinic receptors in mouse atria were examined by pharmacological and molecular biological techniques, using wild-type mice, muscarinic M₂ or M₃ receptor single knockout (M₂KO, M₃KO), and M₂ and M₃ muscarinic receptor double knockout mice (M₂/M₃KO). Real-time quantitative reverse transcriptase-polymerase chain reaction analysis showed that the M₂ receptor mRNA was expressed predominantly in mouse atria but that the M₁, M₃, M₄, and M₅ receptor subtypes were also expressed at low levels. Carbachol (10 nM–30 μM) decreased the spontaneous beating frequency of right atria isolated from wild-type mice. Studies with subtype-preferring antagonists and atria from M₂KO mice confirmed that this activity is mediated by the M₂ receptor subtype. In left atria from wild-type mice, carbachol decreased

the amplitude of electrical field stimulation-evoked contractions (negative inotropic action), but this inhibition was transient and was followed by a gradual increase in contraction amplitude (positive inotropic response). In atria from M₃KO mice, the transient negative inotropic action of carbachol changed to a sustained negative inotropic action. In contrast, in atria from M₂KO mice, carbachol showed only positive inotropic activity. In atria from M₂/M₃ double KO mice, carbachol was devoid of any inotropic activity. These observations, complemented by functional studies with subtype-preferring antagonists, convincingly demonstrate that atrial M₃ muscarinic receptors mediate positive inotropic effects in mouse atria. Physiologically, this activity may serve to dampen the inhibitory effects of M₂ receptor activation on atrial contractility.

Muscarinic receptor stimulation by acetylcholine plays an important role in parasympathetic control of cardiac functions such as heart rate (chronotropic action), conduction velocity (dromotropic action), and contractility (inotropic action). Muscarinic receptors are prototypic members of the superfamily of G protein-coupled receptors, and molecular cloning studies have demonstrated the existence of five distinct mammalian muscarinic receptor subtypes (M₁–M₅) (Caulfield and Birdsall, 1998). Based on their differential G protein-coupling properties, the five receptors can be subdivided into two major functional classes. The M₁, M₃, and M₅ receptors preferentially couple to G_{q/11} proteins, whereas the M₂ and M₄ receptors are selectively linked to G_{i/o} proteins (Caulfield and Birdsall, 1998; Lanzafame et al., 2003). It is well documented that the heart predominantly expresses the M₂ receptor subtype (Brodde and Michel, 1999; Dhein et al., 2001). After activation of cardiac M₂ receptors, the activated α subunit of G_i proteins inhibits adenylate cyclase activity, resulting in a decrease of cytoplasmic cAMP, whereas the βγ subunit of the G_i proteins directly activates the inwardly rectifying muscarinic K⁺ channel (Yamada et al., 1998; Dhein et al., 2001). However, the M₂ receptor is not the only muscarinic receptor subtype present in the heart (Wang et al., 2004). Pharmacological, biochemical, immunohistochemical, and molecular biological studies using whole heart tissues or isolated cardiomyocytes indicate that the heart, es-

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ABBREVIATIONS: M₂KO, M₂ receptor single knockout; M₃KO, M₃ receptor single knockout; M₂/M₃KO, M₂ and M₃ receptors double knockout; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; EFS, electrical field stimulation; AF-DX116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one; 4-DAMP, 4-diphenylacetoxy-N-methyl-piperidine; p-F-HHSD, p-fluorohexahydrosiladiphenidol hydrochloride.

pecially cardiomyocytes, also expresses low levels of non-M₂ muscarinic receptors, including the M₁ and M₃ receptor subtypes (Gallo et al., 1993; Sharma et al., 1996; Hardouin et al., 1998; Hellgren et al., 2000; Wang et al., 2001, 2004; Krejčí and Tucek, 2002; Pönicke et al., 2003; Willmy-Matthes et al., 2003; Pérez et al., 2006; Mysliveček et al., 2008).

At present, little is known about the functional roles of the non-M₂ muscarinic receptors that are present in the heart. It has been suggested that the M₁ receptor causes positive inotropic effects by increasing the activity of the L-type Ca²⁺ currents in guinea pig myocytes (Gallo et al., 1993) or by increasing Ca²⁺ release in rat myocytes (Sharma et al., 1996). It has also been proposed that M₁ or M₃ receptors mediate positive inotropic responses in isolated cardiac muscle strips from human and mouse (Du et al., 1995; Nishimaru et al., 2000). However, studies in this area have been hampered by the lack of small-molecule ligands that can block or activate specific muscarinic receptor subtypes with high selectivity.

Recently, mutant mice lacking M₁ to M₅ muscarinic receptors (knockout mice) have become available as novel experimental tools to study the functional roles of individual muscarinic receptors in the heart and other tissues and organs (Wess, 2004). In spontaneously beating right atria from M₂ receptor knockout (M₂KO) mice, carbachol was devoid of any negative chronotropic activity (Stengel et al., 2000), in agreement with previous evidence indicating that the M₂ receptor mediates the negative chronotropic actions of muscarinic agonists (Caulfield and Birdsall, 1998). In electrically stimulated atria from wild-type mice, acetylcholine was shown to induce both positive and negative inotropic responses (Nishimaru et al., 2000; Tanaka et al., 2001). Pertussis toxin treatment selectively abolished the negative inotropic activity of acetylcholine (Nishimaru et al., 2000), suggesting that one or more of the odd-numbered muscarinic receptors (M₁, M₃, and M₅) that couple to pertussis toxin-insensitive, G_{q/11}-type proteins mediate the positive inotropic action of acetylcholine.

In the present study, we used muscarinic M₂ or M₃ receptor single knockout (M₂KO and M₃KO) and M₂ and M₃ muscarinic receptor double knockout mice (M₂/M₃KO) to study the potential role of the M₃ receptor subtype in modulating atrial contractility. These experiments were complemented by functional studies using subtype-preferring antagonists and real-time qRT-PCR expression studies. Our data clearly demonstrate that atrial M₃ muscarinic receptors mediate the positive inotropic effects of muscarinic agonists in mouse atria. This finding further highlights the usefulness of muscarinic receptor knockout mice to study different aspects of cardiac function.

Materials and Methods

Animals and Tissue Preparations. All experiments were performed in accordance with the institutional guidelines approved by the Animal Ethics Committee of the School of Veterinary Medicine (Rakuno Gakuen University, Ebetsu, Hokkaido, Japan).

Wild-type mice (DDY strain; Sankyo Lab Service, Sapporo, Japan) as well as mice lacking either the M₂ (M₂KO) or the M₃ receptor (M₃KO) or both M₂ and M₃ receptors (M₂/M₃KO), and the corresponding wild-type mice were used in the present experiments (either male or female). The generation of M₂KO, M₃KO, and M₂/M₃KO mice has been described previously (Gomez et al., 1999; Yamada et al., 2001; Struckmann et al., 2003). The genetic backgrounds of the

mice used in the present study were 129J1 (50%) × CF1 (50%) for M₂KO and their corresponding wild-type mice, 129vEv (50%) × CF1 (50%) for M₃KO and their corresponding wild-type mice, and 129J1(25%) × 129SvEv (25%) × CF1 (50%) for M₂/M₃KO mice. The animals were housed in ventilated polycarbonate cages. The temperature of the animal room was maintained at 23 ± 1°C, with a relative humidity of 40 to 60% and a daily light/dark cycle (7:00 AM–7:00 PM). Food (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) and water were given ad libitum.

Adult mice that were more than 3 months old (weight, 23–30 g) were killed by cervical dislocation. The beating heart was isolated from each animal and immersed in warmed bubbling Krebs' solution. Spontaneously beating left and right atria were dissected together from ventricles and their lumens were rinsed well to remove blood. The left atrium was attached with thread to a stationary glass rod, whereas the right atrium was tied with thread to a force displacement transducer. The atria were placed in an organ bath containing 20 ml of Krebs' solution (118 mM NaCl, 4.75 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₂, and 11.5 mM glucose) warmed at 37°C and gassed with 95% O₂ + 5% CO₂. After a 60-min equilibration period, the effect of carbachol on spontaneous beating frequency was investigated to analyze chronotropic activity. To study inotropic responses, the left atrium was isolated rapidly and washed well using a fresh bubbling Krebs' solution. The atrium was placed between a pair of platinum rod electrodes and suspended vertically in an organ bath. The end of the preparation was tied and connected to a force displacement transducer. Electrical field stimulation (EFS; 1 Hz, 2 ms in duration, 1.5× threshold voltage; Nishimaru et al., 2000; Tanaka et al., 2001) was applied by an electrical stimulator. After a steady EFS-induced contraction was established, carbachol was applied cumulatively at 3-min intervals or noncumulatively (single applications at 30-min intervals), and carbachol concentration-response curves for inotropic responses were constructed. The concentration-response curves were analyzed by sigmoidal nonlinear regression fit using Origin 7.0 (OriginLab Corp., Northampton, MA) to determine the carbachol concentrations producing 50% (EC₅₀) of its maximal effect (E_{max}). Because there were no significant differences in heart rate and responsiveness to carbachol among the different wild-type mice examined (M₂WT, M₃WT, and M₂/M₃WT), data obtained with these mice were generally pooled.

We also studied the effects of several muscarinic receptor antagonists on carbachol-induced atrial responses. After obtaining control responses to carbachol, preparations were pretreated with the antagonist, and then carbachol concentration-response curves were constructed in the presence of the antagonist. Antagonist dissociation constants (pK_b) were determined by using the following equation: pK_b = log(CR - 1) - log[antagonist], where [antagonist] denotes the molar concentration of the antagonist, and CR is the ratio of the EC₅₀ value of carbachol in the presence of the antagonist divided by that in the absence of the antagonist.

To examine the involvement of pertussis toxin-sensitive G proteins in the carbachol-induced negative chronotropic and negative inotropic actions, DDY mice were injected with pertussis toxin (300 µg/kg i.p.) 96 h before experiments. Treatment with pertussis toxin did not cause any obvious behavioral changes or toxic effects. After 96 h, atria were isolated, and the inotropic and chronotropic responses to carbachol were investigated and compared with those of control atria from nonpertussis toxin-treated DDY mice.

Analysis of the mRNA Expression of Muscarinic Receptor Subtypes. Isolated cardiac ventricles and atria from DDY mice were cut into small pieces and were immediately immersed in RNAlater (RNA stabilization solution; Takara, Kyoto, Japan) for 12 h at 4°C and then stored at -30°C until use. Total RNA was extracted by the acid-guanidine-phenol-chloroform method (TRIZOL reagent; Invitrogen, Carlsbad, CA). For quantitative analysis of muscarinic receptor mRNA expression levels, real-time PCR was conducted using SYBR Green. All primer pairs used were custom-synthesized, and their

sequences were 5'-TCAGGACTCCTCTGGCTTC-3' (forward) and 5'-CCGGGTTTCACTCTCTGTCT-3' (reverse) for the M₁ receptor (GenBank NM_007698); 5'-CCGGTGTCTCCAGTCTAGT-3' (forward) and 5'-CAGACGTGGAGTCATTGGAG-3' (reverse) for the M₂ receptor (GenBank NM_203491); 5'-ACCAAGCTACCCTCCTCAGA-3' (forward) and 5'-GACAGTTGTCACGGTCATCC-3' (reverse) for the M₃ receptor (GenBank NM_033269); 5'-ATGGTGTTCATTGCGCAGT-3' (forward) and 5'-GACTGTCTGCAACTGCCTGT-3' (reverse) for the M₄ receptor (GenBank NM_007699); and 5'-CGATCATGATGCAGCCCTCT-3' (forward) and 5'-GACTGTCTGCAACTGCCTGT-3' (reverse) for the M₅ receptor (GenBank NM_205783). PCR amplification was carried out in a total volume of 20 μ l containing Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The assay was performed using an Opticon Chromo 4 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA), and the PCR cycling program consisted of 2 min at 50°C, 2 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 61.4°C. Plate reading was carried out at 61.4°C. Melting curve analysis after PCR amplification confirmed the specificity of the primers by detection of a single PCR product of the expected size on 2% agarose gels. PCR reactions were carried out in duplicate in 96-well plates. Standard curves were obtained using PCR fragments that had been isolated using a PCR purification kit, resuspended in 10 mM Tris-EDTA buffer, pH 8.0, and quantified with a spectrophotometer to calculate cDNA concentration. Standard curves of five points consisted of 10-fold serial dilutions. Sample concentrations calculated from the standard curves were converted into copies of receptor cDNA per 1 μ g of RNA.

Chemicals. The following chemicals were used: atropine sulfate (Wako Pure Chemicals, Osaka, Japan), carbamylcholine chloride (carbachol; Sigma-Aldrich, St. Louis, MO), AF-DX116 (Tocris Bioscience, Ellisville, MO), 4-diphenylacetoxy-*N*-methyl-piperidine (4-DAMP; Tocris Bioscience), *p*-fluorohexahydrosiladiphenidol hydrochloride (p-F-HHSiD; Tocris Bioscience), indomethacin (Sigma-Aldrich), and pertussis toxin (Wako Pure Chemicals). AF-DX116 and indomethacin were dissolved in dimethyl sulfoxide and diluted with distilled water. The maximum concentration of dimethyl sulfoxide in the bathing solution was kept below 0.05%, a concentration at which vehicle did not change spontaneous atrial beating frequency and EFS-induced contractions. Pertussis toxin was dissolved in sterilized saline and was given to mice (300 μ g/kg i.p.) in a volume of 0.2 ml.

Statistics. Data are expressed as means \pm S.E.M. of at least four independent experiments using left and right atria isolated from different mice. The significance of differences between two or more groups was determined by Student's *t* test (paired and unpaired) or one-way analysis of variance using post hoc Bonferroni's test. The significance of differences was determined at $p < 0.05$.

Results

Expression of Muscarinic Receptors in the Mouse Heart. We carried out real-time qRT-PCR studies to examine the expression of M₁ to M₅ muscarinic receptor mRNAs in the mouse heart. As shown in Table 1, M₂ receptor mRNA expression was predominant both in the ventricle and atrium, but M₁, M₃, M₄, and M₅ receptor mRNAs were also expressed at low levels. Total receptor mRNA expression levels were similar in atria and ventricles.

Effects of Carbachol on Spontaneously Beating Atria from Wild-Type (DDY) Mice. In spontaneously beating right atria of DDY mice (380 \pm 25 beats/min; $n = 7$), cumulative administration of carbachol (10 nM–10 μ M) caused concentration-dependent inhibition of spontaneous beating frequency and finally abolished atrial beating activity (EC₅₀ = 429 \pm 96 nM; $n = 7$) (Fig. 1). The inhibition of spontaneous beating by carbachol lasted for 15 to 20 min without recovery.

TABLE 1

Expression of muscarinic receptor mRNAs in the mouse atrium (left and right) or ventricle studied by real-time qRT-PCR

Values are means \pm S.E.M. of four tissues from different animals. Total copies of muscarinic receptor transcripts in the atrium were not significantly different from those in the ventricle. For details, see *Materials and Methods*.

	Receptor Subtype	Copies/Microgram RNA	% Expression
Atrium	M ₁	1044 \pm 290	0.03
	M ₂	3,436,720 \pm 198,800	99.87
	M ₃	1622 \pm 1009	0.05
	M ₄	1038 \pm 133	0.03
	M ₅	902 \pm 154	0.02
Ventricle	M ₁	2433 \pm 478	0.06
	M ₂	3,416,230 \pm 883,540	99.8
	M ₃	2470 \pm 542	0.06
	M ₄	1994 \pm 858	0.05
	M ₅	1288 \pm 505	0.03

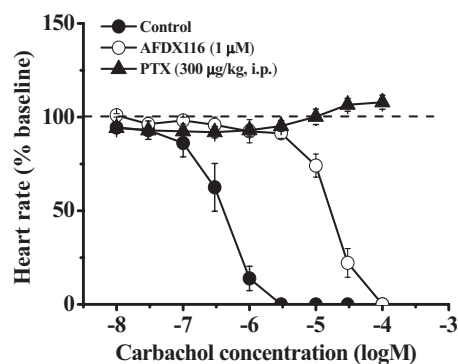


Fig. 1. Chronotropic responses to carbachol in spontaneously beating right atria of DDY mice. Concentration-chronotropic response relationships of carbachol were constructed in right atria isolated from non-treated (control; ●) and pertussis toxin-treated (PTX; ▲) wild-type mice. AF-DX116 (1 μ M) competitively antagonized the negative chronotropic response to carbachol (○). Pertussis toxin treatment did not change spontaneous beating frequency (control, 380 \pm 25, $n = 7$; pertussis toxin, 364 \pm 24, $n = 8$). Ordinate, heart rate expressed as percentage of baseline. Abscissa, carbachol concentration (log M). Symbols and vertical bars are means and S.E.M. of five to eight experiments.

AF-DX116 (1 μ M) shifted the concentration-response curves of carbachol to the right (EC₅₀ = 13,570 \pm 3640 nM; $n = 5$) without change in E_{max} values (calculated pK_b , 7.54 \pm 0.1; $n = 5$) (Fig. 1). We also examined negative chronotropic responses to carbachol in atria isolated from pertussis toxin-treated DDY mice. Pertussis toxin treatment had no significant effect on spontaneous beating frequency (364 \pm 24 beats/min; $n = 8$) but abolished the negative chronotropic responses to carbachol. The spontaneous beating frequency tended to increase at high concentrations of carbachol (10–100 μ M), but this effect was not statistically significant, compared with the control values (Fig. 1).

Effects of Carbachol on EFS-Induced Contractions of Atria from Wild-Type (DDY) Mice. Left atria from wild-type (DDY) mice were stimulated electrically via EFS, and the effect of carbachol on the magnitude of EFS-evoked contractions was examined. Carbachol (10 nM–100 μ M) inhibited EFS-induced atrial contractions in a concentration-dependent manner (Fig. 2A). The EC₅₀ and E_{max} values were 750 \pm 238 nM and 55.8 \pm 2.6% ($n = 5$), respectively (Fig. 2D). However, as shown in Fig. 2, A and C, the responses to carbachol were transient, and the amplitude of the EFS-induced contraction recovered to control levels within 7 to 10 min. E_{max} values (maximally inhibited contractions) and the magnitudes of contractions at 15 min after application of

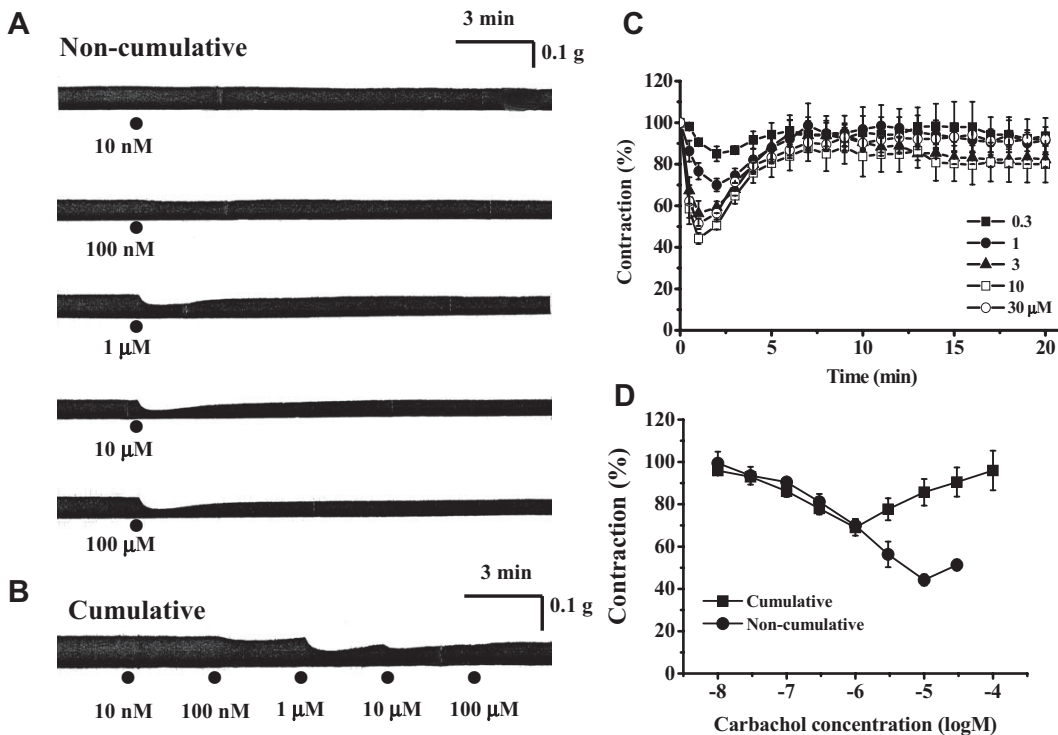


Fig. 2. Effects of carbachol on EFS-induced contractions of left atria from wild-type (DDY) mice. A, typical traces of inotropic responses to 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM carbachol applied noncumulatively. B, typical traces of inotropic response to carbachol applied cumulatively at 3-min intervals (10 nM, 100 nM, 1 μM, 10 μM, and 100 μM). C, time course of inotropic responses to carbachol (300 nM, 1 μM, 3 μM, 10 μM, and 30 μM). Ordinate, relative amplitude of EFS-induced contraction expressed as a percentage of the amplitude just before application of carbachol. Abscissa, incubation time with carbachol. D, comparison of concentration response curves for carbachol constructed from cumulative (■) and noncumulative application of carbachol (●). Ordinate, relative amplitude of EFS-induced contraction expressed as a percentage of the amplitude just before application of carbachol. Abscissa, concentration of carbachol (logM). Values are means and S.E.M. of five to six experiments.

carbachol (1–30 μM) were 76.6 ± 3.1 and $93 \pm 6.7\%$ ($n = 5$) at 1 μM, 56.3 ± 6.1 and $83 \pm 3.1\%$ ($n = 5$) at 3 μM, 44.2 ± 2.6 and $80.3 \pm 8.5\%$ ($n = 5$) at 10 μM, and 51 ± 2.5 and $93 \pm 2.4\%$ ($n = 5$) at 30 μM, respectively (Fig. 2C). Because carbachol-induced inhibition of contractile responses reached its maximum within 3 min, concentration-response curves for the negative inotropic actions of carbachol were also constructed using the results obtained from cumulative carbachol application at 3-min intervals (Fig. 2, B and D). The

concentration-inhibition relationships were virtually identical at carbachol concentrations below 1 μM, but the concentration-response curve obtained after cumulative carbachol application was not sigmoid but V-shaped at higher concentrations, probably due to slowly developed positive inotropic actions (3–30 μM) (Fig. 2D).

Strikingly, in left atria isolated from pertussis toxin-treated wild-type (DDY) mice, carbachol (1 μM–100 μM) increased the amplitude of EFS-induced contractions in a

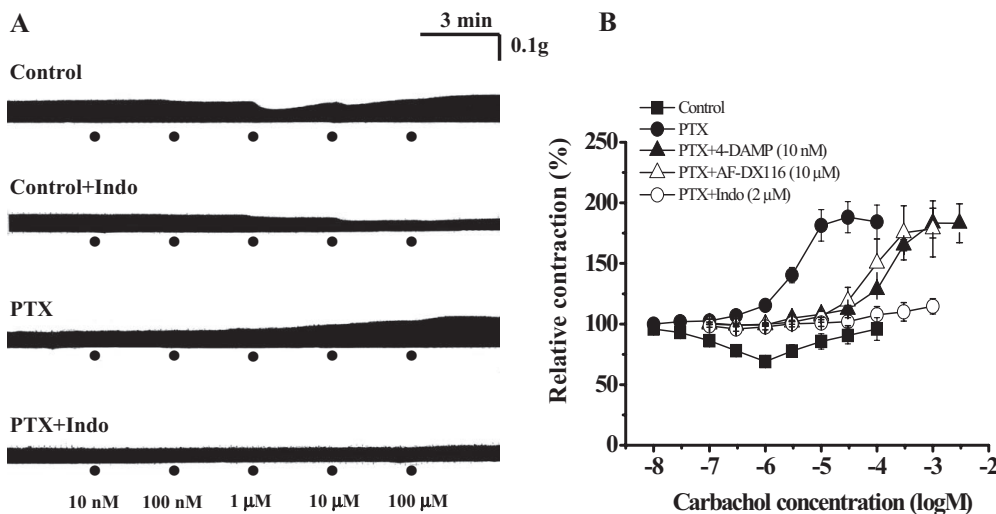


Fig. 3. Inotropic responses to carbachol in left atria of pertussis toxin-treated wild-type (DDY) mice. A, typical inotropic responses to carbachol in left atria of control and pertussis toxin (PTX) (300 μg/kg i.p., 96-h)-treated wild-type mice. In atria of pertussis toxin-treated mice, the biphasic inotropic response pattern was changed to a positive inotropic response. In both control and pertussis toxin-treated mice, the positive inotropic responses to carbachol were decreased by indomethacin (2 μM; 30 min). B, concentration-inotropic response relationships of carbachol in electrically stimulated left atria of control (■) and pertussis toxin-treated mice (●). 4-DAMP (10 nM; ▲) and AF-DX116 (10 μM; ▽) competitively antagonized the positive inotropic responses to carbachol. In atria from pertussis toxin-treated mice, indomethacin (2 μM; 30 min; ◇) virtually abolished the positive inotropic responses to carbachol. In all experiments, carbachol was applied cumulatively at 3-min intervals. Ordinate, relative amplitude of EFS-induced contraction expressed as a percentage of the contraction just before application of carbachol. Abscissa, concentration of carbachol (logM). Symbols and vertical bars are means and S.E.M. of 4 to 11 experiments.

concentration-dependent manner (Fig. 3A). The carbachol EC₅₀ and E_{max} values were $3.3 \pm 0.9 \mu\text{M}$ and $184 \pm 13.9\%$ ($n = 11$), respectively (Fig. 3). Because the positive inotropic activity of carbachol was atropine-sensitive (100 nM; data not shown), the muscarinic receptor subtype involved in this activity was initially characterized by the use of three muscarinic receptor subtype-preferring antagonists: 4-DAMP (10 nM), AF-DX116 (10 μM), and p-F-HHSiD (1 μM). All three antagonists inhibited the positive inotropic action of carbachol, shifting carbachol concentration-response curves to the right in a parallel manner without change in E_{max} values. The estimated pK_b values were 9.66 ± 0.21 ($n = 4$) for 4-DAMP, 6.28 ± 0.07 ($n = 5$) for AF-DX116, and 7.74 ± 0.16 ($n = 4$) for p-F-HHSiD (Fig. 3B). These pK_b values were consistent with those for affinity to M₃ muscarinic receptor (4-DAMP, 8.9–9.3; AF-DX116, 5.9–6.6; p-F-HHSiD, 7.8–8.2) (Dhein et al., 2001).

The positive inotropic responses to carbachol observed with atria from pertussis toxin-treated mice were nearly abolished by indomethacin (2 μM for 30 min) (Fig. 3). Indomethacin (2 μM for 30 min) also decreased the positive inotropic activity of carbachol in atria from nonpertussis toxin-treated control mice, and the biphasic inotropic responses were changed to a monophasic negative inotropic response (Fig. 3A).

Chronotropic and Inotropic Effects of Carbachol on Atria from Muscarinic Receptor KO Mice. The effects of carbachol on spontaneously beating right atria were compared between atria obtained from wild-type, M₂KO, M₃KO, and M₂/M₃KO mice. Spontaneous atrial beating frequencies (beats/min) were similar for wild-type mice (378 ± 22 ; $n = 5$) and muscarinic receptor KO mice (M₂KO: 371 ± 36 , $n = 7$; M₃KO: 420 ± 32 , $n = 5$; and M₂/M₃KO: 385 ± 35 , $n = 5$). Carbachol concentration-dependently decreased spontaneous beating frequency of atria from wild-type and M₃KO mice and finally abolished spontaneous beating at 10 to 30 μM (Fig. 4). The carbachol EC₅₀ values were $1.4 \pm 0.53 \mu\text{M}$ ($n = 5$) for wild-type and $1.2 \pm 0.39 \mu\text{M}$ ($n = 5$) for M₃KO preparations, respectively. Conversely, in atria from M₂KO mice,

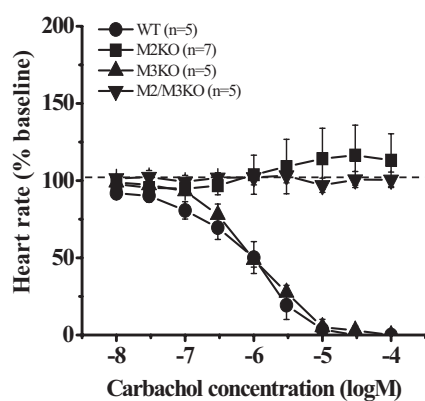


Fig. 4. Comparison of chronotropic responses to carbachol in isolated right atria from wild-type and muscarinic receptor KO mice (M₂KO, M₃KO and M₂/M₃KO). Heart rates (beats/min) of isolated atria were 378 ± 22 (wild-type; $n = 5$), 371 ± 36 (M₂KO; $n = 7$), 420 ± 32 (M₃KO; $n = 5$), and 385 ± 35 (M₂/M₃KO; $n = 5$). Increasing concentrations of carbachol (10 nM, 30 nM, 100 nM, 300 nM, 1 μM , 3 μM , 10 μM , 30 μM , and 100 μM) were applied cumulatively at 3-min intervals, and concentration-response curves were constructed. Ordinate, heart rate expressed as percentage of baseline just before application of carbachol. Abscissa, concentration of carbachol (logM). Symbols and vertical bars are means and S.E.M. of five to seven experiments.

the negative chronotropic actions of carbachol were abolished. Carbachol seemed to slightly increase heart rate at high concentrations (30–100 μM), but this effect was not statistically significant (Fig. 4). Carbachol had no effect at all on the spontaneous beating frequency of right atria from M₂/M₃KO mice (Fig. 4).

We next compared the inotropic responses to carbachol in left atria from wild-type and muscarinic receptor KO mice. Carbachol inhibited the EFS-induced contraction of wild-type atria in a concentration-dependent manner, but the inhibition was transient, as observed with atrial preparation from DDY mice (Figs. 5 and 6A). The EC₅₀ and maximal inhibition were $700 \pm 110 \text{ nM}$ and $58 \pm 3.4\%$ ($n = 6$), respectively, when carbachol was applied in a noncumulative manner (Figs. 5 and 6A). In isolated atria from M₃KO mice, carbachol caused negative inotropic actions with similar potency and efficacy as observed with wild-type atria (EC₅₀ and maximal inhibition: $1.5 \pm 0.5 \mu\text{M}$ and $56 \pm 3.7\%$, respectively; $n = 5$) (Fig. 6A). However, in atria from M₃KO mice, the inhibition was not transient but sustained (Figs. 5 and 6B).

Although the maximal inhibition by carbachol (30 μM) was almost the same in wild-type and M₃KO atria (minimum amplitude of EFS-induced contractions: $46 \pm 3.9\%$ for wild-type mice, $n = 6$; $45 \pm 5.5\%$ for M₃KO mice, $n = 5$; $p = 0.95$), the relative amplitudes of contraction were significantly reduced in M₃KO atria at 5, 10, 15, and 20 min after application of carbachol (Fig. 6B). The following values were obtained in wild-type and M₃KO atria: 63.1 ± 5.1 and $50 \pm 4.5\%$ (5 min; $p = 0.09$), 73 ± 6.0 and $54 \pm 4.2\%$ (10 min; $p = 0.028$), 72.4 ± 5.4 and $56 \pm 4.5\%$ (15 min; $p = 0.038$), and 75 ± 5.6 and $55 \pm 5.5\%$ (20 min; $p = 0.04$), respectively (Fig. 6B). Alternatively, carbachol did not decrease the EFS-induced contraction in atria from M₂KO mice but significantly increased the EFS-induced contraction in a concentration-dependent manner (EC₅₀ = $3.7 \pm 1.6 \mu\text{M}$; maximal response obtained at 100 μM , $119 \pm 2.1\%$; $n = 5$) (Fig. 6A). This positive inotropic action reached a peak within 3 to 5 min after carbachol application and then declined slowly (Fig. 6B). Carbachol had no effect at all on the EFS-induced contraction of atria from M₂/M₃KO mice (Figs. 5 and 6).

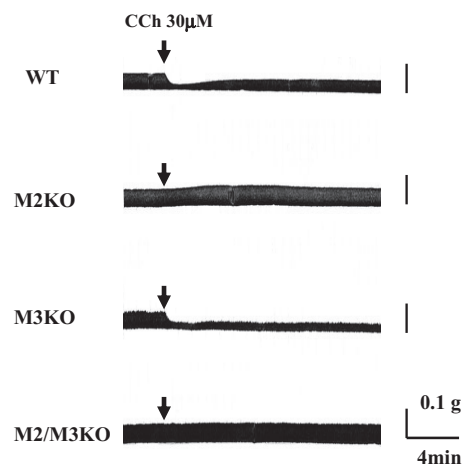


Fig. 5. Typical traces of inotropic responses to carbachol in left atria isolated from wild-type (WT), M₂KO, M₃KO, and M₂/M₃KO mice. After establishment of steady EFS-induced contractions, carbachol (30 μM) was applied as indicated by arrows.

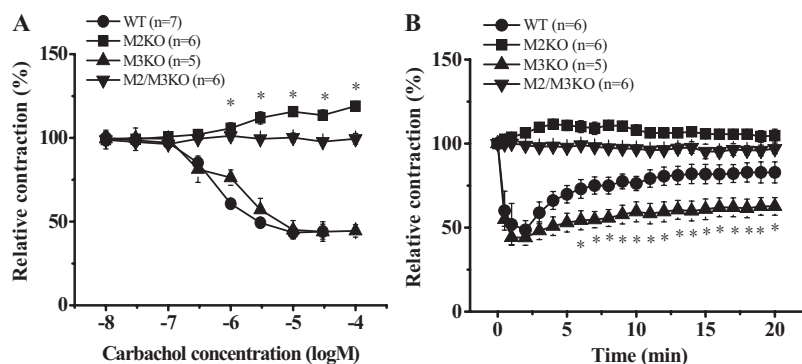


Fig. 6. Comparison of inotropic responses to carbachol in left atria isolated from wild-type (WT), M_2 KO, M_3 KO, and M_2/M_3 KO mice. A, after establishing reproducible EFS-induced atrial contractions, carbachol (10 nM–100 μ M) was applied noncumulatively at 30-min intervals, and concentration-response relationships were constructed. The asterisk (*) indicates significant increase compared with the values before application of carbachol. B, time courses of inotropic responses to carbachol in left atria isolated from WT, M_2 KO, M_3 KO, and M_2/M_3 KO mice. After stable EFS-induced contractions had been obtained, carbachol (30 μ M) was applied to the organ bath, and inotropic responses were observed for 20 min. Ordinate, relative amplitude of EFS-induced contraction expressed as a percentage of the amplitude just before application of carbachol. Abscissa, concentration of carbachol (log M) (A) and incubation time with 30 μ M carbachol (min) (B). Values are means and S.E.M. of five to seven experiments. B, the asterisk (*) indicates significantly different compared with WT values.

Discussion

It is well documented that muscarinic agonists can induce both negative and positive inotropic responses in cardiac atria (Eglen et al., 1988; Du et al., 1995; Nishimaru et al., 2000; Tanaka et al., 2001). Overwhelming evidence indicates that the negative inotropic effects are mediated by the M_2 muscarinic receptor subtype (Caulfield and Birdsall, 1998; Dhein et al., 2001). However, the nature of the muscarinic receptor subtype(s) mediating the positive inotropic activity of muscarinic agonists is a matter of argument and remains to be identified.

We demonstrated, by using real-time qRT-PCR expression analysis, that the M_2 receptor mRNA is the predominant muscarinic receptor transcript found in mouse atria and ventricles but that the M_1 , M_3 , M_4 , and M_5 receptor subtypes are also expressed at low levels in these tissues. This observation is consistent with the outcome of several previously published studies (Hardouin et al., 1998; Hellgren et al., 2000; Krejci and Tucek, 2002; Wang et al., 2001; Pérez et al., 2006). Functional studies with right atria prepared from different muscarinic receptor knockout mice, complemented by studies with M_2 muscarinic receptor subtype-preferring antagonists, indicated that muscarinic agonist-induced reduction in heart rate is mediated by the M_2 receptor, in agreement with previous results (Caulfield and Birdsall, 1998; Stengel et al., 2000; Dhein et al., 2001). In the present study, carbachol was unable to produce statistically significant positive chronotropic responses in right atria from M_2 KO mice and pertussis toxin-treated mice. These results suggest that the M_3 receptor lacks chronotropic activity in right atria of mice under the experimental conditions used (360–380 beats/min).

In left atria from wild-type mice, carbachol inhibited EFS-induced contractions followed by a gradual increase in contraction amplitude. After *in vivo* pertussis toxin treatment of wild-type mice, carbachol did not show any negative inotropic activity in atrial preparations but only positive inotropic responses. This observation indicated that the positive inotropic effects of carbachol are mediated by either M_1 , M_3 , or M_5 receptors (or a combination of two or all of these receptors), which couple to pertussis toxin-insensitive G proteins (G proteins of the $G_{q/11}$ family). Studies with muscarinic

receptor subtype-preferring antagonists suggested that the positive inotropic effects of muscarinic agonists in mice may be mediated by the M_3 receptor (Nishimaru et al., 2000; Tanaka et al., 2001). However, the antagonists used in these studies show a rather limited degree of receptor subtype selectivity (M_3 and M_5 receptors, e.g., show a very similar ligand binding pharmacology; Caulfield and Birdsall, 1998). Moreover, as indicated above, transcripts for all five muscarinic receptor subtypes are found in mouse atria, further complicating the interpretation of classical pharmacological studies of the roles of non- M_2 muscarinic receptor subtypes in atrial function.

To identify the molecular nature of the muscarinic receptor subtype mediating the positive inotropic effects of muscarinic agonist, we used muscarinic receptor knockout mice as novel experimental tools (Wess, 2004). In atria from M_3 KO mice, the maximal negative inotropic activity of carbachol was comparable with that found with wild-type atria. However, the time course of carbachol-mediated inotropic effects was different in atria from M_3 KO mice. The slow increase in EFS-induced contractions observed in atria from wild-type mice was significantly decreased in atria from M_3 KO mice. The biphasic inotropic effects of carbachol seen with wild-type atria were changed to an almost monophasic sustained negative inotropic response in M_3 KO atria. Conversely, in atria from M_2 KO mice, carbachol was devoid of negative inotropic activity and only induced positive inotropic effects. In atria from M_2/M_3 double KO mice, carbachol had neither positive nor negative inotropic effects. These data clearly indicate that the M_2 receptor mediates the negative inotropic action and that the M_3 receptor mediates the positive inotropic effects of carbachol in mouse atria. One possible mechanism for the observed transient negative inotropic action of carbachol in wild-type atria may involve M_2 receptor desensitization. However, in M_3 KO atria, the negative inotropic action of carbachol was sustained for 20 min, suggesting that M_2 receptor desensitization does not play a significant role under the experimental conditions used in this study.

At present, it remains unclear whether the M_3 muscarinic receptors mediating the positive inotropic effects of muscarinic agonists are localized on cardiomyocytes or on other

cardiac cell types such as neurons or endothelial cells (note that the samples for real-time qRT-PCR were obtained from whole atria or ventricles in the present study). An immunohistochemical study indicated that M₃ receptors are present on cardiomyocytes (Wang et al., 2001). In addition, M₃ receptor-mediated increases in inositol phosphate formation have been observed with dispersed cardiomyocytes (Pönicke et al., 2003; Myslivecek et al., 2008). Alternatively, because M₃ receptor-mediated positive inotropic effects were abolished by treatment with 1% Triton X-100 or indomethacin, it has been proposed that M₃ receptors present on the endocardial endothelium stimulate the production of endogenous prostaglandins eliciting positive inotropic actions (Tanaka et al., 2001). Hassall et al. (1993) reported the expression of M₁ to M₄ receptor mRNA in intrinsic neurons of rat and guinea pig hearts. In the present study, we demonstrated that indomethacin inhibited the positive inotropic actions of carbachol in atria from pertussis toxin-treated mice, in agreement with a previous report (Tanaka et al., 2001). Clearly, however, more detailed studies are needed to clarify the precise localization of the M₃ muscarinic receptors mediating the positive inotropic actions in the mouse atrium.

Muscarinic receptor agonists are known to cause positive inotropic effects in several species (rat, guinea pig, mouse, human, and chicken). It has been demonstrated that the M₁ receptor can stimulate L-type Ca²⁺ currents in guinea pig ventricular cells (Gallo et al., 1993) and Ca²⁺ release in rat ventricular cells (Sharma et al., 1996), causing positive inotropic effects. The M₁ receptor has also been implicated in mediating positive inotropic responses in isolated human (Du et al., 1995) and chicken myocardium (Nouchi et al., 2007). Conversely, Protas et al. (1998) reported that the M₂ receptor, the same receptor subtype mediating inhibition of cardiac contractility, caused positive inotropic effects through activation of Na⁺-Ca²⁺ exchange and myofilament Ca²⁺ sensitivity in guinea pigs. In the present study, we clearly demonstrated that the M₃ receptor mediates the positive inotropic action of carbachol in mouse atria. Taken together, these findings suggest that the molecular nature of the muscarinic receptor subtypes mediating cardiac positive inotropic effects and the underlying mechanisms may differ from species to species.

In conclusion, consistent with previous studies, we found that the negative chronotropic and negative inotropic actions of muscarinic receptor agonists in mouse atria are mediated by the M₂ muscarinic receptor. In addition, we provide unambiguous evidence, by using atria prepared from muscarinic receptor knockout mice, that the positive inotropic actions of muscarinic agonists are mediated by the M₃ muscarinic receptor subtype. We speculate that this activity serves to counteract or dampen the inhibitory inotropic effects observed after M₂ receptor activation in cardiac atria.

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