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Excitatory Effect of M_1 Muscarinic Acetylcholine Receptor on Automaticity of Mouse Heart

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We have investigated the effects of relatively high concentration of carbachol (CCh), an agonist of muscarinic acetylcholine receptor (mAChR), on cardiac automaticity in mouse heart. Action potentials from automatically beating right atria of mice were measured with conventional microelectrodes. When atria were treated with 100 μM CCh, atrial beating was immediately arrested and diastolic membrane potential (DMP) was depolarized. After exposure of the atria to CCh for ~4 min, action potentials were regenerated. The regenerated action potentials had lower frequency and shorter duration when compared with the control. When atria were pre-exposed to pirenzepine (1 μM), an M_1 mAChR antagonist, there was complete inhibition of CCh-induced depolarization of DMP and regeneration of action potentials. Pre-exposure to AFDX-116 (11({2-[(diethylamino)-methyl]-1-piperidyl}acetyl)-5,11-dihydro-6*H*-pyridol[2,3-b][1,4] benzodiazepine-6-one base, 1 μM), an M_2 mAChR antagonist, failed to block CCh-induced arrest of the beating. However, prolonged exposure to CCh elicited gradual depolarization of DMP and slight acceleration in beating rate. Our data indicate that high concentration of CCh depolarizes membrane potential and recovers right atrial automaticity *via* M_1 mAChR, providing functional evidence for the role of M_1 mAChR in the atrial myocytes.

Key words: Muscarinic acetylcholine receptor, Automatic action potential, Mouse atrium, Carbachol

INTRODUCTION

Stimulation of parasympathetic postganglionic neurons in the heart causes the release of acetylcholine (ACh), that acts on muscarinic ACh receptors (mAChRs) localized on the cardiac myocyte membrane. The mAChRs mediate a variety of cellular responses, including inhibition of adenylate cyclase, increased breakdown of phosphoinositide, and modulation of K^+ and Ca^{2+} channels (Hartzell, 1988; Schimerlik, 1989; Hosey, 1992; Caulfield, 1993). The diverse effects of mAChR activation elicit both inhibitory and stimulatory effects in the heart (Korth and Kühlkamp 1985; Gilmour & Zipes, 1985; Pappano, 1991; Caulfield, 1993). The negative inotropic and chronotropic effects of cholinergic agonists are observed only at lower concentrations (<10 μM) whereas the positive inotropic effect is observed at higher concentrations (Korth and Kühlkamp, 1985; Kohl *et al.*, 1990; Gilmour & Zipes, 1985). It is assumed that the dual effects of mAChR activation in cardiac ventricular muscles may be due to the presence of multiple subtypes of mAChRs.

Because of limited selectivity of the current pharmacological agents to distinguish one subtype of mAChR in the presence of others, classification of the mAChR subtypes present in heart is still controversial. Nevertheless it is thought that major subtype of mAChR which is expressed in the cell membrane of mammalian ventricular myocytes is M_2 mAChR. Activation of M_2 mAChRs inhibits the activity of adenylate cyclase, closes Ca^{2+} channels, lowers the hyperpolarization-activated pacemaker current, and activates an inwardly rectifying potassium channel (Brann *et al.*, 1993; Caulfield, 1993). These changes lead to both negative chronotropic and inotropic effects on the heart (Caulfield, 1993). Another major mAChR subtype which is expressed in the ventricular myocytes is M_1 mAChR (Watson *et al.*, 1983; Sharma *et al.*, 1996). The M_1 mAChRs were considered to be responsible for the

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stimulatory effects on Ca^{2+} transients in rat ventricular myocytes in the presence of high concentrations of CCh (>10 μM, Sharma *et al.*, 1996). It has also been reported that the rate of spontaneous Ca^{2+} transients occurring in cultured neonatal rat ventricular myocytes was accelerated by high concentration of CCh *via* the activation of M_1 mAChRs (Colecraft *et al.*, 1998).

Expression of multiple subtypes of mAChRs in cardiac *atrium* is somewhat inconsistent and is different amongst species. In human atrial myocytes, all five subtypes (M_1-M_5) have been detected by RT-PCR (Wang *et al.*, 2001). In canine atrial myocytes, M_2, M_3, and M_4 were identified by RT-PCR (Shi *et al.*, 1999). In mouse atria, one group of researchers has identified five subtypes of mAChRs by RT-PCR and have reported much stronger expressions of M_2 and M_1 mAChRs (Cho *et al.*, 2002) while another group detected only M_2 subtype by using the same technique (Hardouin *et al.*, 2002). Despite the varying identification of mAChR subtypes between species and among reports, functional evidence of each subtype except M_2 receptor is largely limited in intact atrial cells. There are previous reports which have stated that M_3 and M_4 receptors regulate different K^+ channels in the canine atrial myocytes (Shi *et al.*, 1999), and also that activation of M_1 mAChR reduces maximum upstroke velocity of action potential in mouse atrium (Islam *et al.*, 1998). However there is no report on the role of M_1 mAChRs in the regulation of electrical automaticity in the atrium.

In the present study, we have investigated the effects of relatively high concentration of CCh (100 μM) on automatic membrane potentials of right atria and beating rate in mice. We observed that CCh induces depolarization of diastolic membrane potential (DMP) and resumption of automaticity *via* activation of M_1 mAChRs.

MATERIALS AND MERHODS

Tissue preparation and superfusion

Mice were anesthetized with 25 mg kg^{-1} of *i.p.* pentobarbital. After a surgical level of anesthesia was confirmed, a thoracotomy was performed, the heart was removed, and the animal was killed by exsanguination. All the studies were carried out according to institutional guidelines and were approved by the institutional animal care. A small region (width, about 1 mm; length, about 2 mm) of the right atrium containing sino-atrial node was dissected from the heart of mouse. The tissue strip was mounted on a chamber with volume of 200 μL which was located on the inverted microscope (Nikon, Japan). The tissue was then continuously superfused with 4-(2-hydroxyethyl)-1-piperazine-ethansulphonic acid (HEPES) Tyrode solution (see below for composition) at a rate of 2.5 mL min^{-1}. Extracellular solution was exchanged by miniature solenoid valves (LFAA1201618H, Lee Products, Ltd., Bucks, U.K.). All the experiments were carried out at 37°C.

Measurement of automatic membrane potentials

Conventional microelectrodes were made with thin-wall filamented glass capillaries by using a horizontal puller (PD-5, Narishige, Tokyo, Japan). The electrodes were filled with 300 mM KCl and possessed a resistance of 45-55 MΩ. After compensating for electrode resistance and capacitance, the microelectrode was inserted into single atrial myocyte. Membrane potential was amplified by appropriate amplifier, Axoclamp 2A (Axon Instruments, Foster City, Calif., U.S.A.). The amplified signals were recorded on a chart recorder (Model 30-V7412-11; Gould Electronics Ltd., U.S.A.) or were digitized by computer software ("WCP", written and supplied by John Dempster of Strathclyde University) *via* A/D converter (CED 1401; Cambridge Electronic Design, Cambridge, U.K.). Although nodal action potentials, exhibiting a spontaneous depolarization, were sometimes detected, they were not included for analysis.

Solutions

Normal Tyrode solution was comprised of (in mM) NaCl 140, HEPES 10, glucose 10, KCl 4.4, $CaCl_2$ 1.8, $MgCl_2$ 1, titrated to pH 7.4 with NaOH. CCh and pirenzepine were purchased from Sigma (St. Louis, MO, U.S.A.). AFDX-116 was obtained from TOCRIS (Ballwin, MO, U.S.A.). One molar stock solutions of CCh or AFDX-116 (11({2-[(diethylamino)-methyl]-1-piperidyl}acetyl)-5,11-dihydro-6*H*-pyridol [2,3-b][1,4]benzodiazepine-6-one base) were prepared in dimethyl sulpfoxide (DMSO). The stock solutions were diluted in Tyrode solution to attain the final concentration of drug. Pirenzepine was initially dissolved in Tyrode solution as a stock solution, which was diluted to obtain the final concentration.

Statistics

The results in the text and in the figures are presented as means ± standard error (SE). Statistical analyses were performed by using the Student's *t*-test. The difference between two groups was considered to be significant when $P < 0.05$.

RESULTS

Effect of CCh on automatic action potentials in mice atria

In ventricle, it is known that a relatively high concentration (>10 μM) of ACh generates M_1 receptor-mediated positive inotropic effect (Korth & Kühlkamp, 1985). To choose the optimal conditions for the experiments (using *atrium*) we treated the mice atrial preparations with CCh for 0.5-1 min

at concentrations ranging from 10 nM to 500 μM. Low dose (10 nM-1 μM) of CCh produced hyperpolarization, negative chronotropic effect and shortening of action potential duration (APD) in a dose dependent manner, which is a well-known M_2 mAChR-mediated effect (Brann *et al.*, 1993; Caulfield, 1993). Relatively high concentration of CCh (10 μM) induced an arrest of beat within approximately 10 s after the application, although the time taken for arresting the beat was varied depending on preparations. Concentrations of CCh higher than 50 μM elicited an immediate arrest of the spontaneous beating in most of the preparations and 100 μM CCh induced an immediate arrest of beating in all of the atria. Thus, we chose 100 μM CCh for consistent observation of the immediate arrest of the beat, and carried out experiments to examine possible stimulatory effect of CCh on atrial automaticity.

Fig. 1 illustrates the representative effects of muscarinic stimulation by CCh on automatic action potential (V_m) in beating mouse right atrial myocyte. The exposure to 100 μM CCh induced an immediate arrest of action potential and depolarization of DMP (Fig. 1a; 6.2 ± 0.4 mV, n = 12, $P < 0.05$). The CCh-induced depolarization was preceded by slight transient hyperpolarization in some of the tested cells. Interestingly, approximately 4 min after the onset of CCh exposure, automatic action potentials were resumed in the continued presence of CCh. The regeneration of action potentials appeared to be associated with membrane depolarization, which was seen at intervals of every 20-40 seconds (Fig. 1a). The oscillatory reoccurrence of action potentials became regular with longer treatment of CCh (>10 min).

The regenerated action potentials had lower frequency and shorter duration (Fig. 1b-ii & -iii) when compared with the control action potentials (Fig. 1b-i). Table I summarizes the average effects of CCh on beating rate and the properties of action potentials. When the action potential

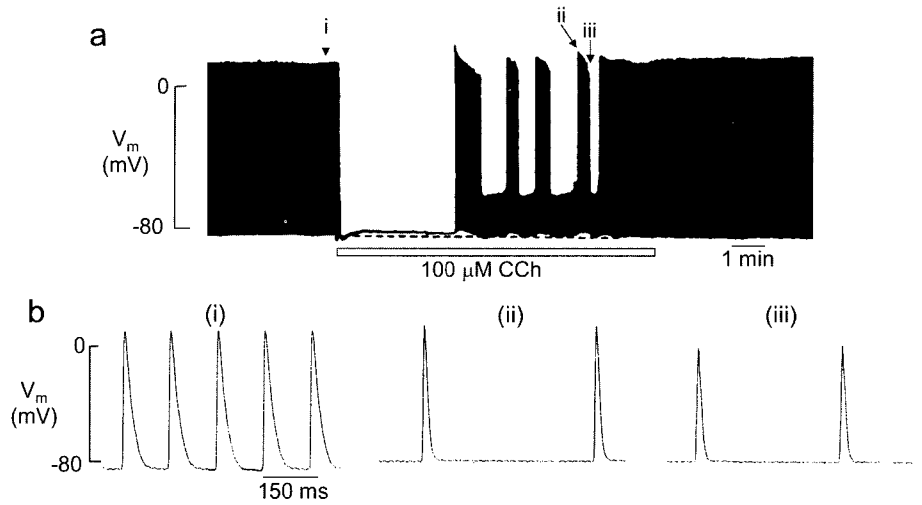

Fig. 1. Effects of 100 μM CCh on automatic action potentials in mouse atrium. (a) Continuous recording of action potentials (V_m). The base line of control diastolic action potential is indicated by *dashed line*. (b) Expanded traces of action potentials (*i*, *ii*, and *iii*), recorded at the corresponding time points in the panel (a).

Table I. Comparisons of the properties of automaticity and action potential parameters in the absence or presence of drug interventions in mouse right atrial tissue

Treatment	Control					CCh (100 μM)				
	beating rate (beats min^{-1})	APD_{90} (ms)	APD_{50} (ms)	APA (mV)	n	beating rate (beats min^{-1})	APD_{90} (ms)	APD_{50} (ms)	APA (mV)	n
none	442 ± 23	81 ± 6.9	22 ± 2.5	88 ± 2.3	12	113 ± 17**	27 ± 7.6**	7.5 ± 1.3*	89 ± 1.9	12
pirenzepine										
(100 nM)	459 ± 28	76 ± 7.9	20 ± 3.0	87 ± 4.6	7	0	(no AP)	(no AP)	(no AP)	7
(1 μM)	437 ± 30	71 ± 9.0	19 ± 5.4	88 ± 3.2	3	0	(no AP)	(no AP)	(no AP)	3
AFDX-116										
(1 μM)	444 ± 25	79 ± 8.1	21 ± 5.1	84 ± 6.4	6	263 ± 17*	71 ± 4.7	19 ± 5.7	88 ± 6.0	6

Data are expressed as mean ± SE. "*n*" indicates number of trials. APD_{90}, APD_{50}, and *APA* indicate action potential duration at 90% repolarization, action potential duration at 50% repolarization and action potential amplitude, respectively. Effects of CCh (100 mM) were evaluated at intervals of 10 min following the onset of treatment. * $P < 0.05$, ** $P < 0.01$ were compared with corresponding values in the control (prior to the application of CCh).

frequency became regular after the treatment with CCh for about 10 min, beating rate was ~4-fold slower than the control rate, and APD at 90 (APD_{90}) and 50% (APD_{50}) repolarization were reduced by about 65% (Table I). Although amplitudes of the regenerated action potentials (APA) were somewhat varying in the beginning (Fig. 1b-iii), they were not significantly different when stabilized after ~10 min-exposure to CCh, when compared with the control (Table I). After withdrawal of CCh from the bath solution, the beating rate and shape of the action potentials were fully recovered.

M_1 mAChR blockade removes CCh-induced excitatory effects

We further examined a possibility that the CCh-mediated excitatory effects were mediated by M_1 mAChRs in mouse atrial tissue. Fig. 2 shows the effects of 100 μM CCh on automatic action potentials in mouse atrium, which were pre-incubated with pirenzepine (1 μM, an antagonist of M_1 mAChR) for 20 min. When tissues were pre-treated with pirenzepine before the second application of CCh, the configuration of action potential and beating rate of right atria were not significantly changed (Table I). In the presence of pirenzepine, the exposure to CCh stopped heartbeat, and hyperpolarized DMP by 11 ± 1.7 mV (n = 7; Fig. 2). However, pre-exposure to pirenzepine completely antagonized the CCh-induced depolarization and the recovery of heartbeat (Fig. 2), indicating that CCh brings about excitatory effects on the automatic action potentials via the activation of M_1 mAChR.

No effect of M_2 mAChR inhibition on CCh-induced stimulatory action

To determine whether the CCh-induced excitatory effect on atrial automaticity is independent of the action of M_2 mAChR; we tested the effects of CCh in atria, which werepre-treated with an M_2 mAChR antagonist, AFDX-116. When 1 μM AFDX-116 was applied for 20 min before adding CCh (100 μM), there were no significant changes in the action potential parameters (Table I). Pre-exposure to AFDX-116 inhibited CCh-mediated arrest of action potential (Fig. 3), confirming the previous idea that CCh-induced arrest of heartbeat is mediated by M_2 mAChR (Levy & Martin, 1984). However, M_2 mAChR-mediated hyperpolarization of DMP (11 ± 1.8 mV, n = 6) and decrease in the beating rate (Table I; Del Castillo & Katz, 1955; Toda & West, 1965) were still observed (Fig. 3a and Fig. 3b-ii), suggesting incomplete inhibition of M_2 mAChRs by 1 μM AFDX-116. Reduction in APD_{90} and APD_{50} by CCh was antagonized by the presence of 1 μM AFDX-116 (Fig. 3b, Table I). It should be noted that exposure to

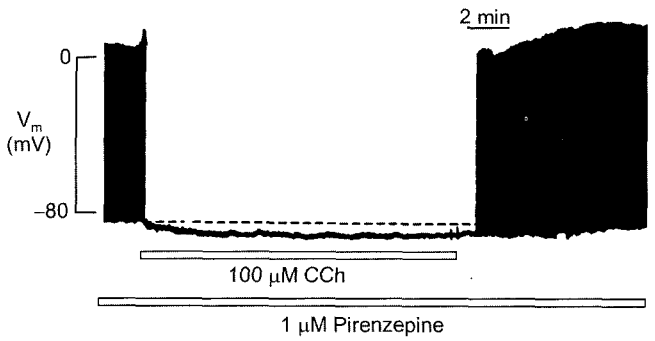

Fig. 2. Effect of M_1 mAChR antagonist on the response of atrial cell to CCh. Representative automatic action potentials (V_m) were continuously measured in mouse atrial cell by using conventional microelectrode. When mouse atria were pre-treated with pirenzepine (1 μM), an M_1 mAChR antagonist, the CCh-induced membrane depolarization and regeneration of automaticity were completely inhibited.

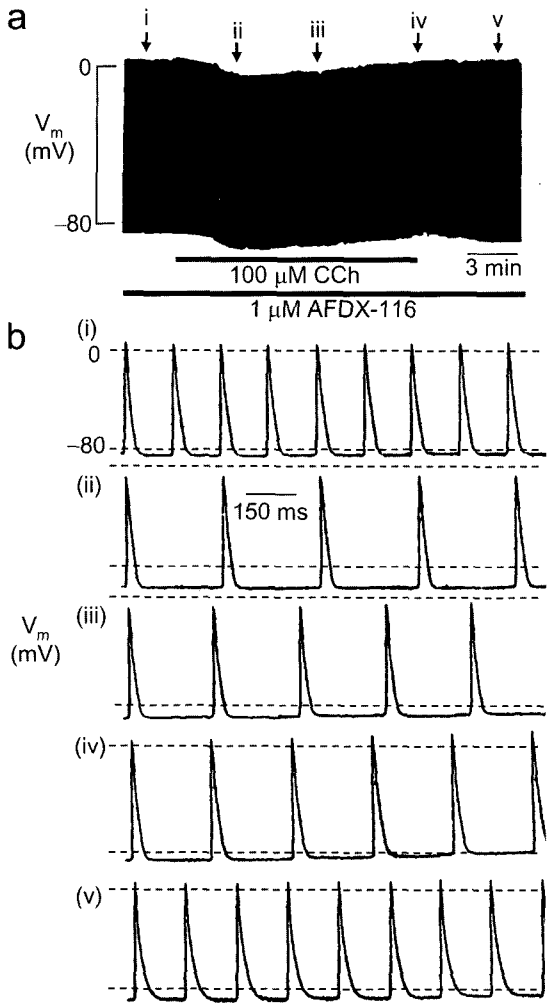

Fig. 3. Effect of M_2 mAChR antagonist on the atrial cell responses to CCh. (a) Continuous recording of automatic action potentials (V_m) in mouse right atrial cell. When atrial tissue was pre-exposed to AFDX-116 (1 μM), an M_2 mAChR antagonist, CCh failed to arrest heartbeat but still produced gradual depolarization (iii and iv) following the first hyperpolarization (ii). (b) Expanded recordings of action potentials measured at the times (i-v) as indicated in the panel (a).

>1 μM AFDX-116 usually induced unstable recordings of the action potentials (gradual depolarizations). A closer analysis of the action potential traces revealed that CCh produced biphasic changes in the diastolic membrane potential in AFDX-116 pre-treated tissue: hyperpolarization followed by gradual depolarization (1.05 ± 0.2 mV min^{-1}; Fig. 3a, n = 6). In addition, beating was gradually accelerated with the depolarization of DMP (Fig. 3b-iii). The data indicates that the excitatory effects of CCh on action potentials may not be directly related to M_2 mAChR signaling.

DISCUSSION

In this paper, we have shown that activation of mAChRs with relatively high concentration of CCh produced depolarization of diastolic membrane potentials, late resumption of automatic action potentials, and immediate arrest of heartbeat in mice atria. The CCh-induced membrane depolarization and the late recovery were specifically antagonized by the blockade of M_1 mAChRs, but not M_2 AChRs. These results provide functional evidence for the M_1 mAChR in mouse atrial myocytes and on the role of M_1 mAChR in mediating the regulation of membrane potential and automaticity in the heart.

In the present study we observed that high concentration of CCh produces M_1 mAChR-mediated regeneration of automatic action potentials within 4 min and also even in its continued presence (Fig. 1). This result is novel and clearly distinct with the previous reports on the effects of M_1 mAChR activation on cardiac functions. Previous studies with anti-sense oligonucleotides specific for M_1 mAChRs suggested a role for M_1 receptors in the CCh-induced increases of spontaneous Ca^{2+} transients in cultured neonatal ventricular myocytes (Colecraft *et al.*, 1998). It should be noted that the acceleration of Ca^{2+} transient rate does not fully account for acceleration of action potential rate, since Ca^{2+} transients could be induced without action potentials (Jaconi *et al.*, 2000).

The M_1 mAChR-mediated regeneration of automaticity appeared to be associated with membrane depolarization (Fig. 1). The depolarization of diastolic membrane potentials induced by CCh was simultaneously observed with the arrest of heartbeat. The CCh-induced arrest of heartbeat appeared to be mediated by M_2 mAChR, since the effect was inhibited by M_2 receptor antagonist (Fig. 3). The M_2 mAChR-mediated inhibition of automaticity may be caused by inhibition of Ca^{2+} channels and hyperpolarization-activated pacemaker channels, and by activation of inwardly rectifying potassium channels (Brann *et al.*, 1993; Caulfield, 1993). Specific mechanism for the M_1 mAChR-mediated depolarization still remains unclear. One possible mechanism to explain the CCh-elicited depolarization may be activation of phospholipase C, linked to M_1 mAChR (Kim *et al.*, 1997), which requires further investigation. It has been recently reported that atrial G-protein gated inwardly rectifying K^+ currents evoked by ACh *via* M_2 muscarinic receptor (I_{KACh}) are inhibited by depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2, Cho *et al.*, 2001; Meyer *et al.*, 2001).

The present data provides functional evidence on the previously detected M_1 mAChR in isolated mouse atrial myocytes (Cho *et al.*, 2002). However, our data is not consistent with a previous report on the absence of biochemical evidence for the M_1 mAChR in the mouse atrial tissue (Hardouin *et al.*, 2002). The reason for the inconsistent detection of the receptor subtypes in the same species is not clear. One of the experimental difference between the two previous reports is that the latter study was carried out with whole heart tissues, and not with isolated cardiac myocytes.

The action potentials, regenerated by the long exposure to CCh, showed significantly shorter duration and lower frequency (Table I). Interestingly, however, the reoccurring action potentials became regular in the continued presence of CCh. The CCh-induced action potential shortening was suppressed by the M_2 mAChRs antagonist (Fig. 3), suggesting a continued activation of M_2 mAChRs. The increase in the rate of repolarization of action potential was most likely to be caused by the M_2 receptor-mediated activation of K^+ channels (Kubo *et al.*, 1993). In the presence of M_2 mAChR antagonist, it was observed that CCh slightly increased APD with membrane depolarization (Fig. 3, detailed data not shown). The observation is somewhat consistent with the previous report that CCh enhances L-type Ca^{2+} current in ventricular myocytes *via* M_1 mAChRs (Gallo *et al.*, 1993).

The opposing actions of M_1 and M_2 muscarinic receptor subtypes during stimulation of parasympathetic neuron in the heart may simultaneously occur with β-adrenergic stimulation. There may also be a cross-talk between the signal transductions mediated by the several types of receptors. In such circumstances, cardiac automaticity may be precisely tuned by a net effect of several different receptor signalings. The M_1 receptor-mediated excitatory effects on the right atrium may contribute to protect large suppression of cardiac automaticity, especially when the β-adrenergic receptor signaling is compromised.

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