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Research Paper

Different signal molecules involved in the muscarinic modulation of pacemaker current I_f on the heart of mouse embryo in different developmental stages

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Abstract: We isolated mouse embryonic cardiomyocytes derived from timed-pregnant females at different periods and used patch-clamp technique to investigate the muscarinic cholinergic modulation of pacemaker current I_f in different developmental stages. In early development stage (EDS), muscarinic agonist carbachol (CCh) significantly decreased the magnitude of the pacemaker current I_f but had no effect in late development stage (LDS). Forskolin (a direct adenylate cyclase activator) and IBMX (a non-selective phosphodiesterase inhibitor) increased I_f in both EDS and LDS cells. Interestingly, although both forskolin and IBMX increased basal I_f, their effects on CCh-inhibited I_f were different. Forskolin did not reverse the inhibitory action of CCh until intermediate development stage (IDS). In contrast, IBMX reversed the inhibitory action of CCh on I_f in EDS but not in IDS. It is suggested that a decrease in intracellular cAMP is a possible mechanism for CCh to modulate I_f. During the EDS and IDS CCh controls the cytoplasmic cAMP level by different pathways: In EDS, CCh modulates I_f possibly by activating PDE which accelerates the breakdown of cAMP, but in IDS possibly by inhibiting adenylate cyclase (AC) which then reduces the synthesis of cAMP.

Key words: muscarinic cholinergic modulation; patch clamp; pacemaker current

不同信号分子参与 M 胆碱能受体激动剂对不同胚胎发育阶段小鼠心肌细胞起搏电流的调控

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摘 要：应用全细胞膜片钳技术，研究了 M 胆碱能对不同孕期的胚胎小鼠心肌细胞的起搏电流(I_f)的调节。我们发现，在胚胎发育的早期阶段, M 胆碱能受体激动剂(muscarinic agonist carbachol, CCh)明显抑制 I_f，但在胚胎发育的晚期阶段，CCh 对 I_f 的抑制作用消失。腺苷酸环化酶(adeinylate cyclase, AC)激动剂毛喉素 Forskolin 和非选择性磷酸二酯酶(PDE)抑制剂 IBMX 均可增强发育早期阶段和晚期阶段的 I_f。但有趣的是，尽管 Forskolin 和 IBMX 可增加基础 I_f，它们对 CCh 抑制的 I_f 的作用却大不相同。在胚胎发育的早期阶段，Forskolin 不能拮抗 CCh 对 I_f 的抑制作用，但 IBMX 可以。在发育的中期阶段 Forskolin 可以拮抗 CCh 的抑制作用，但 IBMX 不可以。因此，我们推断，CCh 可能是通过调控细胞内的 cAMP 水平来调节 I_f 的。但是在胚胎发育的早期阶段和中期阶段，CCh 可能通过不同的信号转导通路来实现对胞内 cAMP 的水平调控。在发育的早期阶段，CCh 主要是通过增强 PDE 的活性，加速 cAMP 的降解而实现对 I_f 的调控。而在发育的中期阶段，CCh 则主要通过与 AC 耦联，抑制其活性，通过减慢 cAMP 的合成而实现对 I_f 的调控。

关键词: M 胆碱能调控; 膜片钳; 起搏电流
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The pacemaker current I_f is a kind of hyperpolarization activated nonselective inward cation current. In heart, I_f current has been detected mainly in the conductive system such as frog sinus venosus[1], rabbit sino-atiral node[2], rabbit atrioventricular node[3] , and rabbit purkinje fibers[4]. It has also been demonstrated in embryonic stem cell (ES-cell) derived cardiomyocytes[5] and embryonic cardiomyocytes[6]. The pacemaker current I_f seems to be involved in the generation of the autorhythmicity in heart caused by the spontaneous action potential generated in the sinus-atrial node which spreads through the conductive system and causes the heart to contract spontaneously and harmoniously[7,8].

In the recent decade, the abnormally re-expression of early embryonic genes[9] and I_f in pathologically altered cardiac myocytes has been extensively noted. In spontaneously hypertensive rats, I_f density was linearly related to the severity of cardiac hypertrophy and was found to be significantly larger than that in healthy control animals[10]. Thus, it was suggested that the overexpression of I_f might contribute to the increased propensity of arrhythmias in hypertrophied rat ventricular myocardium[10]. In human ventricular myocytes of patients with end-stage heart failure, a trend of I_f density increase compared with the non-failure controls was also observed[11].

Detailed regulatory mechanisms of I_f in adult cardiac myocytes have been investigated. It is well known that I_f is regulated by sympathetic and parasympathetic neurotransmitters. Indeed, the β-adrenoceptor agonist isoprenaline (ISO) stimulates I_f by shifting the activation curve to more positive voltages whereas the vagal neurotransmitter acetylcholine (ACh) inhibits I_f by shifting the activation curve to more negative potentials[12]. In adult cardiac myocytes, I_f regulation involves the modulation of adenylate cyclase activity[13,14], the direct binding of cAMP to I_f-channels[15] as well as the phosphorylation of the channels via cAMP-dependent pathway[16]. In mouse embryonic heart, however, the regulation of I_f in cardiomyogensis differs dramatically in early and terminal differentiation stages. It has been reported that in early development stage (EDS) intrinsic adenylate cyclase (AC) and phosphodiesterase (PDE) activities are high, whereas β-adrenoceptors are not yet functionally coupled with AC[17,18]. So ISO can merely increase the amplitude of I_f in late development stage (LDS) but not in EDS in mouse embryonic heart[6] and ES-cell derived cardiomyocytes[5]. The stimulatory effect of ISO on I_f in LDS was mediated by phosphorylation via the cAMP-dependent protein kinase (PKA)[5,6]. Muscarinic cholinergic modulation of I_f in cardiomyogenesis is still obscure, however. It has been demonstrated that muscarinic agonist carbachol (CCh) inhibits I_f current in early stage of ES-cell derived cardiomyocytes. In LDS, however, CCh can not affect the current[5]. The detailed mechanisms of this phenomenon are not clear. In mouse embryonic heart little is known about the muscarinic cholinergic modulation on I_f.

In this experiment, we investigated the effects of muscarinic agonist CCh on I_f current in different developmental stages of mouse embryonic heart, and studied the possible regulatory mechanisms of CCh in different developmental stages.

1 MATERIALS AND METHODS

1.1 Cell isolation and culture

Pregnant mice of different development stages were killed by cervical dislocation and embryos were removed quickly. Then hearts were dissected from the embryos. And the ventricular tissues were then placed in Eppendorf tubes with enzyme-containing solution (1 mg/ml collagenase B, Roche Molecular Biochemicals, Mannheimm, Germany) to digest for 35~37 min at 37°C. Isolated cells were cultured in glass culture dishes with sterile, gelatin-coated glass cover slips containing 2~3 ml culture medium (see below for composition) for 18~48 h before current recording. In our study 8.5~11.5, 12.5~15.5 and 16.5~19.5 d after coitus were considered as EDS, IDS and LDS respectively.

1.2 Solutions

Modified Tyrode's solution was composed of (mmol/L): NaCl 140, NaOH 2.3, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 10, glucose 10, $CdCl_2$ 0.5, $BaCl_2$ 1,4-aminopyridine 2 (pH was adjusted to 7.4 with NaOH). $CdCl_2$, $BaCl_2$, and 4-aminopyridine was added to reduce the interference of other currents, i.e., slow inward calcium current, inward rectifier potassium current and transient outward current. Culture medium contained DMEM (Dulbecco's modified Eagle's medium, Gibco), 20% fetal bovine serum. Pipette solution was composed of (mmol/L): NaCl 10, potassium aspartate 130, Na_2ATP 2, EGTA 1, $MgCl_2$ 2, Na_2GTP 0.1, Hepes 10 (pH was adjusted to 7.2 with KOH).

1.3 Electrophysiology

The glass cover slips with cultured cells were placed in a temperature-controlled [(37±0.3)°C] recording chamber mounted on the stage of an inverted microscope (Zeiss, Germany) and continuously superfused with the modified

Tyrode's solution by gravity at a rate of 1 ml/min. Extracellular application of drugs was performed by superfusing cells with Tyrode's solution containing the drugs.

In our investigation only single cells that are spontaneously beating were used. Experimental data shown in this article were obtained using patch-clamp procedures in conventional whole-cell configuration[19]. The cells were held in voltage-clamp mode using an Axopatch 200-A amplifier (Axon Instruments, CA, USA) driven by ISO2 software (MFK, Frankfurt, FRG). I_f current was elicited by a hyperpolarizing pulse to –110 mV (lasting for 3 000 ms) from a holding potential of –35 mV (200 ms, to inactive sodium current). Patch pipettes were prepared from glass capillary tubes (Liuhe Laboratory Apparatus Factory, Nanjing, China) by means of a two-step vertical puller (David Kopf Instruments, Germany) .The resistance was 2~3 MΩ when filled with the pipette solution. Data were collected at a sampling rate of 10 kHz, filtered at 3 kHz, stored on hard disk and analyzed off-line using the ISO2 analysis software package.

1.4 Data analysis

The amplitude of I_f was measured as the difference between the instantaneous current at the beginning of the hyperpolarizing pulse and the steady-state current at the end of hyperpolarization[20]. When substances were applied, the instantaneous current amplitude in the presence of the substance was taken as the reference value for the estimation of I_f. Data are presented as mean±SEM when appropriate. Statistical analysis was performed using Student's paired or unpaired *t* tests and values of $P<0.05$ were considered significant. Graphics and statistical data analysis were carried out by ORIGIN 6.0 software (Microcal).

1.5 Reagents

The following reagents were all purchased from Sigma: CCh, forskolin, Hepes, isobutylmethyl-xanthine (IBMX), and 4-aminopyridine. DMEM and fetal bovine serum were purchased from Gibco. Forskolin was dissolved in DMSO (final DMSO concentration 0.01%), stored frozen at –20°C. Aliquots were thawed immediately before use and diluted in the bath solution to the concentration desired.

And other chemicals, if not stated, were all purchased from Chinese reagent companies.

2 RESULTS

2.1 Effect of CCh on I_f in EDS and LDS cells

It is well established that muscarinic agonist inhibits I_f[12]. In ES-cell derived cardiomyocytes, it is also reported that CCh inhibits basal I_f in EDS but not in LDS[5]. Our study in embryonic cardiomyocytes showed that CCh (μmol/L) depressed basal I_f current by (30.40±3.74)% [from (399.78±58.21) to (275.84±45.49) pA, $P<0.05$, $n=25$], (19.61±4.47)% [from (369.28±34.14) to (298.89±38.56), $P<0.05$ $n=27$] and (5.53±2.24)% [from (358.79±43.36) to (339.25±46.34) pA, $P>0.05$, $n=23$] in EDS, IDS, and LDS, respectively (Fig. 1*B*). So with heart development, the effect of CCh on I_f gradually diminished. Current traces recorded from a typical EDS cell showed that CCh profoundly inhibited I_f (Fig. 1*A*). But CCh almost had no effect on I_f in LDS (data not show).

2.2 Effect of forskolin and IBMX on I_f current

In our experiment either forskolin (1 μmol/L) or IBMX (50 μmol/L) enhanced the basal I_f current in both EDS and LDS.

Forskolin (1 μmol/L) increased the amplitude of I_f by (28.34±2.56)% [from (403.92±73.56) to (519.04±62.74) pA, $P<0.01$, $n=18$], and (27.16±7.14)% [from (347.64± 39.97) to (487.23 ±69.81) pA, $P<0.01$, $n=20$] in EDS and LDS cells, respectively.

Similar with the effect of forskolin, IBMX (50 μmol/L) also enhanced the amplitude of I_f by (23.46±3.40)% [from (385.67±62.78) to (478.39±53.96) pA, $P<0.01$, $n=18$] and (21.53±2.18)% [from (364.31±83.94) to (442.86±69.42) pA, $P<0.01$, $n=16$] in EDS and LDS cells, respectively.

2.3 Effect of forskolin and IBMX on CCh-inhibited I_f current

To evaluate the effect of forskolin and IBMX on CCh-inhibited I_f current, forskolin or IBMX was administered after I_f current had been inhibited by CCh.

In EDS, administration of CCh (1 μmol/L) inhibited the current from (403.19±45.34) to (282.42±29.68) pA. Consequent co-administration of forskolin (1 μmol/L) and CCh (1 μmol/L) had no effect on the CCh-inhibited current, only changed the current from (282.42±29.68) to (290.59±29.74) pA, ($P>0.05$, $n=21$). In IDS, however, forskolin (1 μmol/L) abolished the inhibitory action of CCh on I_f. After the current had been inhibited by CCh (1 μmol/L) from (371.14±35.34) to (296.95±18.21) pA, consequent co-administration of forskolin (1 μmol/L) and CCh (1 μmol/L) recovered the current to (356.59±37.58) pA ($P<0.05$, Fig. 2*C*). Figure 2*A* and 2*B* illustrated the results from a typical EDS and IDS cell, respectively.

The action of IBMX, however, was different from that of forskolin. In EDS, after I_f current had been inhibited by CCh (1 μmol/L) from (381.30±42.14) pA to (255.27±

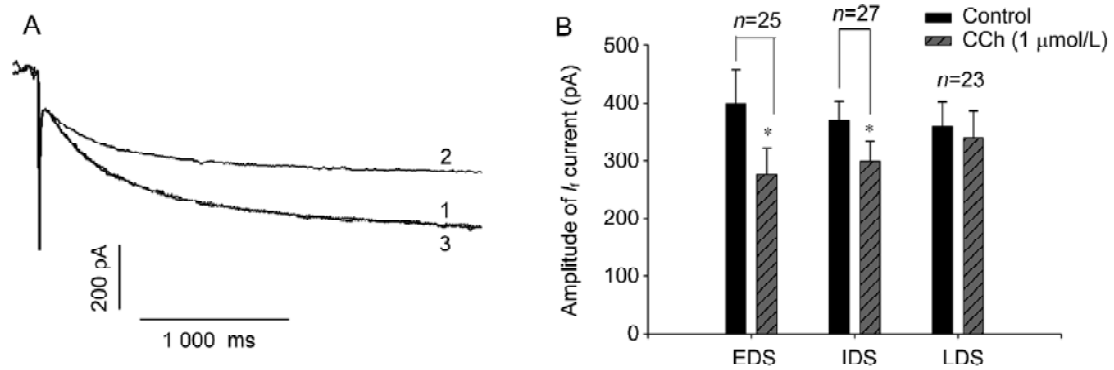

Fig.1. Effect of muscarinic agonist CCh on mouse embryonic cardiomyocytes. *A*: A typical result recorded from an individual EDS cell. Mark 1, 2, and 3 present control, application of CCh (1 μmol/L) and washout, respectively. *B*: Pooled data of the effect of CCh (1 μmol/L). $^*P<0.05$.

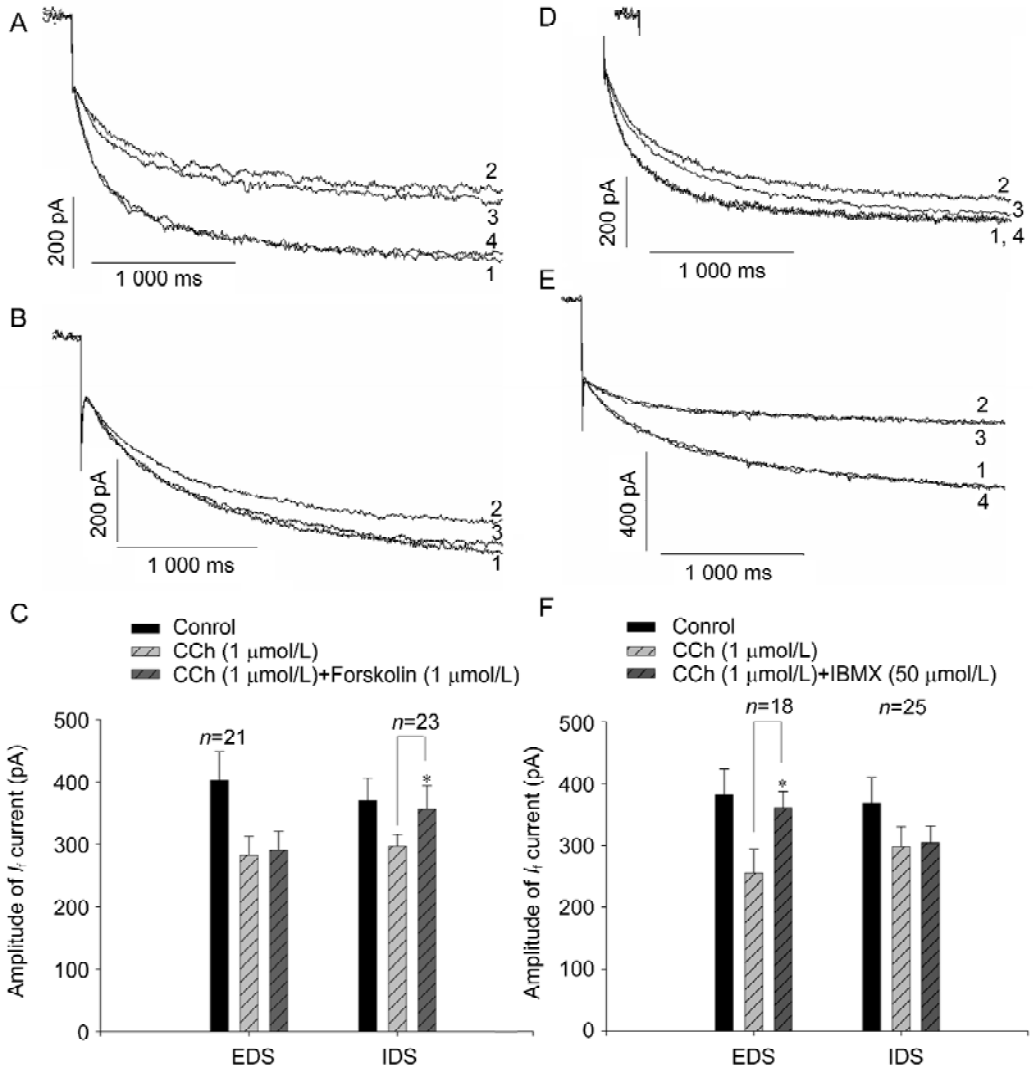

Fig.2. Different effects of forskolin (1μmol/L) and IBMX (50 μmol/L) on CCh-inhibited I_f current in EDS and IDS. *A-C*: Effect of forskolin (1 μmol/L) on CCh-inhibited I_f current in EDS and IDS. *A*: Typical tracings in an EDS cell. 1, control; 2, application of CCh (1 μmol/L); 3, superimposing forskolin (1 μmol/L) in the CCh (1 μmol/L) containing bath solution; 4, washout. *B*: Typical tracings in an IDS cell. 1, control; 2, application of CCh (1 μmol/L); 3: superimposing forskolin (1 μmol/L) in the CCh (1 μmol/L) containing bath solution. *C*: Pooled data of the effect of forskolin. $^*P<0.05$. *D-F*: Effect of IBMX (1 μmol/L) on CCh-inhibited I_f current in EDS and IDS. *D*: Typical tracings in an EDS cell. 1, control; 2, application of CCh (1 μmol/L); 3, superimposing IBMX (50 μmol/L) in the CCh (1 μmol/L) containing bath solution; 4, washout. *E*: Typical tracings in an IDS cell. 1, control; 2, application of CCh (1 μmol/L); 3, superimposing IBMX (50 μmol/L) in the CCh (1 μmol/L) containing bath solution; 4, washout. *F*: Pooled data of the effect of IBMX. $^*P<0.05$.

38.76) pA, co-administration of CCh (1 μmol/L) and IBMX (50 μmol/L) recovered the current from (255.27±38.76) to (360.84±26.14) pA, (P<0.05, n=18). In IDS, after I_f current had been inhibited from (368.73±41.19) to (298.08±31.82) pA by CCh (1 μmol/L), co-administration of IBMX (50 μmol/L) and CCh (1 μmol/L) could not recover the current, and changed the current from (298.08±31.82) pA to (303.64±27.08) pA (P>0.05, Fig. 2*F*). Figure 2*D* and 2*E* illustrated the results from a typical EDS and IDS cell, respectively.

3 DISSCUSION

Regulation of the heart autorhythmicity by the sympathetic and parasympathetic nerves is in part mediated by the effects of the transmitters they released on pacemaker current I_f[21]. In canine Purkinje fibers it has been demonstrated that, β-adrenergic stimulation increases I_f by raising the intracellular cAMP levels[22] and the elevation of cAMP can activate the cAMP-dependent protein kinase (PKA) which in turn activates the I_f channel by protein phosphorylation[16]. In ES-cell derived cardiomyocytes[5] and mouse embryonic heart[6] β-adrenergic agonist ISO has also been demonstrated to activate I_f via AC-cAMP-PKA pathway in LDS. The muscarinic cholinergic effect on I_f is paradoxical. In rabbit sino-atrial node, Ach-induced I_f depression is due to the inhibition of AC, but this action of ACh is not mediated via PDE[12]. In adult canine cardiac purkinje fibers CCh can not inhibit I_f directly, but only depresses adrenergic agonist ISO-enhanced I_f current[23]. In ES-derived cardiomyocytes CCh depresses I_f in EDS but has no effect in LDS[5].

Our study in embryonic cardiomyocytes indicates that muscarinic agonist CCh can strongly inhibit I_f current [(34.4±3.74)%, n=25] in EDS. Compared with EDS, the inhibitory effect of CCh becomes almost negligible [(5.53±2.24)%, n=23] in LDS. The result is partly in line with the findings in ES-cell derived cardiomyocytes[5] in which CCh has no effect on I_f in LDS.

To study the possible regulatory mechanism of CCh on I_f, we firstly evaluated the effects of AC activator forskolin and PDE inhibitor IBMX on I_f. In our study, either forskolin or IBMX increased I_f in both EDS and LDS. These results indicate that cAMP is involved in the modulation of I_f.

Althouth forskolin and IBMX have similar effect on I_f, their effect on CCh-inhibited I_f current are different. In EDS forskolin had no effect on CCh-inhibited I_f current, but IBMX abolished the inhibitory effect of CCh on I_f. These results suggest that in EDS, CCh can inhibit I_f via enhancing the activity of PDE, since PDE inhibitor IBMX can abolish CCh's effect on I_f in this stage.

In IDS, however, forskolin antagonized the inhibitory action of CCh on I_f, but IBMX had no effect on CCh-inhibited I_f in this stage. This suggests that in this developmental stage CCh may inhibit I_f by inhibiting the activity of AC, because AC activator forskolin can antagonize the inhibitory of CCh on I_f.

We conclude that cAMP-dependent pathway is involved in the modulation of I_f and muscarinc agonist CCh modulates I_f possibly via different signal transduction pathways in EDS and IDS in mouse embryonic heart. In EDS, CCh may depress I_f mainly by modulating the activity of PDE. In IDS, however, AC may be involved in the muscarinic modulation of I_f.

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