Muscarinic cholinergic regulation of L-type calcium channel in heart of embryonic mice at different developmental stages

Hua-min LIANG2, Ming TANG2-4, Chang-jin LIU2, Hong-yan LUO2, Yuan-long SONG2, Xin-wu HU2, Jiao-ya XI2, Lin-lin GAO2, Bin NIE2, Su-yun LI2, Ling-ling LAI2, Juergen HESCHELER3

2Department of Physiology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China; 3Institute of Neurophysiology, University of Cologne, D-50931 Cologne, Germany

KEY WORDS 8-bromo cyclic adenosine monophosphate; calcium channels; cell differentiation; embryo and fetal development; forskolin; heart; mice; patch-clamp techniques; muscarinic receptors

ABSTRACT

AIM: To investigate the muscarinic regulation of L-type calcium current (I_{Ca-L}) during development. METHODS: The whole cell patch-clamp technique was used to record I_{Ca-L} in mice embryonic cardiomyocytes at different stages (the early developmental stage, EDS; the intermediate developmental stage, IDS; and the late developmental stage, LDS). Carbachol (CCh) was used to stimulate M-receptor in the embryonic cardiomyocytes of mice. RESULTS: The expression of I_{Ca-L} density did not change in different developmental stages (P>0.05). There was no difference in the sensitivity of I_{Ca-L} to CCh during development (P>0.05). This inhibitory action of CCh was mediated by inhibition of cyclic AMP since 8-bromo-cAMP completely reversed the muscarinic inhibitory action. IBMX, a non-selective inhibitor of phosphodiesterase (PDE), reversed the inhibitory action of M-receptor on I_{Ca-L} current by 71.2 %±9.2 % (n=8) and 11.3 %±2.5 % (n=9) in EDS and LDS respectively. However forskolin, an agonist of adenylyl cyclase (AC), reversed the action of CCh by 14.5 %±3.5 % (n=5) and 82.7 %±10.4 % (n=7) in EDS and LDS respectively. CONCLUSION: The inhibitory action of CCh on I_{Ca-L} current was mediated in different pathways: in EDS, the inhibitory action of M-receptor on I_{Ca-L} channel mainly depended on the stimulation of PDE. However, in LDS, the regulation by M-receptor on I_{Ca-L} channel mainly depended on the inactivation of AC.

INTRODUCTION

More and more researches were focused on the developmental changes both in the ion channels and in their regulations in cardiomyocyte[1-4]. In adult mammalian cardiomyocytes, the heart was regulated by stimulating β-adrenergic receptor and M-receptor with sympathetic and parasympathetic neurotransmitters (noradrenaline and acetylcholine), respectively. L-type calcium current (I_{Ca-L}) was regulated by M-receptor through the signal transduction that included several steps of interaction of functionally coupled downstream components. Binding of the agonist to the M-receptor activated an inhibitory pertussis-toxin-sensitive guanine nucleotide-binding G protein (Gi), which triggered the inactivation of adenylyl cyclase (AC) and, in turn,
decreased the level of cytosolic cAMP. It was followed by the dephosphorylation of the Ca\(^{2+}\) channel through the inactivation of cAMP-dependent protein kinase A (PKA)\(^{5,6}\).

However, nitric oxide (NO) might be involved in the muscarinic regulation of the cardiomyocytes. It was proposed that NO signalling activated by ACh might also participate in the inhibitory effects of ACh, although this idea remained controversial\(^{7,8}\). This suggested another possible pathway for the muscarinic regulation of PDEs increased basal or pre-stimulated this idea remained controversial\(^{7,8}\). This suggested another possible pathway for the muscarinic regulation of the cardiomyocytes. It was proposed that NO signalling activated by ACh might coexist in the heart muscle\(^{11,12}\). Studies of the regulation of \(I_{\text{Ca-L}}\) showed that the inhibition of different kinds of PDEs increased basal or pre-stimulated \(I_{\text{Ca-L}}\). Fischmeister et al reported that PTX-sensitive G protein could activate NOS, then activated the NO-sensitive GC\(^{11,13}\). CCh action was blocked by L-NMA and L-NAME on cardiomyocytes derived from ES cells, but the blockade was prevented by excess L-Arg\(^{4}\).

Ca\(^{2+}\) channel was modulated during embryonic and postnatal development\(^{16,17}\), indicating that the modulation of \(I_{\text{Ca-L}}\) changed during development. That caused a new interest of the research on the cardiomyocytes. However, all these researches mainly resulted from the embryonic stem (ES) cell derived cardiomyocytes, and it was unclear whether the muscarinic regulation of \(I_{\text{Ca-L}}\) in naturally pregnant and differentiated embryonic cardiomyocytes was similar to that in the ES derived cardiomyocytes.

The present study was to investigate the muscarinic regulation of the \(I_{\text{Ca-L}}\) channel in the heart of embryonic mice to determine the nature and origin of the muscarinic inhibitory pathway.

**MATERIALS AND METHODS**

**Reagents** The following chemicals were all purchased from Sigma Chemical Co: CCh, forskolin, HEPES, IBMX, 8-Br-cAMP, and 4-aminopyridine. The substances for cell culture were purchased from Gibco Company. Forskalin and IBMX was dissolved in Me\(_2\)SO at final concentration of 0.01 %, stored at -20 °C.

The other chemicals, if not stated, were of analytical grade and purchased from Shanghai Chemical Reagent Plant.

**Cell isolation and culture** Female pregnant Kunming mice of 4-6 week old were supplied by the Medical Experimental Animal Center, Tongji Medical College of Huazhong University of Science and Technology (Grade II, Certificate No 19-023). Embryos of 9.5+1-3 d, 9.5+4-6 d, 9.5+7-9 d were considered as EDS (early developmental stage), IDS (intermediate developmental stage), and LDS (late developmental stage) respectively. Briefly, pregnant mice were killed by cervical dislocation, and embryos at different developmental stages were taken out from the uterus and placed in low calcium solution containing (mmol/L): NaCl 120, KCl 5.4, MgSO\(_4\) 5, sodium pyruvate 5, glucose 20, taurine 20, HEPES 10 (pH was adjusted to 6.9 with NaOH) at 4 °C. Then hearts were dissected from the embryos, and then placed in Eppendorf tubes with enzyme-containing solution (containing collagenase B 1 g/L, Roche Molecular Biochemicals, Mannheim, Germany) to digest at 37 °C for 36±1 min in quiescence. The digestion process was terminated by adding KB solution containing (mmol/L): KCl 85, K\(_2\)HPO\(_4\) 30, MgSO\(_4\) 5, edetic acid 1, Na\(_2\)ATP 2, sodium pyruvate 5, creatine 5, taurine 20, and glucose 20 (pH was adjusted to 7.2 with KOH). Isolated cells were cultured on gelatin (0.1 %)-coated glass cover slips in culture medium containing Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 20 % fetal bovine serum in 3 %-5 % CO\(_2\) incubator for 18-48 h before current recording, as previously described by Song GL et al\(^{31}\).

**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 Tyrode’s solution containing (mmol/L): NaCl 140, NaOH 2.3, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1, HEPES 10, glucose 10, and 4-aminopyridine 5 (pH was adjusted to 7.4 with NaOH). Extracellular application of drugs was performed by superfusing cells with Tyrode’s solution containing the drugs. Substances were applied by replacing the solution in the chamber. A 90 % volume exchange was achieved within approximately 20 s. In our investigation only single spontaneously beating cells were used. The cells were held in voltage-clamp mode using an Axopatch 200A amplifier (Axon Instruments, CA, USA) driven by ISO2 software (MFK, Frankfurt, FRG). The whole-cell configuration of the patch-clamp technique was used throughout the present study\(^{18}\), \(I_{\text{Ca-L}}\) was elicited by a depolarizing pulse to 0 mV (lasting 300 ms) from a hold-
ing potential of -40 mV (100 ms, to inactive sodium current). The intracellular ATP concentration was raised to 5 mmol/L to minimize $I_{\text{Ca,L}}$ rundown\(^{[19]}\). The internal and external solutions contained Cs\(^+\) and tetra-ethylammonium, respectively, to effectively block K\(^+\) current. Patch pipettes were prepared from glass capillary tubes (Liuhe Laboratory Apparatus Factory, Nanjing, China) by a two-step vertical puller (David Kopf Instruments, Germany). The resistance was 0.5-3 M\(\Omega\) when filled with the pipette solution (mmol/L): CsCl 115, HEPES 10, 3-ethyl acid 10, Tea-Cl 20, MgATP 5, Tris-GTP 0.1, phosphocreatine 10, and CaCl\(_2\) 1 (pH was adjusted to 7.4 with CsOH at 36 °C). Cell membrane capacitance (Cm) was determined on-line using the ISO2 acquisition software program. 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.

**Data analysis** The amplitude of $I_{Ca,L}$ was determined by measuring the absolute magnitude of the peak inward current during the step depolarization to 0 mV. The effect of drugs on peak $I_{Ca,L}$ magnitude was calculated by averaging the amplitudes of currents evoked by three consecutive stimuli just before drug was added and it was repeated after the drug effect reached steady state\(^{[8]}\). Currents were normalized to membrane capacitance to calculate current densities. The inhibition or stimulation of $I_{Ca,L}$ was reported in terms of the percentage of the decrease or increase in $I_{Ca,L}$ density. Data were presented as mean±SD. Statistical analysis of $I_{Ca,L}$ was performed using a two-way ANOVA and Bonferroni’s test for multiple comparisons. The results were considered significant if $P<0.05$ and graphically presented using SigmaPlot software.

**RESULTS**

**Effect of CCh on $I_{Ca,L}$ in different developmental stages of cardiomyocytes** $I_{Ca,L}$ densities were 20±5 pA/pF, 19±3 pA/pF, and 18±5 pA/pF ($n=25$) in EDS, IDS, and LDS, respectively ($P<0.01$, Fig 1A). These results suggested that the expression of $I_{Ca,L}$ in cardiomyocytes did not change during development.

To measure the muscarinic action on $I_{Ca,L}$, CCh 1 µmol/L, an agonist of M-receptor, was superfused extracellularly. $I_{Ca,L}$ was suppressed by the application of CCh: the $I_{Ca,L}$ densities decreased from 20±5 pA/pF to 13±3 pA/pF ($n=27$, $P<0.01$), from 19±3 pA/pF to 12±3 pA/pF ($n=23$, $P<0.01$), and from 18±5 to 11.3±2.8 pA/pF ($n=27$, $P<0.01$) in EDS, IDS, and LDS, respectively (Fig 1A). $I_{Ca,L}$ densities decreased by 36.8 %±4.4 % in EDS, 37.0 %±5.7 % in IDS (Fig 1B), and 37.0 %±5.4 % in LDS, indicating that there was no difference in the sensitivity of $I_{Ca,L}$ to CCh during development ($P>0.05$, ANOVA).

**Effect of cyclic AMP in muscarinic action on $I_{Ca,L}$ in cardiomyocytes during development** To study the role of cyclic AMP in the muscarinic action on $I_{Ca,L}$ at different developmental stages, 8-Br-cAMP, a membrane-permeable analogue of cAMP, 400 µmol/L was superfused together with CCh after the inhibitory action of CCh appeared steadily. The muscarinic inhibitory actions on $I_{Ca,L}$ were completely reversed by 8-Br-cAMP in EDS ($n=10$, $P<0.01$), IDS ($n=9$, $P<0.01$), and LDS ($n=14$, $P<0.01$) separately (Fig 2A, data not shown). Changes in $I_{Ca,L}$ densities indicated that the muscarinic inhibitory action on $I_{Ca,L}$ was mediated by the decrease in cAMP (Fig 2B).

**Effect of forskolin on muscarinic inhibitory action on $I_{Ca,L}$ during development** To determine the role of AC in the muscarinic regulation, co-superfusion of forskolin (agonist of AC, 1 µmol/L) plus CCh was applied in the same way as 8-Br-cAMP. Forskolin had weak effect to reverse the inhibitory action of CCh on $I_{Ca,L}$ (14.5 %±3.5 %, $n=5$). The reversed percentage was 44.2 %±37.7 % in IDS and 82.7 %±10.4 % in LDS, respectively ($n=7$, $P<0.05$, Fig 3). These results suggested that the muscarinic inhibitory action on $I_{Ca,L}$ was gradually due to the muscarinic inactivation of AC during development.

**Effect of IBMX on muscarinic inhibitory action on $I_{Ca,L}$ during development** The above results suggested that the muscarinic inhibitory action on $I_{Ca,L}$ in EDS depended on other mechanism than inactivation of AC. So we superfused the cells with IBMX (a non-selective inhibitor of PDE, 50 µmol/L) instead of forskolin together with CCh. In contrast to forskolin, IBMX significantly reversed the action of CCh in EDS, but its effect decreased gradually in IDS and LDS ($P<0.05$). The muscarinic inhibitory action on $I_{Ca,L}$ in EDS was mainly due to the stimulation of PDEs (Fig 4).

**DISCUSSION**

We demonstrated here that CCh had inhibitory action on $I_{Ca,L}$ in cardiomyocytes derived from the em-
bryonic mice. The inhibitory action existed in all developmental stages, and there was no difference between different stages, which was slightly different from that discovered by Hescheler et al\(^1\). Their findings suggested that muscarinic agonists depressed \( I_{Ca-L} \) by 58\%±3\% in early stage of cardiomyocytes but had no effect on basal \( I_{Ca-L} \) but antagonized the \( \beta \)-adrenoceptor-stimulated \( I_{Ca-L} \) by 43\%±4\% in LDS. The difference might result from the difference between the naturally pregnant embryonic cardiomyocytes and the cardiomyocytes derived from ES cells.

Our experiments further proved that cyclic AMP played a key role in the muscarinic regulation of \( I_{Ca-L} \) in cardiomyocytes of embryonic mice during development, because the muscarinic inhibitory action on \( I_{Ca-L} \) was completely reversed by 8-Br-cAMP, which was similar to the findings that cyclic AMP mediated the regulation of \( I_{f} \) channel in cardiomyocytes derived from ES cells and embryonic mice\(^2,3\). Cyclic AMP was demonstrated to mediate the regulation of ion channels in cardiomyocytes by the sympathetic and parasympathetic nerves\(^{20,21}\). The above suggested that cyclic AMP was always the key second messenger to regulate cardiomyocytes since the cardiogenesis of the heart.

In our study, forskolin only reversed the inhibitory action of CCh by 14.5\%±3.5\% in EDS cells and IBMX strongly reversed the inhibitory action of CCh by 71.2\%±9.2\%. Together, these results suggested that in EDS, the inhibitory action of CCh on \( I_{Ca-L} \) was mainly dependent on the stimulation of PDEs, which decreased \( I_{Ca-L} \) by increasing the level of degradation thereby limiting the degree of protein phosphorylation.
In contrast to EDS cells, the inhibitory action of CCh on $I_{\text{Ca-L}}$ was significantly reversed by forskolin, and IBMX only had weak effect. So we concluded that in LDS cells muscarinic control of cAMP concentrations depended on the level of its production via AC rather than its degradation via PDEs.

The muscarinic regulation of $I_{\text{Ca-L}}$ in EDS cells proved to be particularly interesting, because forskolin already had a stimulatory effect on $I_{\text{Ca-L}}$ density\cite{4} but it only reversed the inhibitory action of CCh on $I_{\text{Ca-L}}$ by 14.5%±3.5% in this stage. Probably the G-protein was not functionally coupled to AC, as reported by Slotkin et al\cite{4,22}. Thus the muscarinic regulation of $I_{\text{Ca-L}}$ mainly depended on the degradation but not production of cAMP in EDS cells. Another reason for the changes from activation of PDEs to inactivation of AC might be the declined expression of NOS, as demonstrated by Hescheler et al\cite{1,23}.

In this study, we examined the muscarinic regulation of $I_{\text{Ca-L}}$ in embryonic mice cardiomyocytes during development. Several main conclusions could be drawn from our experiments: (1) muscarinic inhibitory action on $I_{\text{Ca-L}}$ occurred in EDS of cardiomyocytes; (2) this inhibitory action was mediated by inhibition of cyclic AMP; (3) in EDS, the inhibition was mainly dependent on the stimulation of PDEs; (4) in LDS, the inhibition was mainly dependent on the inactivation of AC.

REFERENCES

1. Ji GJ, Fleischmann BK, Bloch W, Feelisch M, Andressen C, Hescheler J, et al. Regulation of the L-type Ca$^{2+}$ channel during cardiomyogenesis: switch from NO to adenylyl...
Fig 3. Effect of forskolin on muscarinic inhibitory action on $I_{Ca-L}$ during development. (A, B, C) Left: $I_{Ca-L}$ current traces were recorded in EDS, IDS, and LDS, respectively. (a) Before the superfusion with CCh 1 µmol/L; (b) After superfusion with CCh 1 µmol/L; (c) After the superfusion with both CCh 1 µmol/L and forskolin 1 µmol/L. Right: The time course of the experiments of the corresponding left-hand panels. (D) Left: percentages of inhibitory action reversed by forskolin in EDS, IDS, and LDS respectively. Right: $I_{Ca-L}$ densities before and after the superfusion with CCh, and after the co-superfusion with forskolin plus CCh. EDS: $n=5$ cells. IDS: $n=7$ cells. LDS: $n=7$ cells. Mean±SD. *$P<0.05$ vs CCh.
Fig 4. Effect of IBMX on muscarinic inhibitory action on $I_{Ca-L}$ during development. (A, B, C) Left: $I_{Ca-L}$ current traces were recorded in EDS, IDS, and LDS, respectively. (a) Before the superfusion with CCh 1 µmol/L; (b) After the superfusion with CCh 1 µmol/L; (c) After the superfusion with both CCh 1 µmol/L and IBMX 50 µmol/L. Right: The time course of the experiments of the corresponding left-hand panels. (D) Left: percentages of inhibitory action reversed by IBMX in EDS, IDS, and LDS, respectively. Right: $I_{Ca-L}$ densities before and after the superfusion with CCh, and after the superfusion with IBMX and CCh. EDS: $n=8$ cells. IDS: $n=6$ cells. LDS: $n=9$ cells. Mean±SD. *$P<0.05$ vs CCh.
7 Han X, Kubota I, Feron O, Opel DJ, Arstall MA, Kelly RA, et al. Muscarinic cholinergic regulation of cardiac myocyte \(I_{Ca-L}\) is absent in mice with targeted disruption of endothelial nitric oxide synthase. Proc Natl Acad Sci USA 1998; 95: 6510-5.
8 Belevych AE, Harvey YR. Muscarinic inhibitory and stimulatory regulation of the \(L\)-type \(Ca^{2+}\) current is not altered in cardiac ventricular myocytes from mice lacking endothelial nitric oxide synthase. J Physiol 2000; 528: 279-89.