Muscarinic cholinergic regulation of L-type calcium channel in heart of embryonic mice at different developmental stages[1]

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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 ($I_{Ca\text{-}L}$) during development. **METHODS:** The whole cell patch-clamp technique was used to record $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ density did not change in different developmental stages ($P>0.05$). There was no difference in the sensitivity of $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ current was mediated in different pathways: in EDS, the inhibitory action of M-receptor on $I_{Ca\text{-}L}$ channel mainly depended on the stimulation of PDE. However, in LDS, the regulation by M-receptor on $I_{Ca\text{-}L}$ 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 ($I_{Ca\text{-}L}$) 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,

[1] Project supported by the National Natural Science Foundation of China (No 30070279).
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Received 2003-12-12 Accepted 2004-05-20

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 $I_{Ca\text{-}L}$. That was, nitric oxide synthase (NOS) was activated after stimulation of M-receptor, which in turn actived guanylate cyclase (GC) and subsequent cyclic GMP-dependent PDEs[9]. Then cAMP broke into 5´-AMP thereby limiting the degree of protein phosphorylation[10,11]. Four different PDEs were demonstrated to coexist in the heart muscle[11,12]. Studies of the regulation of $I_{Ca\text{-}L}$ showed that the inhibition of different kinds of PDEs increased basal or pre-stimulated $I_{Ca\text{-}L}$[11,13]. Fischmeister *et al* reported that PTX-sensitive G protein could activate NOS, then activated the NO-sensitive GC[14,15]. 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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_2SO at final concentration of 0.01 %, stored at -20 ºC.

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 ºC. 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, Mannheimm, Germany) to digest at 37 ºC for 36±1 min in quiescence. The digestion process was terminated by adding KB solution containing (mmol/L): KCl 85, K_2HPO_4 30, $MgSO_4$ 5, edetic acid 1, Na_2ATP 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*[2].

Electrophysiology The glass cover slips with cultured cells were placed in a temperature-controlled (37±0.3 ºC) 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 200-A 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]. $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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 2-3 MΩ, when filled with the pipette solution (mmol/L): CsCl 115, HEPES 10, egtazic 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 ºC). 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ was reported in terms of the percentage of the decrease or increase in $I_{Ca\text{-}L}$ density. Data were presented as mean±SD. Statistical analysis of *t*-test was used to consider the effect of application of drugs, one-way of ANOVA was used to measure the developmental changes. $P<0.05$ were considered significantly different. Graphics and statistical data analysis were carried out by Sigmaplot software.

RESULTS

Effect of CCh on $I_{Ca\text{-}L}$ in different developmental stages of cardiomyocytes $I_{Ca\text{-}L}$ densities were 20±5 pA/pF (n=25), 19±3 pA/pF (n=23), and 18±5 pA/pF (n=27) in EDS, IDS, and LDS, respectively (Fig 1A black bar). There was no difference between them (P>0.05, ANOVA), suggesting that the expression of $I_{Ca\text{-}L}$ in cardiomyocytes did not change during development.

To investigate the muscarinic action on $I_{Ca\text{-}L}$ channel, CCh 1 μmol/L, an agonist of M-receptor, was superfused extracellularly. $I_{Ca\text{-}L}$ was suppressed by the application of CCh: the $I_{Ca\text{-}L}$ 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 1 A). $I_{Ca\text{-}L}$ densities was 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 $I_{Ca\text{-}L}$ to CCh during development (P>0.05, ANOVA).

Effect of cyclic AMP in muscarinic action on $I_{Ca\text{-}L}$ in cardiomyocytes during development To study the role of cyclic AMP in the muscarinic action on $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ densities indicated that the muscarinic inhibitory action on $I_{Ca\text{-}L}$ was mediated by the decrease in cAMP (Fig 2B).

Effect of forskolin on muscarinic inhibitory action on $I_{Ca\text{-}L}$ during development To determine the role of AC in the muscarinic regulation, co-superfusion with 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 $I_{Ca\text{-}L}$ (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 $I_{Ca\text{-}L}$ was gradually due to the muscarinic inactivation of AC during development.

Effect of IBMX on muscarinic inhibitory action on $I_{Ca\text{-}L}$ during development The above results suggested that the muscarinic inhibitory action on $I_{Ca\text{-}L}$ 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 $I_{Ca\text{-}L}$ in EDS was mainly due to the stimulation of PDEs (Fig 4).

DISCUSSION

We demonstrated here that CCh had inhibitory action on $I_{Ca\text{-}L}$ in cardiomyocytes derived from the em-

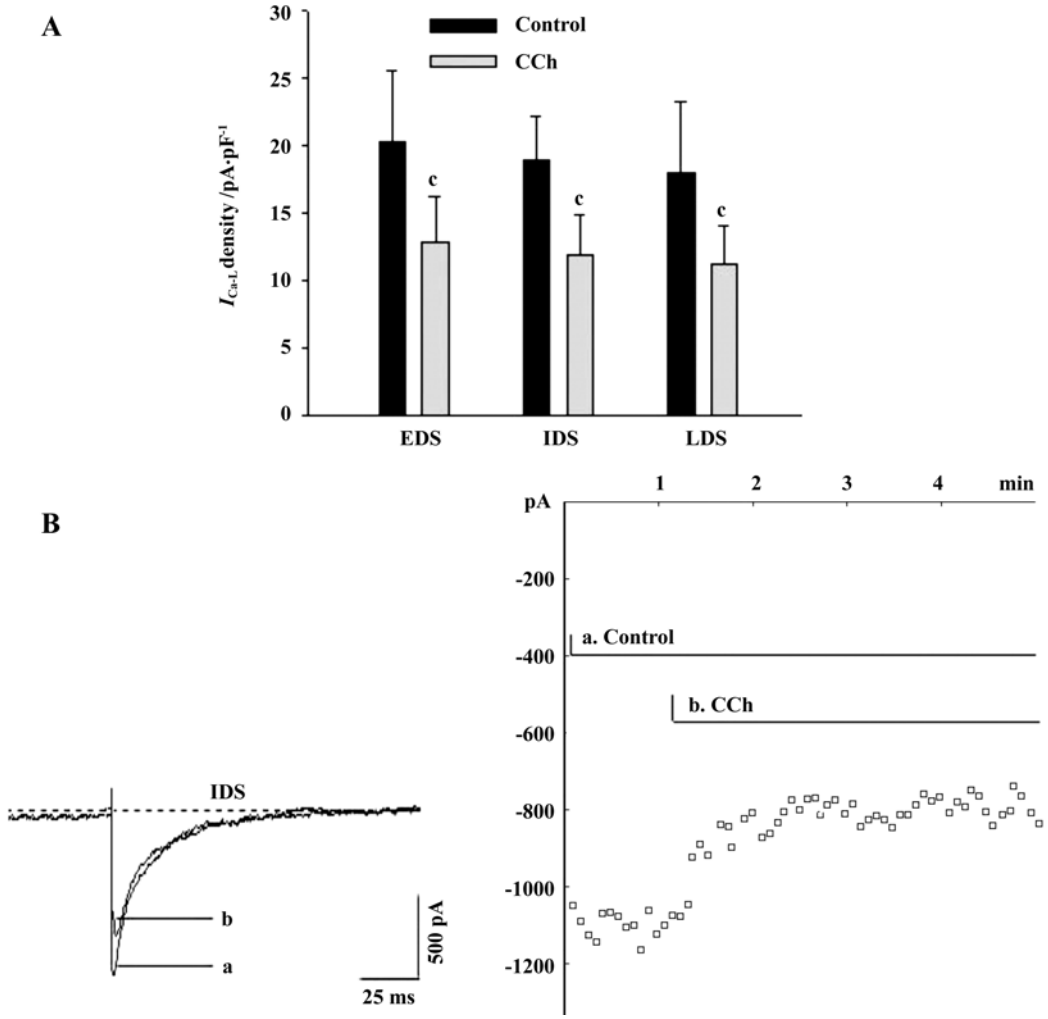

Fig 1. Effect of CCh on $I_{Ca-L.}$ A: the I_{Ca-L} density was suppressed apparently after the application of CCh at different developmental stages. EDS: *n*=27 cells; IDS: *n*=23 cells; LDS: *n*=27 cells. Mean±SD. $^{c}P<0.01$ *vs* control. B: I_{Ca-L} current traces recorded in IDS before (a) and after (b) superfusion with CCh 1 μmol/L (left). Plot of the time course of the experiment (right).

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 β-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.

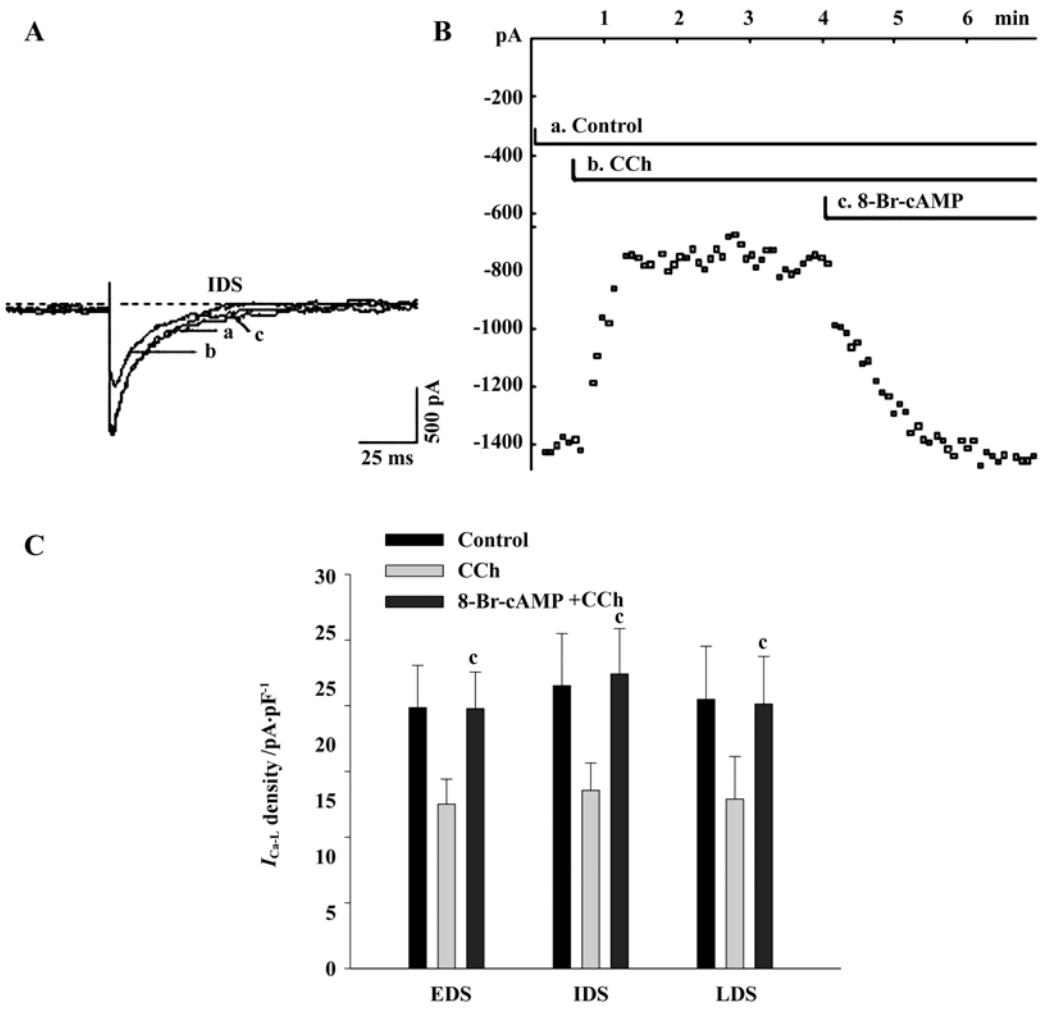

Fig 2. Effect of cyclic AMP in muscarinic action on I_{Ca-L}. A: I_{Ca-L} current trace recorded in IDS. (a) Before superfusion with CCh 1 μmol/L; (b) After superfusion with CCh 1 μmol/L; (c) after the superfusion with both CCh and 8-Br-cAMP. B: The time course of the experiment. C: I_{Ca-L} densities before and after the superfusion with CCh, and after the co-superfusion with 8-Br-cAMP plus CCh. EDS: *n*=10 cells. IDS: *n*=9 cells. LDS: *n*=11 cells. Mean±SD. $^cP<0.01$ *vs* CCh.

In contrast to EDS cells, the inhibitory action of CCh on I_{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_{Ca-L} in EDS cells proved to be particularly interesting, because forskalin already had a stimulatory effect on I_{Ca-L} density[4] but it only reversed the inhibitory action of CCh on I_{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*[4,22]. Thus the muscarinic regulation of I_{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*[1,23].

In this study, we examined the muscarinic regulation of I_{Ca-L} in embryonic mice cardiomyocytes during development. Several main conclusions could be drawn from our experiments: (1) muscarinic inhibitory action on I_{Ca-L} occurred in EDS of cardiomyocyts; (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, Hecheler J, *et al*. Regulation of the *L*-type Ca^{2+} channel during cardiomyogenesis: switch from NO to adenylyl

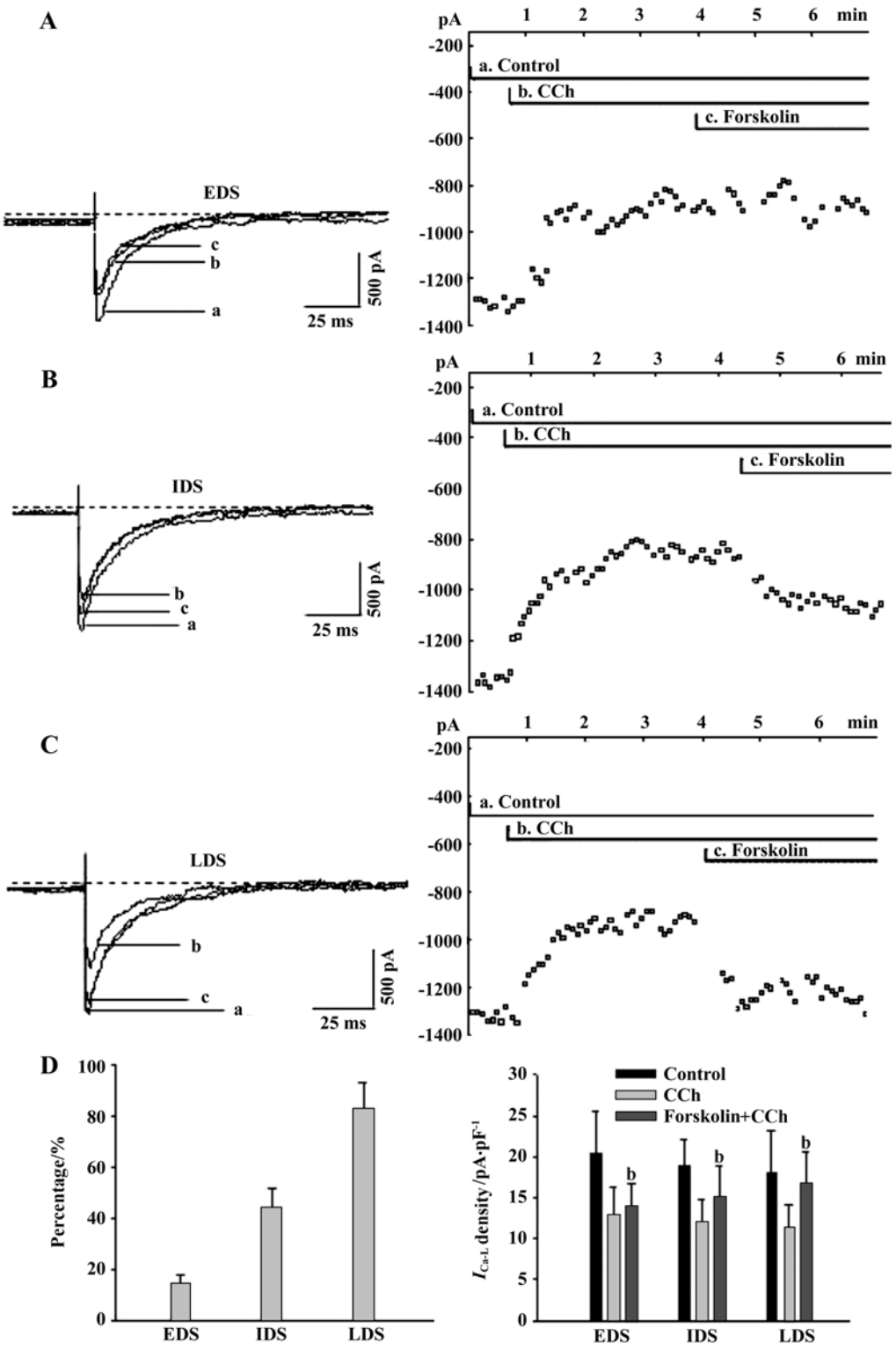

Fig 3. Effect of forskolin on muscarinic inhibitory action on $I_{Ca\text{-}L}$ during development. (A, B, C) Left: $I_{Ca\text{-}L}$ current traces were recorded in EDS, IDS, and LDS, respectively. (a) Before the superfusion with CCh 1 μmol/L; (b) After superfusion with CCh 1 μmol/L; (c) After the superfusion 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: $I_{Ca\text{-}L}$ 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. $^{b}P<0.05$ *vs* CCh.

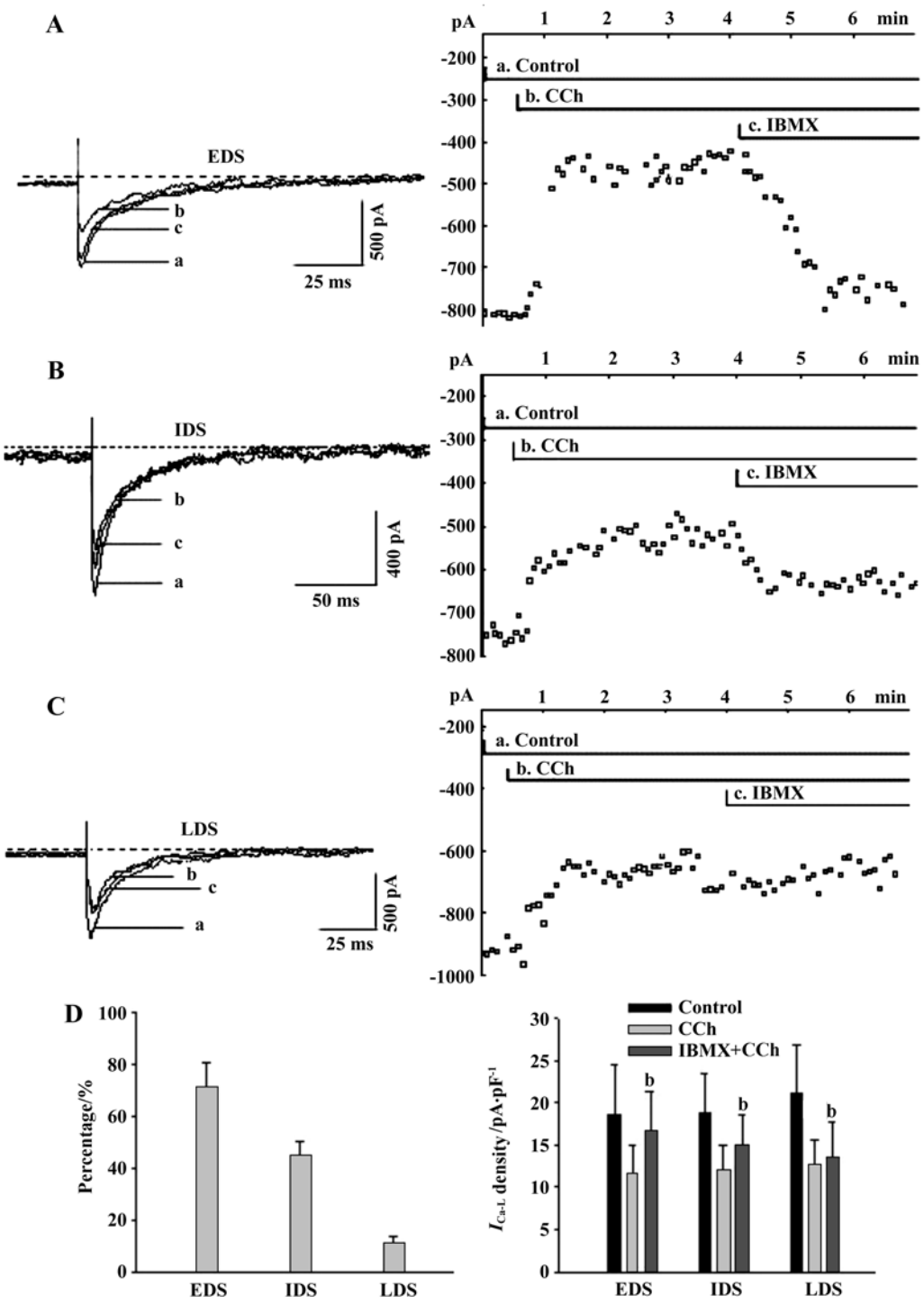

Fig 4. Effect of IBMX on muscarinic inhibitory action on $I_{Ca\text{-}L}$ during development. (A, B, C) Left: $I_{Ca\text{-}L}$ current traces were recorded in EDS, IDS, and LDS, respectively. (a) Before the superfusion with CCh 1 μmol/L; (b) After the superfusion with CCh 1 μmol/L; (c) After the superfusion 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: $I_{Ca\text{-}L}$ 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. $^{b}P<0.05$ *vs* CCh.

cyclase-mediated inhibition. FASEB J 1999; 13: 313-24.

2 Song GL, Tang M, Liu CJ, Luo HY, Liang HM, Hu XW, *et al*. Developmental changes in functional expression and β-adrenergic regulation of I_f in the heart of mouse embryo. Cell Res 2002; 12: 385-94.

3 Abi-Gerges N, Ji GJ, Lu ZJ, Feschmaeister R, Hescheler J, Fleisschmann BK. Functional expression and regulation of the hyperpolarization activated non-selective cation current

in embryonic stem cell-derived cardiomyocytes. J Physiol 2001; 523: 377-89.
4 Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK, Hescheler J. Establishment of β-adrenergic modulation of *L*-type Ca^{2+} current in the early stages of cardiomyocyte development. Circ Res 1999; 84: 136-45.
5 Reuter H. Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 1983; 301: 569-74.
6 Trautwein W, Hescheler J. Regulation of cardiac *L*-type calcium current by phosphorylation and G-proteins. Annu Rev Physiol 1990; 52: 257-74.
7 Han X, Kubota I, Feron O, Opel DJ, Arstall MA, Kelly RA, *et al*. Muscarinic cholinergic regulation of cardiac myocyte $I_{Ca\text{-}L}$ 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.
9 Robert DH, Andriy EB. Muscarinic regulation of cardiac ion channels. Br J Pharmacol 2003; 139: 1074-84.
10 Conti M, Nemoz G, Sette C, Vicini E. Recent progress in understanding the hormonal regulation of phosphodiesterases. Endocr Rev 1995; 16: 370-89.
11 Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995; 75: 725-48.
12 Stoclet JC, Keravis T, Komas N, Lugnier C. Cyclic nucleotide phosphodiesterases as therapeutic targets in cardiovascular diseases. Exp Opin Invest Drugs 1995; 4: 1081-100.
13 Rivet-Bastide M, Vandecasteele G, Hatem S, Verde I, Benardeau A, Mercadier JJ, *et al*. cGMP-stimulated cyclic nucleotide phosphodiesterase regulates the basal calcium current in human atrial myocytes. J Clin Invest 1997; 99: 2710-8.
14 Abi-Gerges N, Fischmeister R, Pierre-François M. G protein-mediated inhibitory effect of a nitric oxide donor on the *L*-type Ca^{2+} current in rat ventricular myocytes. J Physiol 2001; 531: 117-30.
15 Hare JM, Kim B, Flavahan NA, Ricker KM, Peng X, Colmanand L, *et al*. Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. J Clin Invest 1998; 101: 1424-31.
16 An RH, Davies MP, Doevendans PA, Kubalak SW, Bangalore R, Chien KR. Developmental changes in beta-adrenergic modulation of *L*-type Ca^{2+} channels in embryonic mouse heart. Circ Res 1996; 78: 371-8.
17 Osaka T, Joyner RW. Developmental changes in the beta-adrenergic modulation of calcium currents in rabbit ventricular cells. Circ Res 1992; 70: 104-15.
18 Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch clamp techniques for high resolution current recording from cells and cell free membrane patches. Pflügers Arch 1981; 391: 85-100.
19 Belles B, Malecot CO, Hescheler J, Trautwein W. "Run-down" of the Ca current during long whole-cell recordings in guinea pig heart cells: role of phosphorylation and intracellular calcium. Pflügers Arch 1988; 411: 353-60.
20 Hartzell HC, Mery PE, Fischmeister R, Szabo G. Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. Nature (London) 1991; 351: 573-6
21 Habuchi Y, Nishio M, Tanaka H, Yamamoto T, Lu LL, Yoshimura M. Regulation by acetylcholine of Ca^{2+} current in rabbit atrioventricular node cells. Am J Physiol 1996; 271: H2274-82.
22 Slotkin TA, Lau C, Seidler FJ. Beta-adrenergic receptor overexpression in the fetal ratdistribution, receptor subtypes, and coupling to adenylate cyclase activity via G-proteins. Toxicol Appl Pharmacol 1994; 129: 223-34.
23 Bloch W, Fleischmann BK, Lorke DE, Andressen C, Hops B, Hescheler J, *et al*. Nitric oxide synthase expression and role during cardiomyogenesis. Cardiovasc Res 1999; 43: 675-84.