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Developmental changes in functional expression and β-adrenergic regulation of I_f in the heart of mouse embryo

GUI LI SONG[1], MING TANG[1,*], CHANG JIN LIU[1], HONG YAN LUO[1], HUA MIN LIANG[1], XIN WU HU[1], JIAOYA XI[1], LIN LIN GAO[1], BERND FLEISCHMANN[2], JÜRGEN HESCHELER[2]

[1] *Department of Physiology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China*
[2] *Institute of Neurophysiology, University of Cologne, D-50931 Cologne, Germany*

ABSTRACT

The hyperpolarization-activated current (I_f) plays an important role in determining the spontaneous rate of cardiac pacemaker cells. The automatic rhythmicity also exists in working cells of embryonic heart, therefore we studied developmental changes in functional expression and β-adrenergic regulation of I_f in embryonic mouse heart. The expression of I_f is high in early developmental stage (EDS) (10.5 d after coitus) ventricular myocytes, low in intermediate developmental stage (IDS) (13.5 d) atrial or ventricular myocytes and even lower in late developmental stage (LDS) (16.5 d) atrial or ventricular myocytes, indicating that these cells of the EDS embryonic heart have some properties of pacemaker cells. β-adrenergic agonist isoproterenol (ISO) stimulates I_f in LDS but not in EDS cardiomyocytes, indicating that the b-adrenergic regulation of If is not mature in EDS embryonic heart. But forskolin (a direct activator of adenylate cyclase) and 8-Br-cAMP (a membrane-permeable analogue of cAMP) increase the amplitude of I_f in EDS cells, indicating that adenylate cyclase and cAMP function fairly well at early stage of development. Furthermore, the results demonstrate that I_f is modulated by phosphorylation via cAMP dependent PKA both in EDS and LDS cells.

Key words: *embryonic cardiomyocyte, development, I_f, β-adrenergic.*

INTRODUCTION

The hyperpolarization-activated current (I_f) is a nonselective inward current activated on hyperpolarization during the diastolic depolarization period of action potential in heart sino-atrial node and Purkinje system, and plays an important role in the generation and regulation of pacemaker activity in both primary and secondary pacemaker cells[1-3]. The biophysical characteristics of I_f comprise time-dependent activation kinetics, a linear current-voltage (I-V) relationship and voltage-dependent block upon extracellular application of Cs^+. It has been well documented that both sympathetic and parasympathetic control of heart rate involves modulation of I_f[4]. In adult mammalian heart, β-adrenergic agonist isoproterenol (ISO) stimulates I_f by shifting the activation curve to more positive potentials [1], whereas the vagal neurotransmitter acetycholine (ACh) inhibits I_f by shifting the activation curve to more negative potentials[5]. This control is exerted through modulation of adenylate cyclase (AC) and cAMP[6]. Recently, a multigenic family of pacemaker channels has been cloned, the name of which is hyperpolarization-activated and cyclic nucleotide-gated (HCN) channel genes. The I_f channel of heart may be encoded by HCN4 or HCN2[7],[8].

* **Corresponding author:** Prof. Ming TANG
Department of Physiology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China
Tel: 0086-27-83692622, 0086-27-83639950,
E-mail: tangming49@hotmail.com
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Though predominantly expressed in the conductive system of adult heart, I_f is also present in ventricular myocytes of immature heart since it has been recorded in chick embryonic ventricle[9]and neonatal rat ventricle[10]. It is well known that hypertrophy induces a re-expression of genes encoding fetal proteins[11], therefore the currents exist during the fetal life may occur in pathological heart. In fact, I_f is present in ventricular myocytes of hypertensive rat[12] as well as recently in human failing heart [13]. The presence of I_f in ventricular myocytes may have relevance with arrhythmogenesis in hypertrophy and heart failure[28]. Murine embryonic stem (ES) cells are being used by our collaborator to study cardiomyocyte development[14]. The developmental changes in I_f channel are among the major interests. The ES cells differentiation in vitro should be similar to the corresponding process happening in vivo. We studied the functional expression and β-adrenergic regulation of the current I_f in the heart of embryonic mouse instead of ES cell derived cardiomyocytes. Compared with the data resulted from ES cells[15], the present study exposed us to the fact how I_f channel progresses during heart development in vivo. We found that I_f is highly expressed but can not be stimulated by ISO in early stage cardiomyocytes. ISO stimulates I_f at late stage of development, but the expression of the current decreases greatly compared with that of early stage. We further show that I_f is modulated by phosphorylation via protein kinase A (PKA) both in early and late stage cardiomyocytes.

MATERIALS AND METHODS

Preparation of embryonic cardiomyocytes

Embryos were removed from pregnant female mice. Through our study 10.5 d, 13.5 d and 16.5 d after coitus were considered as EDS, IDS and LDS. After hearts were dissected from embryos, atrial and ventricular tissues were separated under a dissecting microscope and incubated in an Eppendorf tube with enzyme-containing solution (1 mg/ml collagenase B, Roche Molecular Biochemicals, Mannheimm, Germany) for 30 min at 37 ±0.3℃. Isolated cells were cultured on sterile, gelatin-coated glass cover slips in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 20% fetal bovine serum for 18-24 h before current recording.

Electrophysiology

The cells were placed in a temperature-controlled (37±0.3℃) recording chamber mounted on the stage of an inverted microscope (Zeiss, Germany) and superfused with the normal Tyrode's solution containing (m*M*): NaCl 140, NaOH 2.3, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 10, Glucose 10, pH 7.4 (adjusted with NaOH). When If was recorded, the normal Tyrode's solution was modified by adding (m*M*): $CdCl_2$ 0.5, $BaCl_2$ 1, 4-aminopyridine 2. Extracellular application of drugs was performed by superfusing cells with Tyrode's solution containing the drugs.

In our experiment only spontaneously beating single cardiomyocyte was selected using the whole-cell configuration of the patch-clamp technique[16]. The cells were held in voltage-clamp or current-clamp mode using an Axopatch 200-A amplifier (Axon Instruments, CA, USA). In voltage-clamp experiments, I_f was elicited by a hyperpolari n 10 mV increments (holding potential -35 mV). For analysis of the I-V relationship, after a 2000 ms hyperpolarizing voltage step to -115 mV, depolarizing voltage steps (1500 ms) from -110 to +50 mV in 10 mV increments were used to elicit tail current.

Patch pipettes prepared from glass capillary tubes (Liuhe Laboratory Apparatus Factory, Nanjing, China) by means of a two-stage vertical puller (David Kopf Instruments) had a resistance of 2-5 MΩ when filled with the pipette solution (m*M*): NaCl 10, potassium aspartate 130, Na_2ATP 2, EGTA 1, $MgCl_2$ 2, Na_2GTP 0.1, Hepes 10, pH 7.2 (adjusted with KOH). Cell membrane capacitance (Cm) was determined on-line using the ISO2 (MFK, Frankfurt, FRG) software program. Data were acquired at a sampling rate of 10 kHz, filtered at 2kHz, stored on hard disk and analyzed off-line using the ISO2 analysis software package.

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 [17]. Currents were normalized to membrane capacitance to calculate current densities. The steady-state activation curve was constructed from the amplitude of time-dependent inward current during hyperpolarizing steps (-40 mV to -140 mV). Specific conductance of If was determined for each cell according to the equation g=I/(Vm-Vrev), where g is the conductance calculated at the membrane potential Vm, I is the current amplitude, and Vrev is calculated from the analysis of tail currents. The values were normalized to the maximal current conductance (g_{max}) and fitted with the Boltzmann equation: $g/g_{max}=1/\{1+\exp[(V1/2-Vm)/S]\}$, where Vm is the membrane voltage, V1/2 is the voltage at half-maximal activation, and S is a slope factor at Vm=V1/2. For I-V relation, the amplitude and reversal potential of currents were analyzed from the tail current measured between peak current (15 ms after depolarization to omit possible interference of sodium or capacitance currents[18]) and maintained current at the end of the clamp pulse. Data are presented as mean±standard error of the mean (S.E.M) when appropriate. Statistical analysis was performed using Student's paired or unpaired t tests and values of $P < 0.05$ were considered significant.

Reagents

The following chemicals were all purchased from Sigma:

protein kinase A inhibitor (PKI), ISO, 8-Br-cAMP, forskolin, Hepes, CsCl, and 4-aminopyridine. PKI was dissolved in pipette solution and stored frozen at -20°C. The PKI aliquots were thawed immediately prior to use and diluted with pipette solution to the desired concentration. After the whole cell configuration was constructed, PKI was dialyzed into the cell through the pipette. 8-Br-cAMP was stored and used in a dark room. The substances for cell culture were purchased from Gibco. And the other chemicals, if not stated, were all from Chinese companies.

RESULTS

Electrophysiological properties of I_f

We used the EDS ventricular myocytes to identify the electrophysiological characteristics of I_f . A serial of hyperpolarizing steps in 10 mV increments from -40 mV to -140 mV (holding potential -35mV) elicited time-dependent and voltage-dependent inward currents. The current traces are showed in Fig 1A, and the amplitudes of I_f were measured in four EDS ventricular myocytes. Specific conductance of I_f was normalized and fitted with the Boltzmann equation,

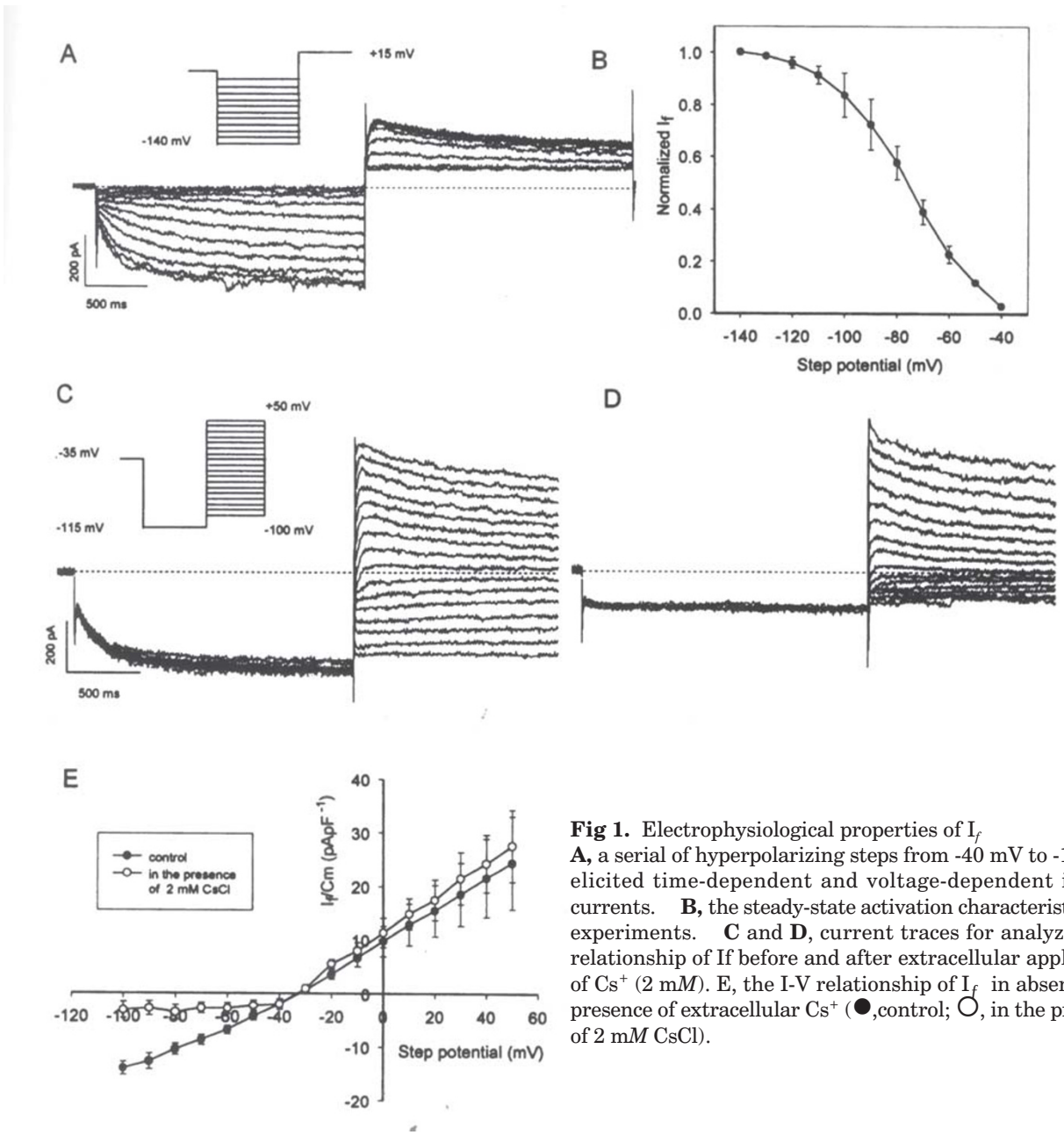

Fig 1. Electrophysiological properties of I_f
A, a serial of hyperpolarizing steps from -40 mV to -140 mV elicited time-dependent and voltage-dependent inward currents. **B,** the steady-state activation characteristics of 4 experiments. **C** and **D**, current traces for analyzing I-V relationship of If before and after extracellular application of Cs^+ (2 m*M*). E, the I-V relationship of I_f in absence and presence of extracellular Cs^+ (●,control; ○, in the presence of 2 m*M* CsCl).

yielding an activation threshold around -40 mV, a half maximal activation voltage ($V_{1/2}$) of -76±2.24 mV (n=4) and a slope factor (S) of -16±1.89 mV^{-1} (n=4) (Fig 1B). Fig 1C and D show the current traces for analyzing I-V relationship of I_f before and after extracellular application of Cs^+ (2 m*M*). Compared with the control condition (Fig 1C), the inward currents were almost completely blocked by 2 m*M* CsCl (Fig 1D). Fig 1E shows the I-V relation of I_f in absence and presence of extracellular Cs^+. Under the control condition, the I-V curve is relatively linear with a reversal potential of -32.6±1.42 mV (n=5). In the presence of Cs^+, at potentials negative to the reversal potential of I_f, the I-V curve demonstrates a voltage dependent inhibition of I_f by Cs^+. But when positive to the reversal potential, the amplitude of I_f evoked by depolarizing voltage steps is unaltered. Our results demonstrate that the electrophysiological properties of I_f are similar to those reported in adult mammalian heart[3],[19],[20],

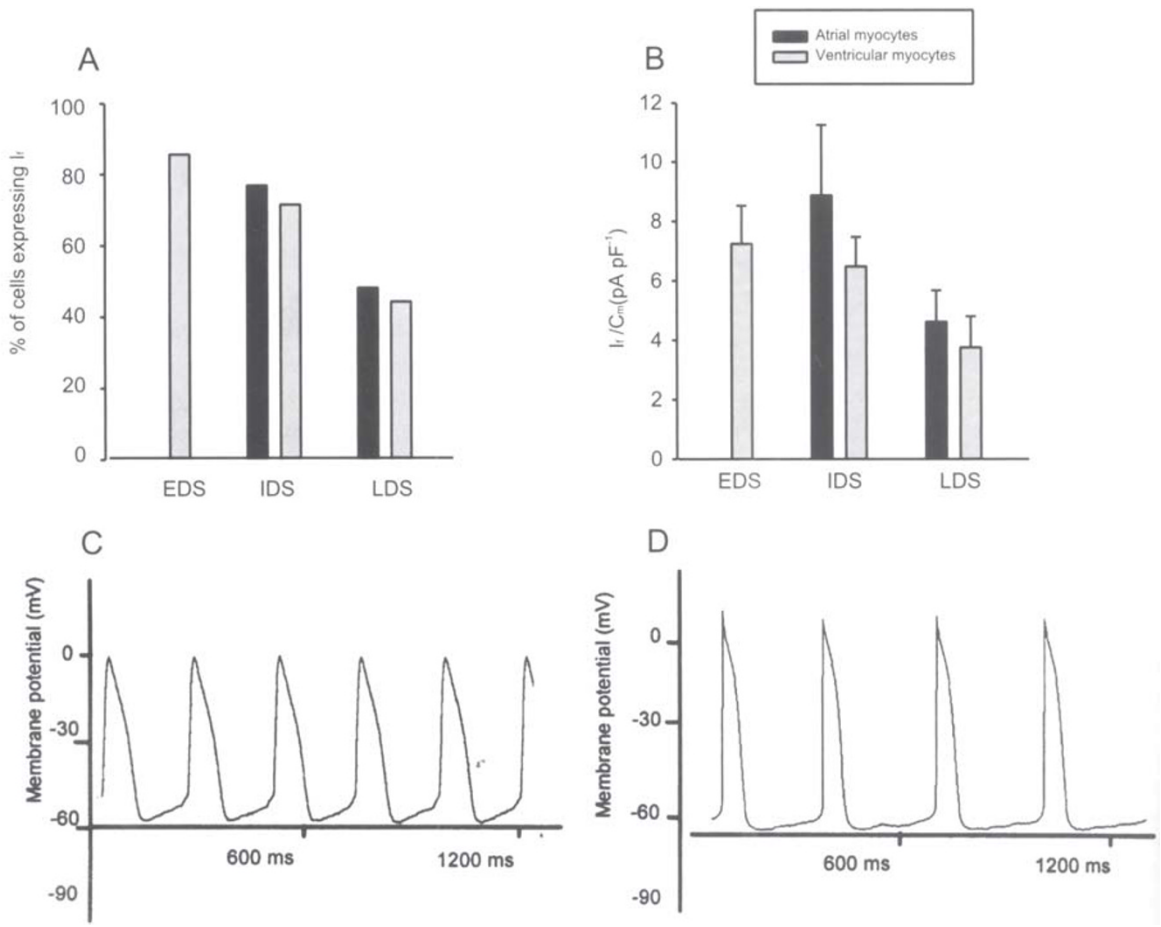

Fig 2. The decreases in expression of I_f during development
A, the percentage of atrial and ventricular myocytes expressing If. **B,** decreases in the current densities (I_f/C_m, where Cm is the membrane capacitance) of I_f during development. *Statistically significant difference between LDS and EDS or IDS at the $P < 0.05$ level was performed using Student's unpaired t test. I_f was evoked at -110 mV, lasting for 2000 ms. **C,** the spontaneous action potentials (AP) recorded from an EDS ventricular myocyte. **D,** the action potentials of a LDS ventricular myocyte.

The decreases in expression of I_f during development

To detect the functional expression of I_f in the heart at different stage of development, we recorded and calculated the densityof I_f and the percentage of cells expressing it. The heart of IDS and LDS is big enough for us to separate atrium from ventricle, but

that of EDS is so small that only ventricles were collected. Fig 2A shows the percentage of atrial and ventricular myocytes expressing I_f at different stage of development. I_f was detected in a large percentage (85.7%, n= 21) of EDS ventricular cells, but fewer (71.6%, n=29) in IDS and fewest (44.4%, n=27) in LDS. Moreover, the percentage of atrial cells expressing I_f also declines from 77% (n=8) in IDS to 48.3% (n=25) in LDS. Fig 2B illustrates decrease in the densities of If during development. The I_f densities were 7.24 ± 1.28 pA pF^{-1} (n=18) and 6.48 ± 0.99 pA pF^{-1} (n=21) in EDS and IDS ventricular myocytes, respectively. LDS ventricular cells display a significantly lower current density of 3.76 ± 1.04

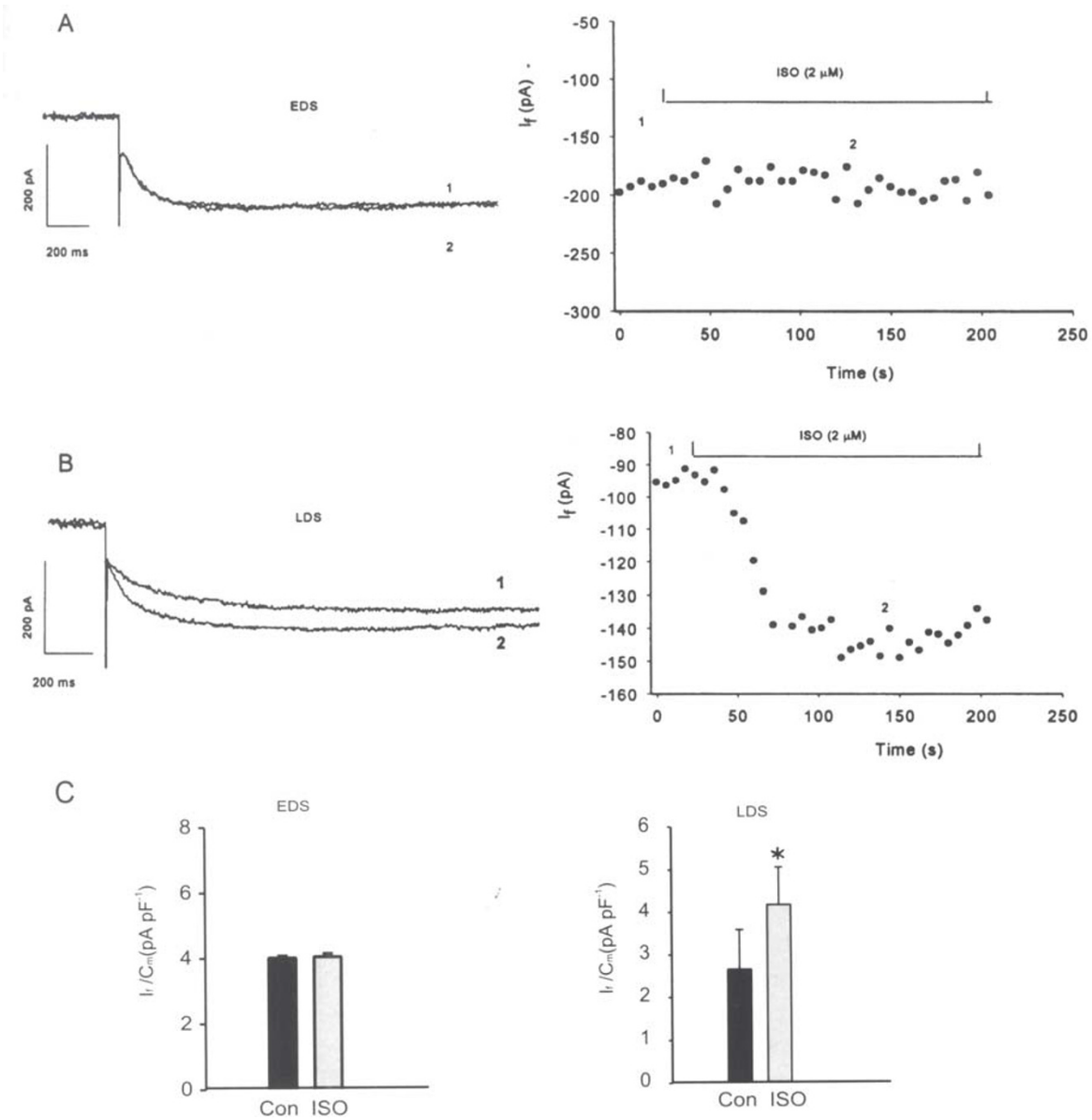

Fig 3. Isoproterenol stimulates I_f in LDS but not EDS ventricular myocytes.
A, the effect of ISO (2 μM) on I_f in an EDS ventricular myocyte. **B,** the effect of ISO (2 μM) on If in a LDS ventricular myocyte. (left panel: the current traces of I_f ; right panel: time course of the experiments, voltage protocol identical to Fig 2). 1, control; 2, ISO (2 μM). **C,** the difference in current densities between the absence and presence of ISO was resulted from the test of EDS and LDS ventricular myocytes. *The difference from the test of LDS ventricular myocytes was statistically significant ($P < 0.05$, paired t test).

pA pF^{-1} (n=12, $P < 0.01$), as compared with EDS and LDS ventricular cells. Current density of atrial cells were also decreased significantly from 8.86±2.34 pA pF^{-1} (n=6) in IDS to 4.62±1.06 pA pF^{-1} (n=12, $P < 0.01$in LDS. Since I_f may play an important role in determining the diastolic depolarization phase of action potential (AP), a typical recording of AP from spontaneously contracting ventricular myocytes was performed in the current-clmap mode. Fig 2C illustrates that in the AP of an EDS ventricular myocyte, there is a slow repolarization after the upstroke and a rapid diastolic depolarization. In a LDS ventricular myocyte (Fig 2D), the diastolic phase of AP is flat, but that in the EDS ventricular cell is fairly steep.

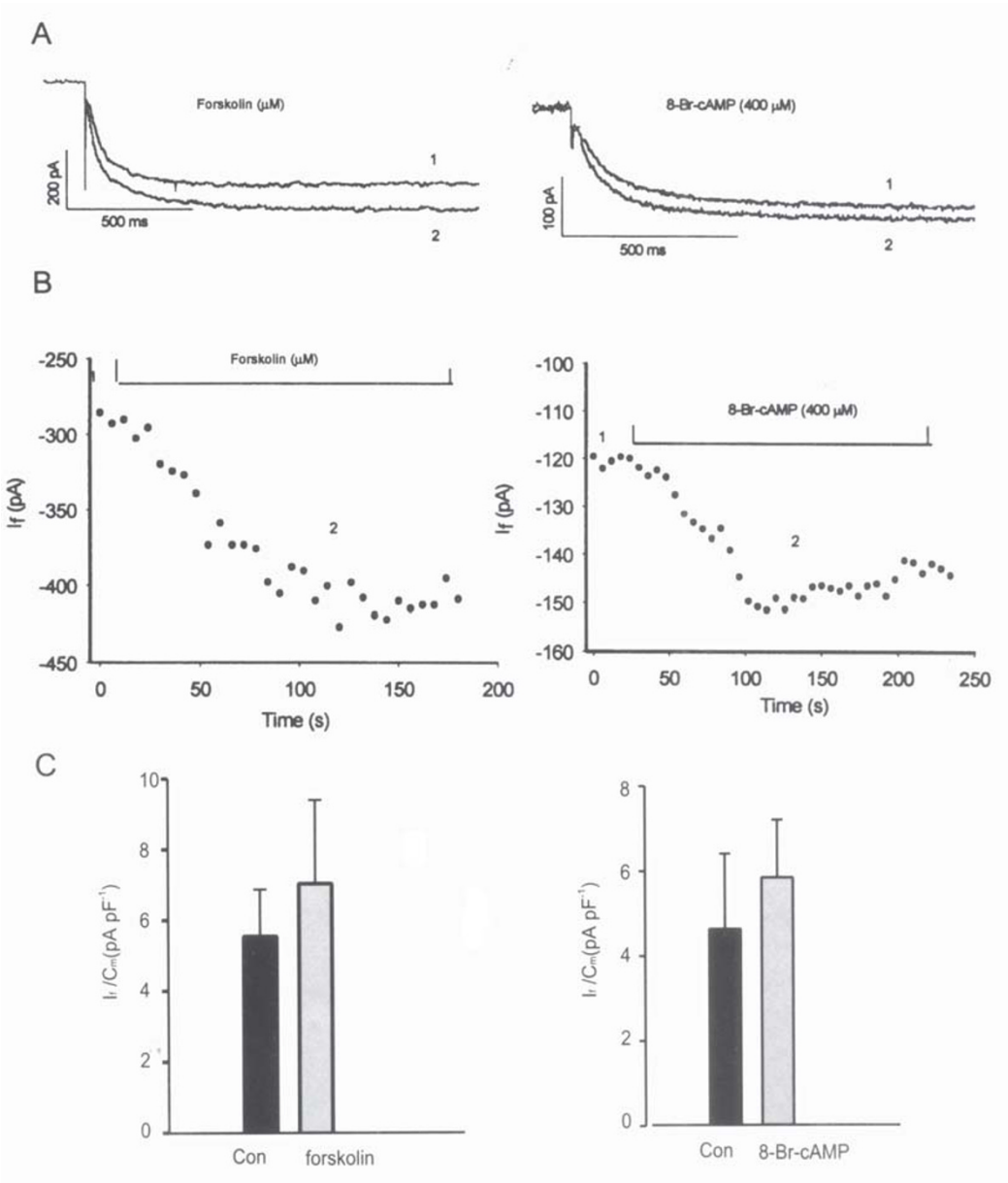

Fig 4. Effects of forskolin and 8-Br-cAMP on I_f in EDS ventricular myocytes.
Both forskolin (μM) and 8-Br-cAMP (400 μM) can increase the size of I_f(voltage protocol identical to Fig 2) in EDS ventricular myocytes, 1, control; 2, application of drugs. **A,** the current traces of I_f. **B,** time course of the experiments. **C,** the I_f densities were increased apparently after application of forskolin or 8-Br-cAMP.

Isoproterenol stimulates I_f in LDS but not EDS cardiomyocytes

It is well know that If increases in response to ISO in adult cardiomyocytes[1],[21]. We tested β-adrenergic regulation of If at -110 mV. Our results show that ISO stimulated I_f in LDS but not EDS cardiomyocytes. In Fig 3A and B, current traces (left panel) and corresponding time course (right panel) of typical experiments are displayed. Shortly after extracellular application of ISO (2 μM), If increased in a LDS ventricular cell (Fig 3B). Fig 3C (right panel) shows that ISO increased the averaged current density significantly from 2.66 ± 0.93 pA pF^{-1} to 4.18 ± 0.88 pA pF^{-1} (n=5, $P < 0.05$) in LDS ventricular cells. The stimulation of I_f by ISO in LDS atrial cells was also recorded. I_f densities in control condition and in the presence of ISO were 4.03 ± 1.82 pA pF^{-1} and 6.34 ± 1.85 pA pF^{-1} (n=6, $P < 0.05$), respectively. But in EDS ventricular cells I_f did not change apparently (Fig 3A). Fig 3C (left panel) shows that I_f densities of EDS ventricular cells before and after ISO application were 4.01 ± 0.06 pA pF^{-1} and 4.05 ± 0.10pA pF^{-1} (n=6).

Effects of forskolin and cAMP on I_f in EDS cardiomyocytes

Because ISO did not stimulate I_f in EDS ventricular cardiomyocytes, we tested the effects of forskolin (a direct activator of AC) and 8-Br-cAMP (a membrane-permeable analogue of cAMP) on I_f at early stage of development. The current traces of I_f (Fig 4A) as well as the time course of experiments (Fig 4B) demonstrate that both forskolin and 8-Br-cAMP increase the amplitude of the current. A summary of these data are showed in Fig 4C, where I_f densities under control conditions and after application of forskolin (3 μM) were 5.54 ± 1.33 pA pF^{-1} and 7.04 ± 2.35 pA pF^{-1} (n=4), respectively. Similarly, after extracellular application of 8-Br-cAMP (400 μM), I_f densities were enhanced from 4.64 ± 1.77 pA pF^{-1} to 5.85 ± 1.35 pA pF^{-1} (n=4). Altogether, these experiments suggest that in the β-adrenergic signaling cascade system, AC and cAMP function fairly well at early stage development.

I_f is stimulated by phosphorylation via PKA in EDS and LDS cardiomyocytes

It is well known that cAMP is the second messenger of β-adrenergic signaling cascade system. The former results show that cAMP increase the amplitude of I_f both in LDS and EDS cardiomyocytes. We further studied whether the effect of cAMP on I_f was mediated through a direct cAMP binding and/or an indirect phosphorylation via cAMP-dependent PKA at these two developmental stages. In order to differentiate between these two regulatory pathways, we test the effects of ISO and 8-Br-cAMP on If again after 15 min of cell dialysis with PKI, a highly selective peptide inhibitor of PKA[22]. Fig 5A shows that the stimulation of ISO (2 μM) on I_f was greatly depressed by intracellular perfusion with PKI (1 mg/ml) in a LDS ventricular myocyte. I_f density under control conditions is 2.30 ± 1.02 pA pF^{-1} (n=3). After 15 min of intracellular dialysis with PKI, the current density in presence of ISO (2 μM) was 2.31 ± 1.14 pA pF^{-1} (n=3). Moreover, ISO had no effect on If under this condition in LDS atrial myocytes (n=3, data not shown). Fig 5B demonstrates that in an EDS ventricular myocyte, 8-Br-cAMP (400 μM) did not stimulate If after intracellular perfusion with PKI (1mg/ml). The I_f densities under control conditions and in presence of 8-Br-cAMP after dialysis with PKI were 3.82 ± 0.28 pA pF^{-1} and 3.99 ± 0.22 pA pF^{-1} (n=4), respectively. As it is noted before, in EDS ventricular myocytes, ISO cannot stimulate I_f, but cAMP can increase the amplitude of the current. Here, the block of PKI on the effect of cAMP in EDS ventricular myocytes was observed.

DISCUSSION

The present results demonstrated that both I_f densities and the percentage of cells expressing it are high in the working cells during early stage development of murine embryo. Since I_f is essential for cell' s pacemaker activity[2-4], the presence of which might imply that there is automatic rhythmicity in these cells. In other word, these cells have not differentiated from pacemaker cells to mature working cells. This point can also be deduced from the AP we recorded from the spontaneously beating ventricular myocytes. The ventricular myocyte of early stage has an AP profile similar to that of adult sino-atrial node cells, characterized by the slow repolarization after the upstroke and the rapid dias-

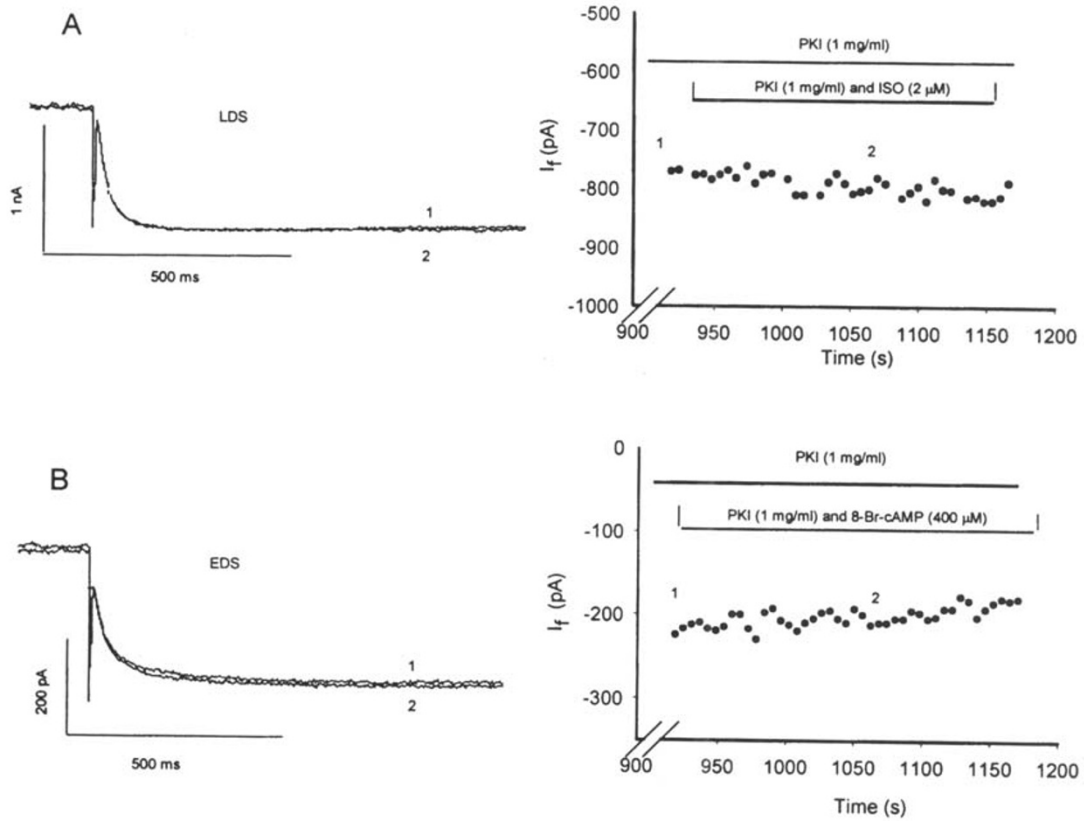

Fig 5. I_f is stimulated by phosphorylation via PKA in EDS and LDS ventricular myocytes.
A, the stimulation of ISO (2 μM) on If current was greatly depressed by intracellular perfusion with PKI (1 mg/ml) in a LDS ventricular myocyte. B, in an EDS ventricular myocyte, 8-Br-cAMP (400 μM) can't stimulate I_f any more under intracellular perfusion with PKI (1 mg/ml). Left panel, the current traces of If. Right panel, time course of the experiments. 1, PKI; 2, PKI and ISO or 8-Br-cAMP. Voltage protocol is identical to Fig 2.

tolic depolarization. At late stage of development, I_f densities and the percentage of cells expressing it are all significantly decreased both in atrial and ventricular myocytes; the AP profile of ventricular myocyte is more like the mature ventricle's. We conclude that I_f exists in most of the early stage cardiomyocytes, which have not differentiated completely. At late stage of development, when atrial and ventricular myocytes develop into mature working cells, which have no automatic rhythmicity, I_f will be greatly downregulated in these cells. But increased current densities were observed by our collaborator during cardiomyogenesis[15]. Maybe, only the cells of heart conduction system had been collected with the procedure they used, so there is some differences from atrial and ventricular myocytes. I_f is re-expressed in the adult ventricular myocytes isolated from heart failure and hypotrophy[11],[13],[23], therefore the automatic rhythmicity may reappear in these ventricular myocytes and the re-expression of I_f may have relevance with arrhythmogenesis in these heart diseases[17],[24]. Therefore, our research on I_f in the embryonic mouse heart is also useful to study pathophysiological phenomena of heart failure and hypertrophy.

It has been documented that f-channel, regulated by the autonomous system, is important for the regulation of heart rhythmicity. In the adult mammalian heart, the binding of β-adrenergic agonist to β-adrenergic receptor (β-AR) is coupled to an intracellular cascade by the stimulatory G protein, Gs. Agonist occupancy activates AC to increase intracellular concentrations of cAMP, which stimulates f-channels directly in sino-atrial node[25],[26]. But in Purkinje fibres, instead of the direct interaction between cAMP and f-channels, the phosphorylation of the channels is involved[27],[28]. Our study demonstrates that ISO stimulates I_f in LDS but not EDS

cardiomyocytes of murine embryo. We conclude that at early stage of development, the β-adrenergic regulation of I_f is not mature; the β-adrenergic signaling cascade is not able to work well until the late stage of development. We further find that foskolin and 8-Br-cAMP are able to increase the amplitude of If in EDS ventricular cells, indicating that AC and cAMP function fairly well. The lack of response to ISO of If in EDS cardiomyocytes could be due to the coupling deficiency precedes AC: either expressions of β-AR and the Gs-protein or their coupling mechanism.

Altogether, there is a cAMP-dependent regulation of I_f both in EDS and LDS embryonic cardiomyocytes. In order to detect whether the effect of cAMP on I_f was mediated through a direct cAMP binding or an indirect phosphorylation via PKA, we used PKI to test the response of I_f to 8-Br-cAMP in EDS ventricular myocytes. The result is that 8-Br-cAMP did not increase the amplitude of I_f after intracellular perfusion with PKI. Likewise, at late stage of development, the stimulation of ISO on I_f was abolished upon intracellular perfusion with PKI. We conclude that at early and late stage of development, the regulation of I_f channels appears to be mediated by phosphorylation via PKA rather than by a direct cAMP binding. This point is identical to our collaborator's finding resulted from ES cell derived cardiomyocytes.

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REFERENCES

[1] DiFrancesco D, Ferroni A, Mazzanti M and Tromba C. Properties of the hyperpolarization-activated current (I_f) in cells isolated from the rabbit sino-atrial node. J Physiol 1986; **377**:61-88.

[2] Irisawa H, Brown HF, Giles WR. Cardiac pacemaking in the sinoatrial node. Physiol Rev 1991; **73**:197-227.

[3] DiFrancesco D. Pacemaker mechanisms in cardiac tissue. Ann Rev Physiol 1993; **55**:455-72.

[4] DiFrancesco D. The onset and autonomic regulation of cardiac pacemaker activity: relevance of the f current. Cardiovasc Res 1995; **29**:449-56.

[5] DiFrancesco D. Rromba C. Muscarinic control of the hyperpolarization-activated current (If) in rabbit sino-atrial node myocytes. J Physiol 1988; **405**:493-510.

[6] DiFrancesco D. Cardiac pacemaker: 15 years of "new" interpretation. Acta Cardiol 1995; **50 (6)**:413-27.

[7] Ludwig A, Zong X, Jeglitsch M, Hofmann F, Biel M. A family of hyperpolarization-activated mammalian cation channels. Nature 1998; **393**:587-91.

[8] Seifert R, Scholten A, Gauss R, Mincheva A, Lichter P, Kaupp UB. Molecular characterization of a slowly gating human hyperpolarization-activated channel predominantly expressed in thalamus, heart, and testis. Proc Natl Acad Sci USA 1999; **96**:9391-6.

[9] Stoh H, Sperelakis N. Identification of the hyperpolarization-activated current in young embryonic chick heart myocytes. J Dev Physiol 1991; **15**:247-52.

[10] Cerbai E, Pino R, Sartiani L, Mugelli A. Influence of postnatal development on I_f occurrence and properties in neonatal rat ventricular myocytes. Cardiovasc Res 1999; **42**:416-23.

[11] Van Bilsen M, Chien KR. Growth and hypertrophy of the heart: towards and understanding of cardiac specific and inducible gene expression. Cardiovasc Res 1993; **27**: 1140-9.

[12] Cerbai E, Barbieri M, Mugelli A. Occurrence and properties of the hyperpolarization-activated current I_f in ventricular myocytes from normotensive and hypertensive rats during aging. Circulation 1996; **94:**1674-81.

[13] Cerbai E, Pino R, Porciatti F et al. Characterization of the hyperpolarization-activated current, I_f, in ventricular myocytes from human failing heart. Circulation 1997; **95**:568-71.

[14] Hescheler J, Fleischmann BK, Lentini S et al. Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. Cardiovasc Res 1997; **36(2)**:149-62.

[15] Abi-Gerges N, Ji GJ, Lu ZJ, Fischmeister R, Hescheler J and Fleischmann BK. Functional expression and regulation of the hyperpolarization activated non-selective cation current in embryonic stem cell-derived cardiomyocytes. J Physiol 2000; **523**:377-89.

. [16] 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. PflÜ1gers Arch 1981; **391**:85-100.

[17] Cerbai E, Barbieri M, Mugelli A. Characterization of the hyperpolarization-activated current, I_f, in ventricular myocytes isolated from hypertensive rats. J Physiol 1994; **481**:585-91.

[18] Hoppe UC, Beuckelmann DJ. Characterization of the hyperpoarization-activated inward current in isolated human atrial myocytes. Cardiovasc Res 1998; **38**:788-801.

[19] DiFrancesco D. Characterization of single pacemaker channels in cardiac sino-atrial node cells. Nature 1986; **324**:470-3.

[20] Callewaert G, Carmeliet E, Vereecke J. Single cardiac Purkinje cells: general electrophysiology and voltage-clamp analysis of the pacemaker current. J Physiol 1984; **349**:643-61.

[21] Zhou Z, Lipsius SL. Properties of the pacemaker current (I_f) in latent pacemaker cells isolated from cat right atrium. J Physiol 1992; **453**:503-23.

[22] Donal A, Walsh, Charles D et al. Purification and charac-

terization of a protein inhibitor of adenosine 3', 5'-monophosphate-dependent protein kinases. J Biol Chem 1971; **246**:1977-85.
[23] Hoppe UC, Jansen E, Sudkamp M, Beuckelmann DJ. Hyperpolarization-activated inward current in ventricular myocytes from normal and failing human hearts. Circulation 1998; **97**:55-65.
[24] Cerbai E, Barbieri M, Porciatti F, Mugelli. Ionic channels in hypertrophy and heart failure relevance for arrthymogenesis. New Trends Arrhyth 1995; **9**:135-9.
[25] DiFrancesco D, Tortora P. Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 1991; **351**:145-7.
[26] Bois P, Renaudon B, Baruscotti M, Lenfant J, Difrancesco D. Activation of f-channels by cAMP analogues in maropatches from rabbit sino-atrial node myocytes. J Physiol 1997; **501**:565-71.
[27] Yu H, Chang F, Cohen IS. Pacemaker current exists in ventricular myocytes. Circ Res 1993; **72**:232-36.
[28] Chang F, Cohen IS. Mechanism of acetylcholine action on pacemaker current (I_f) in canine Purkinje fibers. PflÜ 1gers Arch 1992; **420**:389-92.