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Roles of I(f) and Intracellular Ca^{2+} Release in Spontaneous Activity of Ventricular Cardiomyocytes During Murine Embryonic Development

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ABSTRACT

In recent years, the contribution of I(f), an important pacemaker current, and intracellular Ca^{2+} release (ICR) from sarcoplasmic reticulum to pacemaking and arrhythmia has been intensively studied. However, their functional roles in embryonic heart remain uncertain. Using patch clamp, Ca^{2+} imaging, and RT-PCR, we found that I(f) regulated the firing rate in early and late stage embryonic ventricular cells, as ivabradine (30 μM), a specific blocker of I(f), slowed down action potential (AP) frequency. This inhibitory effect was even stronger in late stage cells, though I(f) was down-regulated. In contrast to I(f), ICR was found to be indispensable for the occurrence of APs in ventricular cells of different stages, because abolishment of ICR with ryanodine and 2-aminoethoxydiphenyl borate (2-APB), specific blockers of ryanodine receptors (RyRs) and inositol trisphosphate receptors (IP3Rs), completely abolished APs. In addition, we noticed that RyR- and IP3R-mediated ICR coexisted in early-stage ventricular cells and RyRs functionally dominated. While at late stage RyRs, but not IP3Rs, mediated ICR. In both early and late stage ventricular cells, Na-Ca exchanger current ($I_{Na/Ca}$) mediated ICR-triggered depolarization of membrane potential and resulted in the initiation of APs. We also observed that different from I(f), which presented as the substantial component of the earlier diastolic depolarization current, application of ryanodine, and/or 2-APB slowed the late phase of diastolic depolarization. Thus, we conclude that in murine embryonic ventricular cells I(f) regulates firing rate, while RyRs and IP3Rs (early stage) or RyRs (late stage)-mediated ICR determines the occurrence of APs. J. Cell. Biochem. 114: 1852–1862, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: ACTION POTENTIAL; Ca^{2+} OSCILLATION; RYANODINE RECEPTOR; IP3 RECEPTOR

Spontaneous electrical activity of cardiomyocytes is critical for the rhythmic beating and arrhythmogenesis. Previous studies on spontaneous electrical activity of cardiomyocytes have been largely limited to membrane-delimited pacemaker mechanisms driven by ion channels or ion carriers, envisioned as a surface "membrane clock," including voltage-gated Ca^{2+} channels, and hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels (also termed I(f), cardiac pacemaker "funny" channel), delayed rectifying K^+ channels, sustained and background Na^+ channels and Na^+–K^+ pump [Hagiwara et al., 1992; Guo et al., 1995; Ju and Allen, 1998; Schram et al., 2002; Baruscotti and Difrancesco, 2004]. The I(f) current, originally identified in sinoatrial node myocytes, is an mixed Na^+–K^+ inward current activated on hyperpolarization to the diastolic range of voltages. Extensive research has shown that I(f) is a very important pacemaking relevant current and plays important role in the regulation of spontaneous activity by participating in diastolic depolarization (DD) in adult sinoatrial nodal cells [Noma et al., 1983; Denyer and Brown, 1990; Choi et al., 1999]. A selective f-channel inhibitor, ivabradine, is commercially available as heart

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rate slowing agent for stable chronic angina. The correlation between I(f) and pacemaking is also evidenced by the finding that mutation to arginine of serine 672, a highly conserved residue in the cyclic nucleotide-binding domain of hHCN4, cause inherited asymptomatic sinus bradycardia in a large Italian family [Milanesi, 2006].

In embryonic ventricular cells, I(f) was found to be relevant to spontaneous electrical activity by an indirect evidence that loss of pacemaker potency is in association with a loss of I(f) current [Yasui et al., 2001; Lakatta and DiFrancesco, 2009]. In recent years, Ca^{2+} cycling-driven events, referred to as an intracellular "Ca^{2+} clock," have provided new insight into the mechanisms that drive pacemaker function and control heart rate. It is suggested that the periodic oscillations of local intracellular Ca^{2+} originated from sarcoplasmic reticulum is critical for the initiation of spontaneous activity of cardiomyocytes during early murine cardiogenesis and adult rabbit sinoatrial nodal cells [Viatchenko-Karpinski et al., 1999; Sasse et al., 2007]. Ryanodine receptors (RyRs) and inositol triphosphate receptors (IP3Rs)-mediated ICR activated the Na–Ca exchanger current ($I_{Na/Ca}$), and subsequently induced membrane potential oscillations, which repeatedly draw membrane potentials toward threshold to fire cardiomyocytes of embryonic Day 9.5 [Sasse et al., 2007]. Mery et al. [2005] reported that intracellular Ca^{2+} oscillations supported by IP3-sensitive stores constituted a pacemaking mechanism in early cardiac development using α-MHC positive cardiac cell derived from embryonic stem cells. In adult sinoatrial nodal cells, CAMP/PKA-dependent local ICR through RyRs permited the occurrence of action potentials (APs) and determines its beating rate [Bogdanov et al., 2001; Vinogradova et al., 2006].

However, during embryonic heart development how I(f) and ICR contribute to the initiation of spontaneous electrical activity remains uncertain. Therefore, the aim of this study was to unravel the functional roles of I(f) current and ICR in spontaneous electrical activity in ventricular cells during embryonic development, to get better understanding of the pacemaking mechanisms during cardiogenesis.

MATERIALS AND METHODS

CELL PREPARATION

Six- to 8-week-old female mice (Kunming mice provided by the Center of Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, China) were superovulated for precise timing of their pregnancy [Fleischmann et al., 1998]. The ventricles were isolated as reported previously [Herr et al., 2001]. Briefly, pregnant mice were sacrificed, the embryos were harvested and primitive ventricles (heart tube at E8.5–9.5 or ventricles (four chambered heart) were dissected. Single ventricular cardiomyocytes from very early development stage (E8.5–9.5), early stage (E10.5–12.5), and late development stage (E16.5–18.5) hearts were obtained using collagenase B (1 mg/ml; Roche, Germany). Cells were cultivated in DMEM supplemented with 20% FCS (selected batches) on gelatine (0.1%) coated cover slips and measured 24–48 h after dissociation. The experiments using mouse tissue were approved by the local ethical committee (Tongji Medical College, Huazhong University of Science and Technology).

ELECTROPHYSIOLOGICAL RECORDINGS

The cells were held in voltage clamp or current clamp mode in a temperature controlled condition ($37 \pm 0.3^{\circ}C$) using an Axopatch 200-A amplifier (Axon Instruments, CA) [Song et al., 2002]. APs of the nonspontaneous cardiac cells were stimulated at 0.33 Hz using 2 nA, 2 ms current pulses applied through the patch pipette. To record I(f), cardiomyocytes were clamped at a holding potential of −35 mV for 200 ms to obtain inactivation of Na^+ currents and then hyperpolarized to −110 mV (2.5 s duration). I(f) was measured as ivabradine sensitive current. Due to the lack of specific inhibitors of $I_{Na/Ca}$, $I_{Na/Ca}$ was measured using voltage ramps before and during application of Ni^{2+}. Voltage ramps from −120 mV (forward mode) to +40 mV (reverse mode) over 1.8 s were applied with an interval of 10 s. The holding potential was set to −30 mV to block T-type Ca^{2+} channels and Na^+ channels [Reppel et al., 2007]. Data are presented as the standard error of the mean (SEM) where appropriate. Statistical analyses were performed using Student's paired or unpaired *t*-tests and $P < 0.05$ was considered significant. Experiments were also performed 6 h after plating at E10.5–12.5 and E16.5–18.5, and the data were pooled since no differences were observed.

$[Ca^{2+}]_i$ IMAGING

Fifty milligrams of Fluo-3-AM (Dojindo, Japan) was dissolved in 50 μl of DMSO and used in 3 h. E10.5–12.5 or E16.5–18.5 embryonic ventricular cardiomyocytes were incubated in 3 ml DMEM containing Fluo-3-AM (final concentration 1 mM) for 30–40 min at 37°C before washout. Ca^{2+} measurements were performed using a Laser scanning confocal microscope FV500 system (Olympus, Japan). Cells were illuminated at 485 nm and Fluo-3-AM fluorescence was detected at 520 nm. Measurements were typically taken at a rate of 25 Hz from individually identified cells. The relative amplitude of fluorescence signal rising to the basal cell fluorescence after dye loading (ΔF/F0) was the measure of response. ΔF represents the synchronous increase in $[Ca^{2+}]_i$-dependent fluorescence associated with spontaneous repetitive APs, whereas F0 indicates the average fluorescence intensity during the diastolic intervals.

RT-PCR

Total RNA was isolated from the embryonic ventricles at different developmental stages (E10.5–12.5, E16.5–18.5) using the RNeasy Mini Kit (Qiagen, Germany). Total RNA was quantified with a spectrophotometer (Eppendorf, Germany). Five hundred nanograms of total RNA was used for one-step RT-PCR following the protocol described in the manual (Takara, Japan). The primers and cycling conditions used in the protocol are listed in Table I. PCR products were separated and analyzed in a 2% horizontal agarose gel. Images were taken with Gel Doc™ EZ system (Bio-Rad) and the densities of bands were analyzed with ImageJ (http://rsb.info.nih.gov/ij/index.html). Negative controls were performed by omitting the RNA templates. The target mRNA amount at E10.5–12.5 or E16.5–18.5 was normalized to the GAPDH mRNA amount at the same stages. The three measurements used for statistical analysis were obtained from three independent RNA preparations.

TABLE I. Sequences of PCR Primers for RyR1-3, IP3R1-3, NCX, and GAPDH and Cycling Conditions

Target sequence	Accession no.[a]	Primers	Sequence (5′–3′)	Position	Amplification length, bp
RyR1	NM_009109	Sense	GTTCCGGTGCTGGGGACATG	1,3911–1,3930	480
		Antisense	TGTGGGCTGTGATCTCCAGA	1,4390–1,4371	
RyR2	NM_023868	Sense	GACGGCAGAAGCCACTCACCTGCG	2,557–2,580	333
		Antisense	CCTGCAGAGAAACTGACAACTGGA	2,889–2,912	
RyR3	NM_177652	Sense	CTTCGCTATCAACTTCATCCTGC	1,3317–1,3339	504
		Antisense	TCTTCTACTGGGCTAAAGTCAAGG	1,3820–1,3843	
IP3R1	NM_010585	Sense	AGTTTGGCCAACGATTTCCTG	7,781–7,801	780
		Antisense	GTTGACATTCATGTGAGGAGG	8,560–8,580	
IP3R2	NM_080586	Sense	GCAGTGGCGATCTGCACATCTATG	6,743–6,766	183
		Antisense	CGGTTTCCCACAAAACTCACCAG	6,925–6,948	
IP3R3	NM_080553	Sense	CCACACGGAGCTGCCACATTT	7,920–7,940	774
		Antisense	TCAGCGGCTCATGCAGTTCTG	8,063–8,083	
NCX	NM_011406	Sense	GTGACTCACGTGAGCGAGAG	1,727–1,746	520
		Antisense	CTCTTGAATGATCACCTCCA	2,247–2,266	
GAPDH	XM_001473623	Sense	TGTCAGCAATGCATCCTGCA	504–525	240
		Antisense	CCGTTCAGCTCTGGGATGAC	724–743	

Cycling conditions: 30 min of cDNA synthesis at 50°C and 2 min of denaturation at 94°C, the samples were subjected to 32 cycles of amplification consisting of 30 s at 94°C, 30 s at 62°C, and 45 s at 72°C, with a final additional extension step at 72°C for 10 min. To amplify NCX and GAPDH, the samples were subjected to 30 cycles as described above for RyRs and IP3Rs, except that the annealing temperature was 58°C.
[a]In GeneBank release.

SOLUTIONS

The solutions used for recording the APs and I(f) were as follows (in mmol/L): NaCl 140, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, *N*-(2-Hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES) 10 (adjusted to pH 7.4 with NaOH, external solution); NaCl 10, potassium aspartate 130, Na_2ATP 2, egtazic acid (EGTA) 1, $MgCl_2$ 2, Na_2GTP 0.1, Hepes 10 (adjusted to pH 7.2 with KOH, internal solution). In some experiments, Na^+ was partially replaced with isomolar concentrations of Li^+. To record $I_{Na/Ca}$, $[Ca^{2+}]_i$ was buffered to 350 nM with BAPTA (calculated with CaBuf software; G. Droogmans, Leuven). The external solution (standard $I_{Na/Ca}$ solution) was K^+-free and contained: (in mmol/L) NaCl 135, $CaCl_2$ 2, $MgCl_2$ 1, Glucose 10, HEPES 10, CsCl 10 (to block the inward rectifier K^+ current, I_{K1}, and the Na^+/K^+ pump); (in μmol/L) niflumic acid 100 (to block Ca^{2+}-activated Cl^- currents), Ouabain 10 (Na^+/K^+ pump inhibitor) and verapamil 10 (dihydropyridine antagonist) (adjusted to pH 7.4 with CsOH, external solution); CsCl 136, NaCl 10, Aspartic acid 42, $MgCl_2$ 3, HEPES 5, tetraethylammonium (TEA) 20, MgATP 10 and 350 nM free $[Ca^{2+}]_i$, (adjusted to pH 7.4 with CsOH, internal solution) as described [Linask et al., 2001]. For some experiments, Ca^{2+} was not buffered, 350 nM free $[Ca^{2+}]_i$ was substituted with 1 EGTA.

All chemicals were purchased from Sigma–Aldrich, except ryanodine (Calbiochem) and Fluo-3-AM (Dojindo). 2-aminoethoxydiphenyl borate (2-APB) and ryanodine were initially dissolved in methnol at 1,000 times the final concentration to make the stock solutions, respectively. All substances were dissolved to their final concentrations in extracellular solution before use.

RESULTS

APs AT DIFFERENT DEVELOPMENTAL STAGES

APs were recorded from E8.5–9.5 cardiomyocytes of tubular hearts, E10.5–12.5 and E16.5–18.5 cardiomyocytes from murine ventricles. As shown in Figure 1A at E8.5–9.5, all cardiac cells are spontaneously active and 60% of them showed primitive spike-like APs without regular diastolic depolarization (DD). After 1 or 2 day's development, most of the spontaneous beating ventricular cells showed regular APs with prominent DD (Fig. 1A). However, with the further development, ventricular cells gradually lost the potential of spontaneous excitation, because as illustrated in Figure 1B, only seven ventricular cells (39%, $n = 16$) at E16.5–18.5 were spontaneously active as compared to E10.5–12.5 cells (100%, $n = 38$), and in accordance with it E16.5–18.5 cells showed much slower AP frequency and lower diastolic depolarization rate than E10.5–12.5 ventricular cells (Fig. 1C). We also found that the maximum diastolic potential gradually hyperpolarized from -59.28 ± 1.21 mV in E8.5–9.5 cells to -69.11 ± 0.99 mV in spontaneous E16.5–18.5 ventricular cells and -76.79 ± 2.74 mV in E16.5–18.5 non-spontaneous cells (Fig. 1D).

FUNCTIONAL EXPRESSION OF I(f) DURING CARDIAC DEVELOPMENT

Next, we tried to figure out how I(f), the most important pacemaker current, contribute to trigger APs in embryonic ventricular cells at different stages. Figure 1A (lower panel) showed current in response to a hyperpolarizing voltage step from a holding potential of -35 mV to a test potential of -110 mV in corresponding cells at different stages. At E8.5–9.5, I(f) was not identified. Just after 1 day's development, I(f) was measured as ivabradine (30 μM) sensitive current and largely identified in 86.8% of the measured E10.5–12.5 cells with a density of 6.43 ± 0.59 pA/pF ($n = 38$, Fig. 2A) [Bucchi et al., 2002]. In accordance with the gradual loss of spontaneous electrical activity, only 30% of E16.5–18.5 cells expressed I(f) with a significantly reduced density of 3.26 ± 0.43 pA/pF ($n = 7$) and nonspontaneous cells did not expressed I(f) ($n = 9$, Fig. 2A). When I(f) current was completely blocked by 30 μM ivabradine, the beating rate of E10.5–12.5 ventricular cells slowed down by $30.2 \pm 7.2\%$ ($n = 15$, $P < 0.05$; paired *t*-test; Fig. 2B,C). Surprisingly, in E16.5–18.5 cells application of 30 μM ivabradine induced a stronger chronotropic effect since it decreased the beating frequency by $49.2 \pm 8.5\%$ ($n = 7$, $P < 0.05$; paired *t*-test; Fig. 2B,D). If we

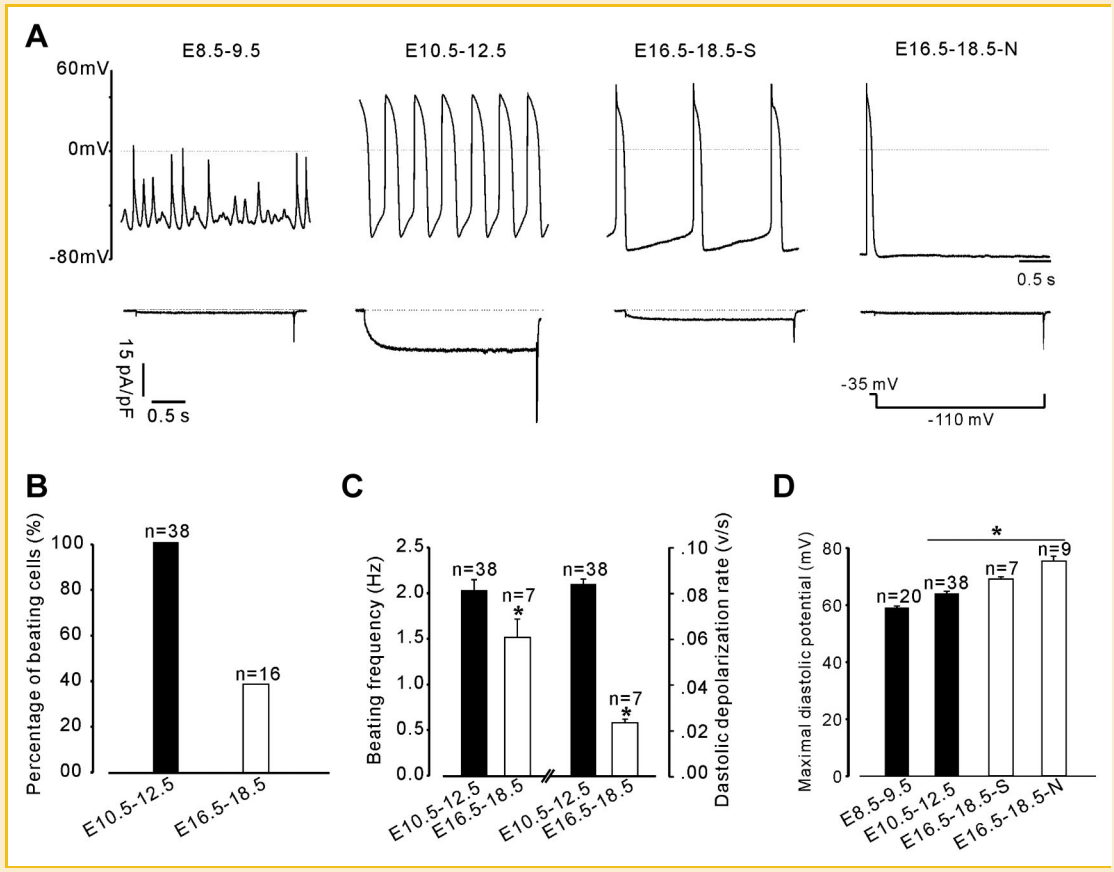

Fig. 1. APs at different developmental stages. A: Spontaneous APs and corresponding I(f) current in E8.5–9.5 cells, E10.5–12.5 ventricular cells, E16.5–18.5 spontaneous beating ventricular cells (E16.5–18.5-S) and E16.5–18.5 nonspontaneous ventricular cells (E16.5–18.5-N). B: Percentage of cells with the spontaneous APs and expression of I(f). (C) and (D) show statistical analysis of AP-associated physiological parameters including beating frequency, DD rate and maximum diastolic potential. E16.5–18.5-N cells were triggered with electrical stimulation. Values are presented as means ± SEM. $^{*}P < 0.05$, unpaired *t*-test.

overlapped the AP traces before and in the presence of ivabradine, we observed that the steepness of DD was reduced from 20.8% and 13% of the control DD length in early- and late stage ventricular cells, respectively (Fig. 2C,D; Table II). Therefore, our data suggest that I(f) participates in regulating spontaneous beating in embryonic ventricular cells by decreasing the DD rate from its earlier phase.

ROLE OF RyR AND IP_3R-MEDIATED ICR IN SLOWING DOWN DIASTOLIC DEPOLARIZATION DURING CARDIAC DEVELOPMENT

To find out how ICR is involved in the initiation of APs in cardiac cells at different developmental stages, we performed current clamp and $[Ca^{2+}]_i$ imaging experiments. We found that, 20 μM ryanodine, an RyR inhibitor [Sutko et al., 1985], significantly decreased the frequency of APs by 52.4 ± 6.8% (n = 13, $P < 0.001$; paired *t*-test; Fig. 3A,E) in E10.5–12.5 ventricular cells. Correspondingly, ryanodine inhibited amplitude and frequency of $[Ca^{2+}]_i$ transient oscillations by 40.5 ± 7.2% ($P < 0.001$; paired *t*-test) and 37.2 ± 6.8% ($P < 0.05$; paired *t*-test) (n = 5, Fig. 3A,F), respectively. By contrast, 2-APB (10 μM), a specific IP_3R antagonist [Gysembergh et al., 1999], only reduced the frequency of APs by 19.7 ± 5.5% (n = 14, $P < 0.001$; paired *t*-test; Fig. 3B,E). With $[Ca^{2+}]_i$ imaging, 2-APB attenuated the amplitude and frequency of $[Ca^{2+}]_i$ transients in E10.5–12.5 cells by 24.7 ± 2.5% ($P < 0.001$; paired *t*-test) and 23.1 ± 3.6% ($P < 0.05$; paired *t*-test) (n = 7, Fig. 3B,F), respectively. Similar experiments were carried out in E16.5–18.5 ventricular cells, and we found that 20 μM ryanodine only decreased the frequency of APs and $[Ca^{2+}]_i$ oscillations by 24.9 ± 6.6% (n = 9, $P < 0.001$; paired *t*-test; Fig. 3C,E) and 17.3 ± 4.3% (n = 10, $P < 0.05$; paired *t*-test; Fig. 3C,F), respectively. However, ryanodine significantly inhibited the amplitude of $[Ca^{2+}]_i$ oscillations in E16.5–18.5 myocytes (74.2 ± 6.1%, n = 10, $P < 0.001$; paired *t*-test; Fig. 3C,F). The contributions of IP_3Rs in APs and ICR were also evaluated. 2-APB failed to induce significant effects on both the firing rate of APs (n = 12, $P > 0.05$; paired *t*-test; Fig. 3D,E) and frequency of $[Ca^{2+}]_i$ oscillations (n = 6, $P > 0.05$; paired *t*-test; Fig. 3D,F). 2-APB only slightly attenuated the amplitude of $[Ca^{2+}]_i$ oscillations in E16.5–18.5 myocytes (11.8 ± 1.2%, n = 6, $P < 0.001$; paired *t*-test; Fig. 3D,F).

Interestingly, we noticed that either ryanodine or 2-APB induced a reduction of DD steepness from about 72.8% (Fig. 3A,B; Table II) of the control DD length which is significantly later than what ivabradine did on DD (Fig. 2C,D; Table II). Surprisingly, although ryanodine or 2-APB alone displayed reduced inhibitory effect on AP and $[Ca^{2+}]_i$ oscillations frequencies during embryonic heart

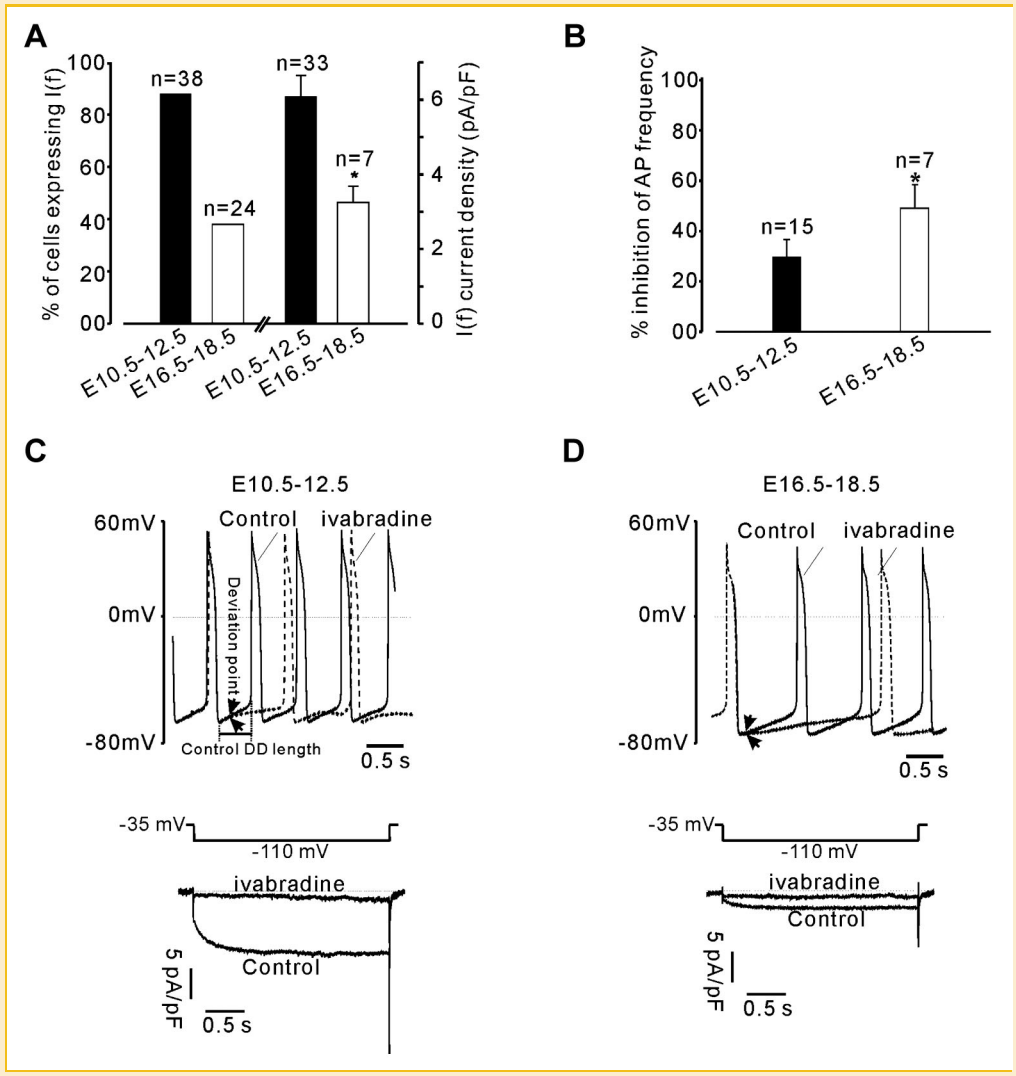

Fig. 2. Functional expression of I(f) and ivabradine-induced chronotropy in early and late stage ventricular cells. A: Percentage of cells expressing I(f) current and current density of I(f) in E10.5–12.5 and E16.5–18.5 ventricular cells. Significant down-regulation of I(f) was observed. B: Statistical analysis of ivabradine induced chronotrpy in both E10.5–12.5 and E16.5–18.5 ventricular cells. C,D: Overlapped APs (upper panel) and corresponding I(f) (lower panel) before and in the presence of ivabradine (30 μM) in E10.5–12.5 (C) and E16.5–18.5 ventricular cells (D) (double arrows indicate the deviation points of the DD in the presence of ivabradine from the control DD). Values are presented as means ± SEM. $^{*}P < 0.05$, E16.5–18.5 versus E10.5–12.5, unpaired *t*-test.

development, co-application of ryanodine and 2-APB still completely abrogated APs and $[Ca^{2+}]_i$ transients in all spontaneous cardiomyocytes at both E10.5–12.5 (n = 11, Fig. 4A,E) and E16.5–18.5 (n = 9, Fig. 4B,F). This observation is collaborated by abolishment of APs when cells were perfused with caffeine to open Ca^{2+} release channels and deplete the intracellular Ca^{2+} stores in E10.5–12.5 (n = 7, Fig. 4C) and E16.5–18.5 cells (n = 4, Fig. 4D). Therefore, we concluded that ICR permitted the initiation of spontaneous electrical activity in embryonic ventricular cells, and there was a mechanism shift from RyR- as well as IP3R mediated ICR to RyR-mediated ICR.

TABLE II. Effect of the Chemicals on the DD of the Action Potentials

	DD length to the deviation point/Control DD length[a]	
	EDS (10.5 dpc)	LDS (17.5 dpc)
Ivabradine	20.86 ± 3.22% (n = 8)	13.27 ± 6.29% (n = 6)
Ryanodine	72.87 ± 4.20% (n = 7)	72.82 ± 5.40% (n = 6)
2-APB	70.55 ± 2.10% (n = 7)	–

[a]DD length is time from the maximal diastolic depolarization to initial point of phase 0 fast depolarization.

Subsequently, we performed RT-PCR to determine the mRNA expression levels of RyR and IP_3R isoforms during cardiac development. As illustrated in Figure 5A,C, type 1 RyR (RyR1) mRNA, which is mainly expressed in skeletal muscle cells, was not detected in the embryonic heart [Ogawa, 1994; Jordan et al., 2004]. While type 3 RyR (RyR3) was transcriptionally repressed, mRNA level of cardiac type 2 RyR (RyR2) was increased (n = 3; $P < 0.05$; paired *t*-test) during cardiac development. Three IP_3R isoforms were

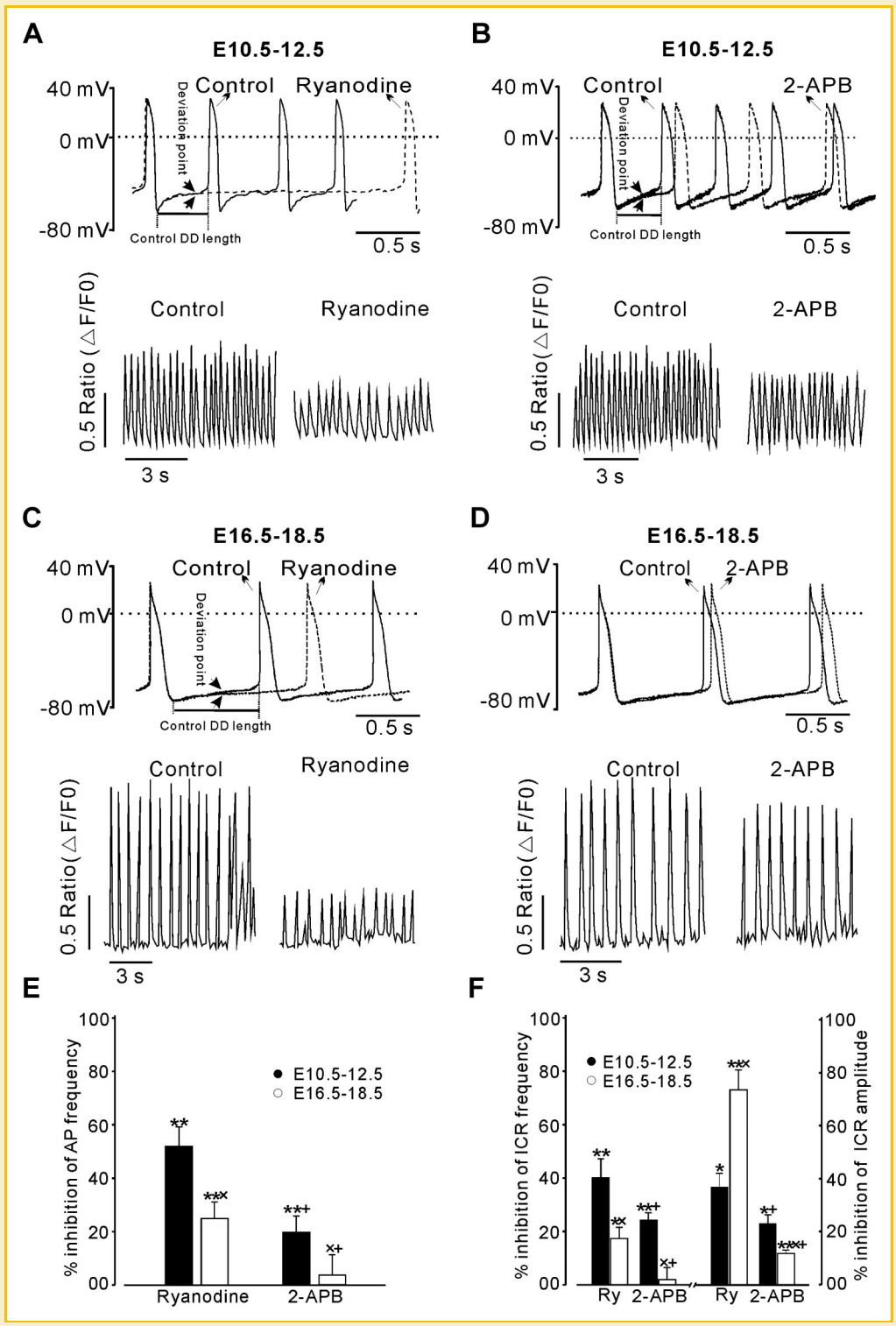

Fig. 3. Role of RyR- and IP3R-mediated ICR in spontaneous electrical activity in E10.5–12.5 and E16.5–18.5 ventricular cells. A,B: Application of 20 μM ryanodine (A) or 10 μM 2-APB (B) decreased the beating frequency of APs (upper panel) and amplitude and frequency of ICR (lower panel) in E10.5–12.5 cells. Ryanodine had more pronounced inhibitory effect (double arrows indicate the deviation points of DD in the presence of ryanodine or 2-APB from the control DD). C,D: Inhibitory effect of ryanodine (C) or 2-APB (D) on APs and ICR at E16.5–18.5 (double arrows indicate the deviation points of DD in the presence of ryanodine or 2-APB from the control DD). E,F: The inhibition on AP frequency (E), amplitude and frequency of ICR (F) upon application of ryanodine or 2-APB were analyzed in E10.5–12.5 and E16.5–18.5 ventricular cells. Values are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.001$ experimental groups (drug treatment) versus control group (before adding drugs), paired *t*-test; $^{+}P < 0.05$ 2-APB versus ryanodine, unpaired *t*-test; and $\times P < 0.05$ E16.5–18.5 versus E10.5–12.5, unpaired *t*-test. Ry, ryanodine.

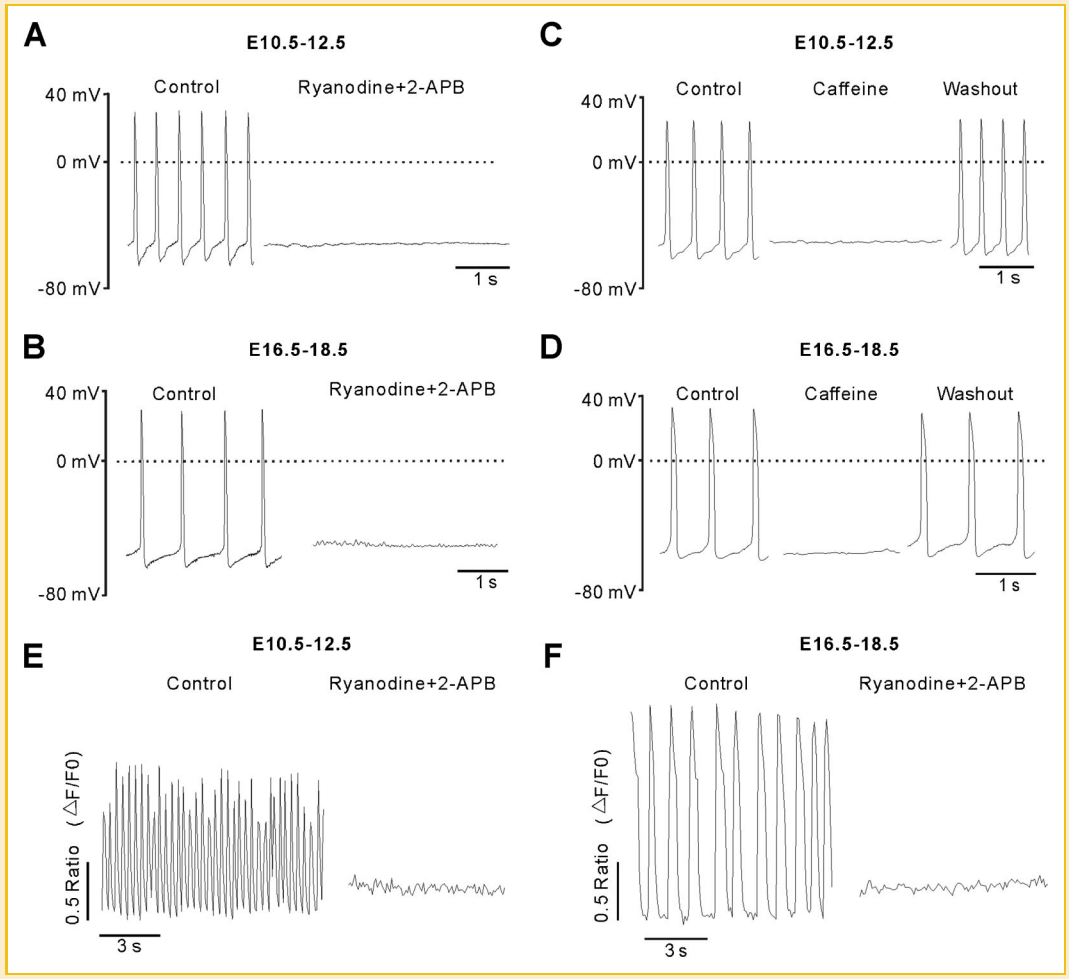

Fig. 4. Role of ICR in spontaneous electrical activity in E10.5–12.5 and E16.5–18.5 ventricular cells. A,B: Co-application of ryanodine (20 μM) and 2-APB (10 μM) led to complete abolishment of the APs in early (A) and late (B) stage embryonic ventricular cells. C,D: Application of caffeine (10 mM) stopped the rhythmic APs in E10.5–12.5 (C) and E16.5–18.5 ventricular cells (D). E,F: Co-application of ryanodine (20 μM) and 2-APB (10 μM) also stopped ICR in early (E) and late (F) stage embryonic ventricular cells.

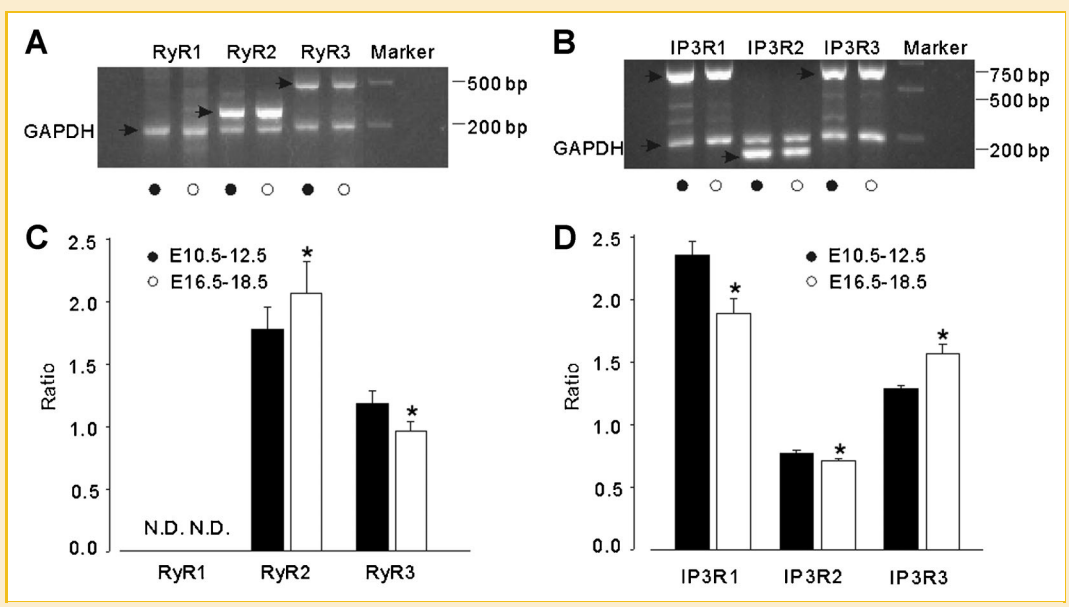

Fig. 5. mRNA expression of RyR1-3 and IP3R1-3 in E10.5–12.5 and E16.5–18.5 ventricles. A,B: mRNA expression level of RyR1-3 (A) and IP3R1-3 (B) were quantified by RT-PCR (arrows indicate the target bands). C,D: Densities of the bands were analyzed with imageJ, and target mRNA amounts were normalized to GAPDH mRNA levels. Values are presented as means ± SEM (n = 3). N.D. indicates below the level of detection. $^{*}P < 0.05$ E16.5–18.5 versus E10.5–12.5.

also identified during cardiac development. Both neuronal type 1 IP_3R (IP_3R1) and cardiac type 2 IP_3R (IP_3R2) are transcriptionally down-regulated ($n = 3$, $P < 0.05$; paired *t*-test, Fig. 5B,D) which is consistent with a previous report [Bootman and Roderick, 2008]. However, mRNA level of type 3 IP_3R (IP_3R3) which is mainly identified in pancreatic β-cells slightly increased ($n = 3$, $P < 0.05$; paired *t*-test, Fig. 5B,D). These data suggested that the contribution of RyRs and IP3Rs to spontaneous activity of ventricular myocytes at different developmental stages might be largely due to their altered mRNA expression during cardiac development.

$I_{Na/Ca}$ IS THE MEDIATOR OF ICR-TRIGGERED APs AT DIFFERENT STAGES OF EMBRYONIC HEART DEVELOPMENT

It has been reported that ICR triggered membrane depolarization by activating $I_{Na/Ca}$ in cardiomyocytes dissociated from E9.5–10.5 embryonic hearts and adult sinoatrial nodal cells [Sasse et al., 2001]. Thus, we determined whether $I_{Na/Ca}$ mediated ICR-triggered APs at different stages of heart development. As illustrated in Figure 6A, caffeine (10 mM) elicited a strong inward current with a density of 1.15 ± 0.23 pA/pF at a constant holding potential of −35 mV, which was strongly reduced to 0.43 ± 0.08 pA/pF ($n = 9$, $P < 0.001$; paired *t*-test) in the presence of Li^+ in E16.5–18.5 cells (data from E10.5–12.5 cells were not shown here). This data clearly indicated that $I_{Na/Ca}$ can be activated by ICR. To know whether $I_{Na/Ca}$ translates ICR into depolarization of membrane potential and thus triggers APs, we used different concentration of $[Na^+]_0$ (the remainder was substituted with isomolar Li^+) to produce different amount of $I_{Na/Ca}$. As shown in Figure 6B, the $I_{Na/Ca}$ density was measured as the subtraction of the currents induced by a ramp protocol before or in the presence of Ni^{2+}. A linear current–voltage relationship of $I_{Na/Ca}$ was revealed as current densities at different voltages from −120 to 40 mV. In the presence of 140 mM $[Na^+]_0$ $I_{Na/Ca}$ was -1.10 ± 0.26 pA/pF at −40 mV, which is significantly larger than that in the presence of 80 mM $[Na^+]_0$ (60 mM $[Na^+]_0$ was replaced with Li^+) ($n = 5$, $P < 0.05$; paired *t*-test). The replacement of $[Na^+]_0$ with Li^+ resulted in complete halts of APs in both early and late stage ventricular cells ($n = 7$, Fig. 6C,D).

Based on these data, we conclude that $I_{Na/Ca}$ activated by ICR depolarizes the membrane potential to reach the threshold for the rapid upstroke of the AP in embryonic ventricular cells of both early and late stages. ICR, the "Ca^{2+} clock," interacts with $I_{Na/Ca}$, one component of the "membrane clock," to control spontaneous electrical activity of embryonic ventricular cells.

DISCUSSION

In this study, we presented experimental data elucidating the mechanism underlying spontaneous electrical activity of murine embryonic ventricular-like cells during heart development. We found that RyR and/or IP_3R-mediated ICR was a major event occurred in the later phase of DD following the activation of $I_{Na/Ca}$, which determined the occurrence of APs of embryonic ventricular-like cells at both E10.5–12.5 and E16.5–18.5. Not as important as ICR, I(f), the pacemaker current, participated in the modulation of the DD slope from its earlier phase and thus regulating firing rate. Besides, we also found that RyRs, as well as IP_3Rs are important for

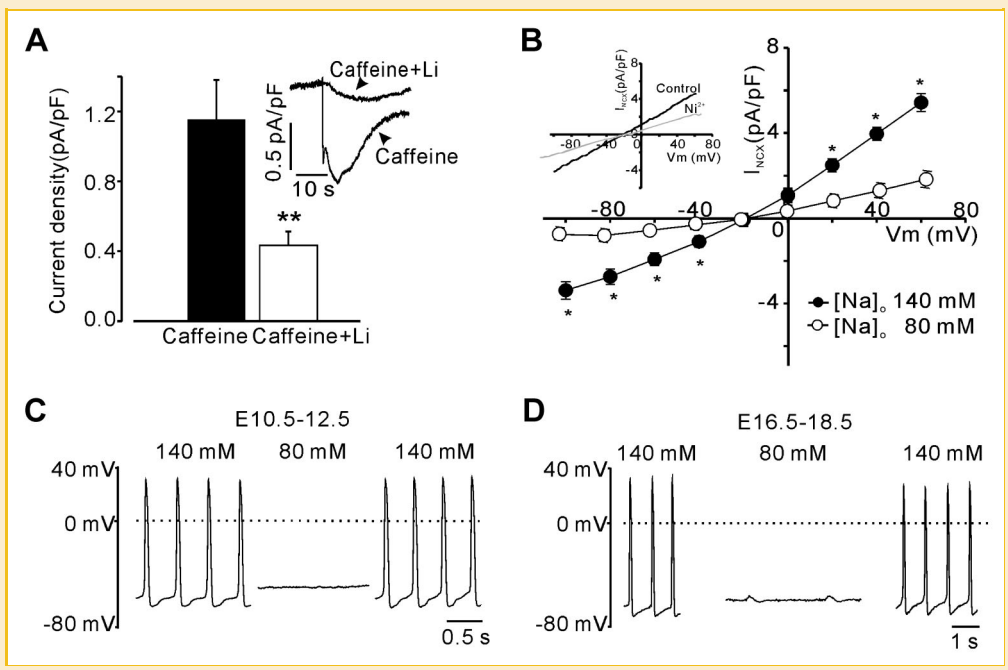

Fig. 6. $I_{Na/Ca}$-mediated ICR induces chronotropy in embryonic ventricular cells. A: Application of caffeine evoked a large inward current, which was significantly reduced by $68.9 \pm 5.5\%$ when Na^+ was replaced with Li^+ (holding potential −35 mV, $n = 9$). B: $I_{Na/Ca}$ density in E16.5–18.5 cells was measured as Ni^{2+} sensitive current and demonstrated with IV curve. In the presence of 80 mM $[Na^+]_0$ (Na^+ was substitute with equal molar of Li^+), $I_{Na/Ca}$ density was significantly reduced as compared to 140 mM $[Na^+]_0$ ($n = 5$). C,D: Perfusion of the embryonic cardiomyocytes with 80 mM $[Na^+]_0$ led to a complete halt of the APs in embryonic ventricular cells, and the APs recovered after washout ($n = 9$).

triggering ICR in the E10.5–12.5 cells, while ICR released through RyRs is the substantial source in E16.5–18.5 cells.

It is generally accepted that the generation of spontaneous electrical activity depends on subthreshold oscillations of membrane potentials that resulted from surface membrane events (membrane o'clock) and pre AP Ca^{2+} oscillations (Ca^{2+} o'clock). The subthreshold oscillations repeatedly draw the resting membrane potential toward threshold of voltage-dependent Ca^{2+} channels and/or Na^{+} channels and to fire cardiomyocytes spontaneously. However, there is presently some degree of uncertainty about the relative roles of pacemaker current—I(f) versus that of intracellular Ca^{2+} oscillations, especially in embryonic heart. At about E8.5, though cardiac cells do not express the full ensemble of ion channels and some channels are expressed at lower densities [Maltsev et al., 1994; Davies et al., 1996], most of the measured cells already displayed spike-like APs (Fig. 1A) which was also observed by Viatchenko-Karpinski et al. [1999] in embryonic stem cell derived early cardiac cells. Apparently, membrane ion channels are not the key mechanism to trigger APs. Spontaneous $[Ca^{2+}]_i$ oscillations rather than channel activities is the primary rhythm generator to initiate APs in early cells with spike-like APs [Sasse et al., 2007]. Starting from E10.5–12.5, most of ventricular cells obtained rhythmic APs with regular phase 4 DD (Fig. 1A). Due to the upregulation of I_{K1} [Davies et al., 1996] and down-regulation of I(f) (Fig. 2A) and $I_{Na/Ca}$ [Reppel et al., 2007], APs of E16.5–18.5 cells showed more hyperpolarized maximum diastolic potential, slower diastolic depolarization rate and lower beating rate as compared to that of E10.5–12.5 ventricular cells (Fig. 1C,D). In these embryonic ventricular cells, our data demonstrated that I(f) did not determine the generation of spontaneous activity, but block of I(f) did have inhibitory effect on the firing rate by reducing steepness from early phase of DD (Fig. 2C,D). The enhanced chronotropic effect in the presence of ivabradine at E16.5–18.5 might be due to the activation of more I(f) channels under more hyperpolarized maximum diastolic potentials which allowed more Na^{+}/K^{+} to move into the cells, and thus contributed more to phase 4 DD though expression of I(f) channels was significantly down-regulated during embryonic heart development (Fig. 2A). More important than I(f), ICR determined the initiation of APs, because our data indicated that blocking ICR abolished spontaneous electrical activity immediately at both embryonic stages (Fig. 4A–F). Obligation of ICR in spontaneous electrical activity was also suggested in sinoatrial nodal cells from rabbit [Bogdanov et al., 2001]. In adult heart, the pacemaker current appears to be absent in working cardiomyocytes and enriched in the sinoatrial nodal cells [Baruscotti and Difrancesco, 2004]. A variety of studies have shown that blocking I(f) pharmacologically decreased spontaneous AP firing rate of sinoatrial nodal cells to moderate extend maximally but could not stop spontaneous AP firing [Noma et al., 1983; Denyer and Brown, 1990; Choi et al., 1999]. In addition, eliminating I(f) genetically in adult mice had minor effects on the average heart rate [Herrmann et al., 2007; Hoesl et al., 2008]. For ICR, Bogdanov et al. [2001] suggested that ICR may be obligatory for pacemaking because 30 μmol/L of ryanodine abolished the spontaneous activity of isolated SANC from rabbit. Taken together, we suggest that in embryonic ventricular cells I(f) contributes to regulate spontaneous electrical activity, and ICR is indispensible for the initiation of spontaneous electrical activity especially in embryonic ventricular cardiomyocytes.

Concerning the molecular mechanism underlying ICR, Mery et al. [2005] provided evidence that IP_3-dependent shuttle of free Ca^{2+} in and out of the endoplasmic reticulum is essential for a proper generation of pacemaker activity during early cardiogenesis using mouse ES cells as an in vitro model. Sasse et al. [2007] found that both RyR- and IP_3R-dependent Ca^{2+} oscillations pace early embryonic heart cells. Our data extended the claim that RyRs were more important than IP3Rs in controlling spontaneous activity at E10.5–12.5 (Fig. 3A,B,E,F), and RyR blocker, but not IP_3R blocker, attenuated the spontaneous activity of E16.5–18.5 cardiomyocytes (Fig. 3C,D,E,F). This dominant function of RyRs is consistent with the finding that RyR2(−/−) ES cell-derived cardiomyocytes at early stage beat at a relatively lower frequency than that in wild-type cells [Takeshima et al., 1998; Yang et al., 2002]. This mechanism shift from RyRs and IP_3Rs mediated chronotropy at E10.5–12.5 to RyRs domination on pacing at E16.5–18.5 could be due to the up-regulation of RyR2 and the down-regulation of IP3R2 (Fig. 5). The much stronger inhibition of ryanodine or 2-APB on Ca^{2+} oscillation amplitude observed in E16.5–18.5 cells is expected if we noticed that RyR- and IP3R-gated ICR recorded by Ca^{2+} imaging technique included pre AP ICR which activated $I_{Na/Ca}$ and subsequently depolarized the membrane potential to the threshold to fire AP and after AP ICR which contributed to trigger cell contraction, and knew that with the development T-tubes gradually mature to set up the connection with surface Ca^{2+} channels and release more Ca^{2+} upon activation of RyR/IP3R [Forbes et al., 1984; Brette and Orchard, 2003]. In addition, we noticed that in E16.5–18.5 ventricular cells co-application of ryanodine and 2-APB stopped APs, which seemed to be not consistent with the observation that individual application of ryanodine or 2-APB had attenuated or even not inhibited APs frequencies. We speculate that at E16.5–18.5 IP3R physiologically does not contribute to ICR. When RyR pathway was completely blocked, IP3R-mediated ICR might be pushed to function more for spontaneous activity. Actually, IP3Rs recently are found to serve as an additional Ca^{2+}-dependent mechanism in modulating cardiac pacemaker activity in sinoatrial nodal cells [Ju et al., 2011].

Finally, our data indicated that ICR activated $I_{Na/Ca}$ which provided sufficient "boost current" to augment late phase of DD and trigger AP in ventricular cells at both early and late stages of embryonic heart development (Fig. 6). Thus, this partner relationship exists in embryonic heart, as well as in adult sinoatrial nodal cells [Bogdanov et al., 2001; Sasse et al., 2007]. Next, we need to confirm the similar function of ICR in adult sinoatrial nodal cells and embryonic ventricular cells.

In pharmacological or pathological conditions, adult cardiomyocytes can recapitulate embryonic genotypes and phenotypes of embryonic cells [Harvey and Leinwand, 2011]. Plenty of studies evidenced that the pacemaking potency of I(f) and ICR can be triggered in terminally differentiated ventricular cardiomyocytes from diseased heart. In fact, I(f) is re-expressed in the adult ventricular myocytes isolated from heart failure and hypotrophic heart patients [Hoppe et al., 1998] and relevant to the arrhythmogenesis that occurs in these diseased heart [Cerbai et al., 1997]. For ICR, spontaneous $[Ca^{2+}]_i$ oscillations-induced $I_{Na/Ca}$ can evoke

delayed after-depolarizations (DADs) in acutely and chronically lesioned adult cardiomyocytes dissociated from dogs and humans [Verkerk et al., 2001; Janse, 2004; Katra and Laurita, 2005]. The DADs are thought to potentially induce life-threatening arrhythmias [Clusin, 2003; Janse, 2004; Rubart and Zipes, 2005; Lehnart et al., 2006]. A better mechanistic understanding of fundamental physiological activities in the embryonic heart may help us find clues for pharmacological interventions in the treatment of arrhythmias. Further extensive basic and translational investigations are needed to develop novel therapeutic strategies for arrhythmia based on our knowledge of the development of electrical activities in the embryonic heart.

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