Electrophysiological Basis of the First Heart Beats

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Key Words

Pacemaker current • Development • Differentiation • Embryogenesis • Ion current • Action potential

Abstract

Aims: Earlier studies on cardiac pacemaker activity were inconclusive regarding the electrophysiological mechanism(s) of first electrical activity generation during cardiac development. We therefore aimed to investigate the role of ion channels in action potential (AP) formation and pacemaker activity during very early murine heart development (E8.5). **Methods and Results:** The voltage clamp mode of the whole-cell patch clamp technique was used to study whole cell ion currents at the single cell level. Extracellular field potentials (FPs) of whole mount hearts were recorded by the use of multi-electrodes arrays (MEAs). RT-PCR was performed to study mRNA expression of the tested ion channels. E8.5 cardiomyocytes displayed membrane potential (MP) oscillations, primitive APs but also more mature-like APs. Cells with primitive APs expressed higher levels of $\text{Ca}_v1.2$, HCN4, $\text{Ca}_v3.2$ and NCX1 than cells with MP oscillations and mature-like cells expressed higher levels of $\text{K}_{ir}3.1$ (P-like) or $\text{K}_{ir}2.1$ (V- and A-like) than

cells with primitive APs. Blockers of I_{CaL} (verapamil+nifedipine), I_{NCX} (KB-R7943 and SEA0400), and I_{CaT} (Ni^{2+}) led to a complete halt of cardiac APs and FPs even when applied alone, while blocker of I_f (Cs^+) only suppressed AP/FP frequency. **Conclusions:** Our data imply that i) The high expression of $\text{Ca}_v1.2$, HCN4, $\text{Ca}_v3.2$, and NCX1 contributed to the shift from MP oscillations to primitive APs at E8.5. ii) Spontaneous AP generation may rely on the sum of transsarcolemmal Ca^{2+} influx through I_{CaL} , I_{NCX} and I_{CaT} since blockage of each individual ion channel/exchanger led to an immediate halt of beating at E8.5.

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Introduction

For embryonic stem (ES) cell-derived cardiomyocytes and native embryonic cardiomyocytes, earlier studies demonstrated that during development the functional role and gene expression of ion channels changes dramatically [1-4]. These reports implied that especially the L-type and T-type Ca^{2+} channel, $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), and HCN pacemaker channel (funny

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current, I_f) may play a major role during early embryomyogenesis. In more detail it was shown for embryonic murine ventricular myocardium that I_{CaL} density increased [4, 5] and that the subtype shifted from $Ca_v1.3$ to $Ca_v1.2$ during development [6] whereas I_{NCX} decreased [7]. Both, mRNA and protein expressions of NCX were significantly higher at very early (VEDS, E8.5-E9.5) and early developmental stages (EDS, E10.5) than at late developmental stages (LDS, E16.5) [7, 8]. I_{CaT} was observed at both stages, VEDS and LDS, with similar current densities. $Ca_v3.2$ encoded I_{CaT} at EDS and switched to $Ca_v3.1$ at the perinatal period [9]. I_f has been shown to be downregulated during development [10, 11] with a subtype switch from HCN4 at EDS to HCN2 at LDS [12]. Taken together, all these ion currents undergo developmental changes, suggesting that they may be involved in regulation of the electrical automaticity in embryonic cardiomyocytes. However, although previous reports hinted to a NCX-mediated initiation of spontaneous contractions [13, 14], the mechanisms underlying the early developmental pacemaker activity and the interplay of the different ion channels are still a matter of debate.

Therefore, the present study aimed at studying mechanisms of AP generation at the earliest stages of murine heart development. We found at E8.5 a significant contribution of I_{CaT} , I_{CaL} and I_{NCX} to AP generation. As blockage of a single type of each of these ion channels/exchanger led to an immediate halt of beating we hypothesize that only ion flux through the entirety of these tested ion channels is sufficient to hold up AP generation and spontaneous activity during very early cardiac development.

Materials and Methods

Harvesting and isolation of embryonic cardiomyocytes
As dramatic developmental changes occur during early cardiomyogenesis, female mice (mouse strain wt HIM:OF1) were superovulated for precise timing of their pregnancy [3]. Female mice were sacrificed by cervical dislocation at E8.5. The embryonic hearts were dissected and enzymatically dissociated to single cells as described [15]. Only spontaneously beating cardiomyocytes were used for functional studies after 24-36 hours of cultivation. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Patch clamp experiments
The whole-cell configuration of the patch-clamp technique was used throughout the study. All experiments were performed at 37°C. The internal pipette solution contained

Primers	Sequence
Ca _v 1.2	F 5'-CAGGAGGTGATGGAGAAGCCA-3' R 5'-CTGCAGGCGGAACCTGTTGTT-3'
Ca _v 1.3	F 5'-GGGGTCCAGCTGTTCAAGGGGGAA-3' R 5'-GCATGATGAGGACGAACATCATG-3'
Ca _v 3.1	F 5'-GGCCCCGGTGGTTCCTTCTA-3' R 5'-TGAGCGGTTCGACGACAC-3'
Ca _v 3.2	F 5'-AGGAAGCGCGCGCGCGC-3' R 5'-GGACAGGAGGACGATGGCCAA-3'
HCN4	F 5'-GCTGGTTTGTGGTAGATTTC-3' R 5'-AAACATCTTGCTTGGTAGC-3'
HCN2	F 5'-GCTGGTTTGTGGTAGATTTC-3' R 5'-GGCTGGAAGACCTCAAATT-3'
NCX1	F 5'-GTGACTCACGTGAGCGAGAG-3' R 5'-CTCTTGAATGATCACCTCCA-3'
SCN5a	F 5'-CTTGCCCAAGATCAACCTGCTCT-3' R 5'-CGGACAGGGCCAAATACTCAATG-3'
SCN1b	F 5'-CGTGTATGGGATGACCTTCAAA-3' R 5'-CAGAGGTGATGGCCAGGTATT-3'
Kir3.1	F 5'-GTGACTCACGTGAGCGAGAG-3' R 5'-GAAGATTTCCTTCCATCCTGG-3'
Kir3.4	F 5'-TACCTTCCCAGTCCCTCCCTT-3' R 5'-TCACATTGAGCCCCTGGT-3'
Kir2.1	F 5'-GCAGGAGCCGCTTTGTGAAGAA-3' R 5'-GGAGGTGAGTCTGTGCTTGTGCTC-3'
GAPDH	F 5'-GTGTTCTACCCCCAATGTG-3' R 5'-CTTGCTCAGTGTCTTGCTG-3'

Table 1. Primers used in the experiments.

(in mM): KCl 50, K-Aspartate 80, MgCl₂ 1, MgATP 3, glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 10, HEPES 10, pH adjusted to 7.40 at 37°C with KOH. The extracellular solution contained (in mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 10, pH adjusted to 7.40 at 37°C with NaOH. APs were recorded as reported [16]. Signals were measured at a sampling rate of 10 kHz, stored on hard disk and analysed off-line using the pulsefit (HEKA, Germany) analysis software package and APanalysis software programmed by Dr. Philipp Sasse (University of Bonn, Germany).

MEA measurements
Murine hearts harvested from E8.5 embryos were directly plated onto a MEA60 System (Multi Channel Systems, Reutlingen, Germany). A standard MEA culture dish contains 60 titanium nitride-coated gold electrodes (30 µm in diameter) arranged in an 8 x 8 electrode grid, with an inter-electrode distance of 200 µm. Raw data were acquired and analyzed as reported [17].

Single cell RT-PCR
Single cell RT-PCR was performed to determine the expression of ion channels in single embryonic cardiomyocytes,

Fig. 1. AP measurements at E8.5. E8.5 cardiomyocytes exhibited diverse electrophysiological activities, including (A) MP oscillations, (B) (a) irregular primitive APs, (b) regular primitive APs, and different phenotypes of mature-like APs: (C) (a) pacemaker-like (P-like), (b) atrial-like APs (triangle-like, A-like), and (c) ventricular-like APs (V-like) with a long plateau phase.

including T-type and L-type Ca^{2+} channels, HCN channels, and NCX. Single cells were picked by the glass pippet and stored together with RNase inhibitor (Invitrogen GmbH, Germany) in PCR tubes and kept at -80°C , followed by standard protocols for further processing [15]. The PCR was processed for 40-43 cycles using the QIAGEN Multiplex PCR Kit (Qiagen GmbH, Germany). The house-keeper rod-GAPDH was used as an internal control. All primer sequences are listed in Table 1. The RNA samples without the reverse transcriptase served as negative control. The PCR processing consisted of 1 min denaturation at 94°C , 1 min annealing at optimal melting temperature (T_m) for different genes, and 1 min extension at 72°C . PCRs were run for 30 cycles and ended with 10 min final extension at 72°C .

Substances

KB-R7943 was purchased from Tocris Bioscience (Bristol, UK), SEA0400 from Saitama (Saitama, Japan). As limitation, for both KB-R7943 and SEA0400 unspecific blokage of other channels cannot be totally excluded. Nickel chloride, verapamil, and cesium chloride were purchased from Sigma Aldrich GmbH (Deisenhofen, Germany). Nifedipine, KB-R7943 and SEA0400 were dissolved in dimethylsulfoxide (DMSO) and others in water as stock solution, frozen and stored at -20°C . Aliquots were thawed immediately before use and diluted in the extracellular solution to the required concentration.

Data analysis

For the analysis of individual electrophysiological experiments the control data were obtained by averaging APs recorded in the last five seconds before the application of the respective ion channel blockers. Data of the experimental group were obtained from the last five seconds of drug presence. If halt of APs occurred, action potential duration (APD), velocity of diastolic depolarisation (Vdd), and maximal velocity of depolarisation (Vmax) in the experimental group were obtained from the last five seconds before AP halt. Results are presented as mean $\pm$ sem. Statistical analysis was done using paired and

AP parameters	P-like (n=33)	V-like (n=54)	A-like (n=59)
MDP (mV)	-58.0 ± 0.9	-64.4 ± 0.7	-62.4 ± 0.6
Amplitude (mV)	80.9 ± 1.7	99.8 ± 1.5	95.6 ± 1.7
Frequency (beats/min)	163.3 ± 35.6	98.0 ± 4.0	127.2 ± 5.2
Vmax (V/s)	12.0 ± 1.3	24.5 ± 1.0	23.8 ± 0.7
Vdd (V/s)	0.072 ± 0.012	0.044 ± 0.02	0.050 ± 0.02
APD90 (ms)	88.1 ± 3.9	117.4 ± 4.1	84.3 ± 3.2
APD90/50	1.66 ± 0.04	1.49 ± 0.03	3.26 ± 0.32

Table 2. AP parameters for P-, A-, and V-like APs at E8.5.

unpaired student's t-test where applicable. P values of <0.05 were considered significant.

Results

Electrophysiological activity at E8.5: Shift from membrane potential oscillations to primitive and mature-like APs

Our data revealed that E8.5 cardiomyocytes exhibited diverse electrophysiological activities, including membrane potential (MP) oscillations, irregular primitive APs, regular primitive APs, and different phenotypes of mature-like cardiac APs: pacemaker-like (P-like), atrial-like APs (triangle-like, A-like), and ventricular-like APs (V-like) with a long plateau phase.

In approximately 70% of E8.5 cardiomyocytes, no real APs but oscillations of the MP were observed

Fig. 2. (A) expression of inwardly directed ion channel subunits in E8.5 cardiomyocytes. (B) expression of potassium channel subunits in E8.5 cardiomyocytes.

(Fig. 1A). The MP showed a mean value of -45.1 ± 2.0 mV ($n=95$). Some cardiomyocytes displayed irregular APs (Fig. 1Ba) that exhibited a short APD₉₀ (40.2 ± 2.5 ms, $n=16$), low amplitude (29.8 ± 3.7 mV) and less negative MDP (-46.5 ± 1.9 mV). With a more negative MDP (-53.9 ± 2.0 mV, $n=17$), regular primitive APs occurred (Fig. 1Bb). These primitive APs were characterized by a short APD₉₀ (46.12 ± 3.2 ms), low amplitude (45.3 ± 2.6 mV), slow V_{max} (6.60 ± 0.65 V/s), high V_{dd} (0.090 ± 0.005 V/s) and high AP frequency (185.4 ± 9.3 beats/min) as compared to mature-like APs. These characteristics suggest that the primitive APs in

Fig. 3. Effect of I_{CaL} , I_P , I_{NCX} , and I_{CaT} blockers on primitive APs. (A) 1 μ M verapamil+nifedipine, (C) 5 μ M KB-R7943, and (D) 1 μ M SEA0400 completely diminished primitive APs, accompanied by a significant depolarization of MDP. 1 mM Cs^+ (B) and 40 μ M Ni^{2+} (E) suppressed AP frequency with a decrease of V_{dd}. The left panel of each figure shows the control traces before drug application, the middle panel depicts APs in the presence of the individual drugs and the right panel of (A), (B), and (E) shows traces after washout of the drugs. Notably, no recovery was observed for KB-R7943 and SEA0400.

E8.5 cardiomyocytes could be a transitional stage from MP oscillations towards mature-like phenotypes of APs.

Only approximately 20% of E8.5 cardiomyocytes displayed typical mature-like AP phenotypes. These APs were characterized by a more negative MDP, and higher amplitude than that of the primitive APs (Fig. 1C). Different phenotypes of APs were defined according to the typical AP morphology [18, 19] and the AP parameters: maximum diastolic potential (MDP), amplitude, frequency, action potential duration at 90% of repolarization (APD₉₀), V_{dd}, V_{max}, and the ratio of APD₉₀/APD₅₀ (Table 2).

	Control	V+N	Control	V+N	Control	V+N
	P (n=3)		V (n=5)		A (n=6)	
MDP (mV)	-60.3±1.4	-53.7±4.0	-63.2±3.8	-54.0±3.0*	-65.0±0.7	-6.2±2.5**
APD90 (ms)	67.8±1.1	65.4±1.7	108.2±13.5	85.7±6.5*	73.7±3.3	68.4±6.9
APD50 (ms)	43.8±0.01	31.1±2.2*	84.6±12.1	39.9±6.4**	41.5±4.2	23.6±4.6**
APD20 (ms)	23.9±0.2	13.1±2.0*	47.1±9.5	18.2±4.8*	17.5±2.9	10.2±2.0**
Vdd (V/s)	0.072±0.005	0.050±0.008	0.044±0.005	0.036±0.006*	0.053±0.003	0.03±0.007*
Vmax (V/s)	12.9±0.7	5.7±0.05*	21.1±4.1	12.8±2.0*	25.6±0.6	13.5±2.0**
Frequency (beat/min)	160±14	0**	91±9	0**	127±13	0**

Table 3. Effect of 1 μ M verapamil+nifedipine on E8.5 mature-like APs. * p <0.05 vs. control; ** p <0.01 vs. control.

Gene expression of ion channels at E8.5

To explore the molecular basis of the striking shift of electrical activity, single cells were collected for RT-PCR after patch clamp measurement to determine the expression of ion channels at the mRNA level. Fig. 2A shows that $Ca_v1.2$, NCX1 and HCN4 subunits were expressed at higher level in cells with primitive APs or mature like APs than in cells with MP oscillations. Cells with primitive APs expressed a higher level of $Ca_v3.2$ than other cell types and $Ca_v1.3$ was stably expressed at similar levels in all cell types. SCN5a and SCN1b were only weakly expressed in V- and A-like cells. $K_{ir3.1}$ was dominant in pacemaker like cells, while $K_{ir2.1}$ was highly expressed in working cardiomyocytes. Both of them were weakly expressed in cells with primitive APs while none of them was detectable in cells with MP oscillations. $K_{ir3.4}$ was weakly expressed in cells with MP oscillations and primitive APs, and upregulated in mature-like cells (Fig. 2B).

Ion Currents underlying primitive APs

To elucidate the contribution of individual ion currents to the generation of APs, blockers of I_{CaL} (1 μ M verapamil+nifedipine), I_T (1 mM Cs^+), I_{NCX} (5 μ M KB-R7943 and 1 μ M SEA0400), and I_{CaT} (40 μ M Ni^{2+}) were applied. Application of verapamil+nifedipine as well as administration of KB-R7943 or SEA0400 led to a fast occurring and complete block of APs, paralleled by a significant depolarization of the MDP. The MDP shifted from -53.51 ± 0.34 mV to -47.32 ± 3.87 mV ($n=4$, $p<0.05$), from -55.85 ± 1.32 mV to -47.98 ± 2.56 mV ($n=4$, $p<0.05$), and from -58.7 ± 1.6 mV to -53.5 ± 2.1 mV ($n=6$, $p<0.05$) for verapamil+nifedipine, KB-R7943 and SEA0400, respectively (Fig. 3A, C, D). Notably, the effects of KB-R7943 and SEA0400 were irreversible.

Cs^+ and Ni^{2+} led to a reduction of the AP frequency (Cs^+ : ~35% reduction, $n=11$, $p<0.01$; Ni^{2+} : ~51% reduction,

Fig. 4. Effect of verapamil+nifedipine on AP generation. (A), 1 μ M verapamil+nifedipine completely abolished the APs regardless of the cell type (a, P-like; b, V-like, and c, A-like). Before the APs halted, the MDP depolarized, the APD shortened, Vdd slowed (especially in the second phase of DD), and Vmax decreased. (B) Original recordings of FPs before and after verapamil+nifedipine application. Verapamil+nifedipine inhibited FP generation in all tested hearts.

$n=9$, $p<0.01$) (Fig. 3B, E). The changes in AP frequency were accompanied by a decrease of Vdd (Cs^+ : ~25% reduction, $p<0.01$; Ni^{2+} : ~33% reduction,

Fig. 5. Effect of KB-R7943 on AP generation. (A), 5 μ M KB-R7943 completely abolished the APs and depolarized the MDP in approximately 50% of the tested cardiomyocytes (a, P-like; b, V-like, and c, A-like). KB-R7943 had the potential to decrease Vdd (last figure in each panel) and decreased APD in V-like cells (b). (B), Original recordings of FPs before and after KB-R7943 application.

Fig. 6. Effect of SEA0400 on AP generation. (A), 1 μ M SEA0400 completely abolished the APs and depolarized the MDP in ~50% of all tested cardiomyocytes. It decreased Vdd in V-like cells (b) while increased in some cells before the halt of APs (c, AP no.2). Note the shortening of the APD. (B), SEA0400 abolished the spontaneous FPs in 100% of E8.5 hearts.

	Control	KB-R7943	Control	KB-R7943	Control	KB-R7943
	P (n=3)		V (n=6 out of 12)		A (n=4 out of 10)	
MDP (mV)	-57.4 $\pm$ 3.7	-52.7 $\pm$ 3.9	-63.2 $\pm$ 1.9	55.5 $\pm$ 2.7*	-60.6 $\pm$ 1.8	-55.6 $\pm$ 1.8*
APD90 (ms)	132.9 $\pm$ 18.2	126.6 $\pm$ 4.9	112.1 $\pm$ 12.1	139.4 $\pm$ 13.1*	86.3 $\pm$ 7.1	95.8 $\pm$ 8.8
APD50 (ms)	79.2 $\pm$ 10.1	69.5 $\pm$ 5.9	75.4 $\pm$ 8.6	72.1 $\pm$ 9.8	32.5 $\pm$ 5.3	33.3 $\pm$ 5.1
APD20 (ms)	40.8 $\pm$ 5.8	35.5 $\pm$ 4.7	36.6 $\pm$ 7.2	30.5 $\pm$ 7.7*	12.2 $\pm$ 2.6	10.7 $\pm$ 2.4
Vdd (V/s)	0.052 $\pm$ 0.006	0.047 $\pm$ 0.015	0.0497 $\pm$ 0.008	0.041 $\pm$ 0.113	0.044 $\pm$ 0.004	0.037 $\pm$ 0.007*
Frequency(beat/min)	190 $\pm$ 16	13 $\pm$ 13*	119 $\pm$ 13	12 $\pm$ 12**	153 $\pm$ 22	0**

Table 4. Effect of 5 μ M KB-R7943 on E8.5 mature-like APs. * p <0.05 vs. control; ** p <0.01 vs. control.

	Control	SEA0400	Control	SEA0400	Control	SEA0400
	P (n=3)		V (n=6 out of 9)		A (n=6 out of 10)	
MDP (mV)	-58.2 $\pm$ 2.8	-59.2 $\pm$ 4.9	-65.3 $\pm$ 2.2	59.7 $\pm$ 1.4**	-64.7 $\pm$ 0.9	-56.9 $\pm$ 3.9**
APD90 (ms)	127.2 $\pm$ 24	111.9 $\pm$ 16.4	116.3 $\pm$ 16.0	100.7 $\pm$ 15.2*	75.5 $\pm$ 9.6	52.9 $\pm$ 10.2*
APD50 (ms)	69.0 $\pm$ 10.6	53.5 $\pm$ 8.8	66.8 $\pm$ 7.7	49.6 $\pm$ 8.3*	27.8 $\pm$ 2.1	19.3 $\pm$ 1.6*
APD20 (ms)	35.7 $\pm$ 4.7	27.1 $\pm$ 4.6	32.0 $\pm$ 5.1	25.9 $\pm$ 3.7*	13.4 $\pm$ 1.3	7.3 $\pm$ 0.4*
Vdd (V/s)	0.064 $\pm$ 0.013	0.055 $\pm$ 0.014	0.043 $\pm$ 0.006	0.037 $\pm$ 0.01	0.040 $\pm$ 0.002	0.054 $\pm$ 0.004*
Frequency(beat/min)	115 $\pm$ 21	0*	91 $\pm$ 13	3 $\pm$ 3**	97 $\pm$ 12	0**

Table 5. Effect of 1 μ M SEA0400 on E8.5 mature-like APs. * p <0.05 vs. control; ** p <0.01 vs. control.

p <0.05). No significant effects on MDP were observed after application of Ni^{2+} or Cs^+ . Taken together, the strong effects of I_{CaL} and I_{NCX} blockage on primitive APs suggest

that I_{CaL} and I_{NCX} may play a crucial role in AP generation at E8.5, while I_{f} and I_{CaT} may contribute less to AP generation.

Fig. 7. Effect of NiCl_2 on AP generation. (A), low concentrations of NiCl_2 abolished the APs regardless of the cell type. Before the AP halted, the MDP depolarized, the APD shortened, Vdd slowed (especially in the second phase of DD), and V_{max} decreased. The right panel shows representative control traces and APs after drug application. (B) Original recordings of FPs before and after Ni^{2+} application. Ni^{2+} exerted negative chronotropic effects on 100% of E8.5 hearts.

Fig. 8. Effect of CsCl on AP generation. (A), application of 1 mM Cs^+ led to a decrease of AP frequency, accompanied by a similar decrease of Vdd in the three cell types. (B) Original recordings of FPs before and after Cs^+ application.

	Control	NiCl_2	Control	NiCl_2	Control	NiCl_2
	P (n=4)		V (n=6 out of 8)		A (n=6 out of 9)	
MDP (mV)	-55.6±1.6	-49.0±2.2	-57.6±2.3	-54.1±2.3*	-57.3±1.9	-51.5±2.2*
APD90 (ms)	72.6±6.5	66.3±1.5	125.0±12.5	102.7±11.2*	90.0±4.1	81.0±4.6
APD50 (ms)	39.3±6.1	31.3±3.9	92.3±11.9	67.3±9.6**	55.5±2.0	46.5±0.7
APD20 (ms)	21.6±3.3	17.3±2.3	53.0±7.7	37.7±5.5**	19.7±4.1	17.3±2.8
Vdd (V/s)	0.066±0.06	0.039±0.07	0.0375±0.007	0.018±0.003*	0.066±0.007	0.039±0.007
V_{max} (V/s)	4.76±0.9	1.73±0.3	13.9±2.8	4.5±1.3*	16.7±2.0	9.7±1.3*
Frequency (beat/min)	134±13	0**	88±11	0*	118±22	0**

Table 6. Effect of 40 μM Ni^{2+} on E8.5 mature-like APs. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control.

Specific roles of inwardly directed ion channels in mature-like cardiomyocytes and whole mount hearts

Role of I_{CaL} - The I_{CaL} specific blockers verapamil+nifedipine completely abolished all APs at E8.5. Before the halt of these APs we noticed that MDP depolarized, APD shortened, Vdd slowed, and V_{max} decreased (Fig. 4A, Table 3), implying that I_{CaL} plays a

role in diastolic depolarization (DD) and upstroke of APs in all cell types. Thus, in fetal cardiomyocytes at E8.5 mainly transsarcolemmal Ca^{2+} flux may contribute to AP generation and membrane potential depolarization [20, 21]. Similarly, MEA measurements of whole mount hearts showed strong negative chronotropic effects (100% reduction, $n=6$, $p < 0.01$) of verapamil+nifedipine (Fig. 4B).

	Control P (n=11 out of 16)	CsCl	Control V (n=6 out of 10)	CsCl	Control A (n=9 out of 14)	CsCl
MDP (mV)	-59.2±2.1	-58.2±1.7	-61.2±2.2	-61.4±2.4	-61.3±2.1	-61.7±2.5
APD90 (ms)	77.3±5.8	71.5±7.1	121.2±16.0	127.8±17.7	65.7±3.4	72.2±8.9
APD50 (ms)	45.8±3.8	38.9±5.7	88.3±13.0	91.2±14.1	28.6±4.3	20.7±2.9*
APD20 (ms)	25.0±2.6	19.5±3.6*	52.3±10.3	55.0±10.3	10.6±2.4	5.5±1.3
Vdd (V/s)	0.065±0.00	0.047±0.004**	0.054±0.015	0.038±0.011*	0.047±0.005	0.021±0.005**
Vmax (V/s)	20.8±1.9	17.6±3.5	29.3±1.5	26.7±2.4	24.6±2.3	25.1±2.7
Frequency(beat/min)	129±10	95±12**	117±25	79±14 *	126±14	63±16**

Table 7. Effect of 5 mM Cs⁺ on E8.5 mature-like APs. *p<0.05 vs. control; **p<0.01 vs. control.

Role of NCX - KB-R7943, inhibiting reverse-mode I_{NCX} and SEA0400, inhibiting reverse- and forward-mode, exerted similar strong negative chronotropic effects. Approximately 50% of P-, V-, and A-like cells responded to KB-R7943 and SEA0400. In these cells, both blockers completely abolished APs and depolarized the MDP (Fig. 5-6A, Table 4, 5). NCX blockage led to decreasing Vdd, shortening of the APD and MDP depolarization (Table 4, 5). Also MEA measurements showed that KB-R7943 led to a ~64% reduction of the FP frequency in all hearts (n=6, p<0.05, Fig. 5B). Likewise, SEA0400 exerted strong effects. It abolished the spontaneous FPs in all four tested hearts (n=4, p<0.05, Fig. 6B).

Role of I_{CaT} - 40 μ M Ni²⁺ led to a halt of all P-, V-, and A-like APs (Fig. 7A, Table 6) and a significant depolarization of the MDP. Moreover, APD decreased in all three cell types in the presence of Ni²⁺. No significant changes of Vmax and MDP were observed. In the presence of Ni²⁺ 50% of hearts stopped spontaneous FP generation (Fig. 7B, n=5, p<0.01). The remaining hearts responded to Ni²⁺ with a decrease of FP frequency (~28% reduction, n=5, p<0.05).

Role of I_f - At E8.5, application of 1 mM Cs⁺ led only to a moderate decrease of AP/FP frequency and a reduction of Vdd (Fig. 8A-B, Table 7) but did not evoke AP/FP blocks.

Discussion

Although in the adult heart different ion channels have been identified that regulate cardiac electrical activity, up to date little is known about the onset/uphold of electrical activity in the embryonic heart. We therefore aimed to test different ion channels for their functional expression and role in AP generation.

The earliest stages of cardiac development studied (E8.5) were characterized by MP oscillations, primitive APs, and mature-like APs implying that embryonic cardiac cells have different differentiation rates. We assume that the primitive APs are a transitional stage from MP oscillations towards mature-like APs. The shift from MP oscillations to primitive APs was paralleled by a higher expression of Ca_v1.2, Ca_v3.2, NCX1 and HCN4. The higher expression of K_{ir}3.1 in P-like cell and higher expression of K_{ir}2.1 in V- and A-like APs may have contributed to the stable and more negative MDP in cells showing mature-like APs.

It is of major importance that blockage of I_{CaL} , I_{CaT} and I_{NCX} led to strong MDP depolarization and finally to a halt of APs and FPs. In line with these data, recent studies showed that early embryonic cardiomyocytes displayed [Ca²⁺]_i oscillations that could lead to small depolarizations of the MP [13, 14]. Only when reaching the threshold potential these oscillations could give rise to typical APs. As also suggested by our data removal of extracellular Ca²⁺ led to cessation of the MP oscillations and AP generation [14].

Studies on murine ES cell-derived cardiomyocytes suggested that early APs were mainly driven by I_{CaL} [18, 19]. Our findings indicate that in native VEDS cardiomyocytes AP upstroke is not only dependent on I_{CaL} but also I_{CaT} .

Another important parameter regulating AP frequency is the diastolic depolarization phase. Although I_{CaL} [22, 23], I_{CaT} [24], I_{NCX} [25, 26] and I_f [27, 28] are well known to be potentially involved in the modulation of Vdd, their specific role during pacemaker activity is still discussed controversially [29-32]. We observed that blockage of either I_{CaT} , I_{NCX} or I_f significantly decreased Vdd. Importantly, blockers of I_{CaL} , I_{CaT} and I_{NCX} finally led to a complete block of AP/FP generation whereas

blockage of I_f only moderately reduced AP/FP frequency. Since blockage of a single type of each of the tested Ca^{2+} channels/exchanger led to an immediate halt of beating at VEDS we hypothesize that only ion flux through the entirety of these tested ion channels is sufficient to hold up AP/FP generation and spontaneous activity. Thus, as compared to the role of Ca^{2+} influx, I_f is of minor importance for AP/FP generation and uphold as well as for the modulation of AP/FP frequency. These data are in contrast to the findings in adult heart tissue where I_f was shown to be one of the major currents contributing to the spontaneous diastolic depolarization of pacemaker cells [27, 28]. In summary, we here show that embryonic cardiomyocytes may have different differentiation rates at E8.5 with differences in ion channel gene expression. AP/FP generation is only possible when Ca^{2+} influx

through I_{CaL} , I_{CaT} and I_{NCX} is ensured. Since the gene expression and functional expression of ion channel currents in the diseased heart resemble to some extent the early embryonic expression pattern [33-35], information about ion channel expression and their function in the embryo/fetus may offer clues for a better understanding of pathophysiological processes in the heart.

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