Acta Physiologica Sinica, December 25, 2020, 72(6): 757–764
DOI: 10.13294/j.aps.2020.0086 http://www.actaps.com.cn

Original Article

Functional roles of sodium-calcium exchange in autorhythmicity and action potential of murine fetal cardiomyocytes at early developmental stage

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Abstract: The aim of the present paper was to study the role of sodium calcium exchanger (NCX) in the generation of action potentials (APs) in cardiomyocytes during early developmental stage (EDS). The precisely dated embryonic hearts of C57 mice were dissected and enzymatically dissociated to single cells. The changes of APs were recorded by whole-cell patch-clamp technique before and after administration of NCX specific blockers KB-R7943 (5 μmol/L) and SEA0400 (1 μmol/L). The results showed that, both KB-R7943 and SEA0400 had potent negative chronotropic effects on APs of pacemaker-like cells, while such effects were only observed in some ventricular-like cardiomyocytes. The negative chronotropic effect of KB-R7943 on ventricular-like cardiomyocytes was accompanied by shortening of AP duration (APD), whereas such an effect of SEA0400 was paralleled by decrease in velocity of diastolic depolarization (Vdd). From embryonic day 9.5 (E9.5) to E10.5, the negative chronotropic effects of KB-R7943 and SEA0400 on ventricular-like APs of embryonic cardiomyocytes gradually disappeared. These results suggest that, in the short-term development of early embryo, the function of NCX may experience developmental changes as evidenced by different roles of NCX in autorhythmicity and APs generation, indicating that NCX function varies with different conditions of cardiomyocytes.

Key words: SEA0400; KB-R7943; sodium calcium exchanger; fetal cardiomyocytes; action potential

钠钙交换在早期小鼠胚胎心肌细胞自律性和动作电位中的作用

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摘 要：本文旨在研究钠钙交换体(sodium calcium exchanger, NCX)在胚胎早期发育阶段心肌细胞动作电位(action potential, AP)产生过程中的作用。酶解法获得C57小鼠胚胎发育早期的单个心肌细胞后，用全细胞膜片钳技术记录NCX特异性阻断剂KB-R7943 (5 μmol/L)和SEA0400 (1 μmol/L)给药前后心肌细胞AP变化。结果显示，KB-R7943和SEA0400对起搏细胞样心肌细胞的AP均具有显著的负性变时作用，但只对部分心室细胞样心肌细胞产生这种作用。KB-R7943对心室细胞样心肌细胞的负性变时作用伴有动作电位时程(action potential duration, APD)缩短；而SEA0400的这种负性变时作用则与舒张期自动去极化速度(velocity of diastolic depolarization, Vdd)降低相关。从胚胎龄9.5天(embryonic day 9.5, E9.5)到E10.5，KB-R7943和SEA0400对心室细胞样胚胎心肌细胞AP负性变时作用逐渐消失。以上结果提示，在胚胎早期的短期发育过程中，NCX在胚胎心肌细胞自律性和AP形成中可能发生发育依赖性变化，表明NCX在不同条件/状态的心肌细胞中具有不同的功能。

关键词：SEA0400；KB-R7943；钠钙交换体；胚胎心肌细胞；动作电位
中图分类号：R33

Received 2019-12-24 Accepted 2020-04-17
This work was supported by the National Natural Science Foundation of China (No. 30700262)
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The sodium calcium exchanger (NCX) has been found present in the heart for more than forty years [1]. It is well accepted that the stoichiometry of cardiac NCX is $3Na^+$:$1Ca^{2+}$ [2–4], which means a net charge transportation across the membrane. The driving force for NCX is E_{NaCa} ($E_{NaCa} = 3E_{Na} - 2E_{Ca}$) [5]. During the action potential (AP), E_{NaCa} changes, thus NCX can act as a forward mode (Ca^{2+} efflux) or a reverse mode (Ca^{2+} influx) [6]. The Ca^{2+} fluxes and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are very sensitive to intracellular Na^+ concentration ($[Na^+]_i$) [7]. For quite a long time, the importance of NCX is centered on its contribution to cardiac contractility [8]. The Ca^{2+} efflux via NCX of the forward mode is the most important mechanism for Ca^{2+} removal [9, 10], and the net Ca^{2+} entry via NCX of the reverse mode contributes to trigger Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) [11, 12].

Contribution of NCX to AP configuration has been fully discussed, but it still remains controversial [6, 10, 13]. The contrary effects of NCX blockage on AP duration (APD) are reported by different researchers [14, 15]. The NCX current via the forward mode may generate a substantial portion of the inward depolarizing current contributing to spontaneous pacemaking. The early evidence for NCX in rabbit pacemaker cells comes from a measurement of two inward currents in response to depolarization, the second one being proposed to be NCX [16, 17]. The experimental evidence supporting a role of NCX in pacemaking has come from several recent studies [18–20]. However, the study from Kang and Hilgemann [3] has raised the possibility that the contribution of NCX to pacemaking may occur not only through the conventionally accepted mode as above mentioned, but also through an additional transport mode. Nevertheless, NCX is still suspected to generate triggered activity in some different models: it is indeed expressed in sinoatrial node and contributes to the autorhythmicity [21] and plays a pivotal role in inward rectifier potassium current (I_{K1}) suppression induced pacemaking activity [22], while NCX blocker KB-R7943 did not prevent aconitine-induced ventricular arrhythmia [23].

All the fetal cardiomyocytes are characterized with autorhythmicity in spite of cell phenotypes [24, 25], and high level of NCX expression is observed in early developmental stage (EDS) cardiomyocytes [26, 27]. Targeting on the above mentioned debates on NCX, the present study applied specific NCX blockers KB-R7943 [28, 29] and SEA0400 [30] to explore the exact role of NCX in the pacemaking activity in EDS fetal cardiomyocytes, which would possibly provide interesting diversities of pacemaking activity under different conditions.

1 MATERIALS AND METHODS

1.1 *Whole-cell patch-clamp technique*

The 6–8-week old female C57 mice (provided by the Center of Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology) were superovulated for precise timing of their pregnancy [31]. They were sacrificed by cervical dislocation at embryonic day 9.5 (E9.5) and E10.5. The embryonic hearts were dissected and incubated in 1 mg/mL collagenase B solution (Roche, Germany) for 20 min at 37 °C to get single cells as previously described [32]. The whole-cell configuration of the patch-clamp technique was used throughout the study. The internal pipette solution contained (in mmol/L): KCl 50, K-Aspartate 80, $MgCl_2$ 1, MgATP 3, EGTA 10, HEPES 10, pH adjusted to 7.40 at 37 °C with KOH. The extracellular solution contained (in mmol/L): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10, pH adjusted to 7.40 at 37 °C with NaOH. The 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). Due to the differential ion channel distribution and morphology of APs[33], the shoulder-like APs were considered as pacemaker-like, and the APs with obvious plateau were taken as ventricular-like. Accordingly, we defined the pacemaker-like APs as following: the faster velocity of diastolic depolarization (Vdd) (> 0.06 V/s), shorter APD (APD50 < 80 ms), and correlatively less negative maximum diastolic potential (MDP). On the contrary, the ventricle-like APs had much slower Vdd (< 0.04 V/s), longer APD (APD50 > 100 ms) and more negative MDP.

1.2 *Chemical reagents and treatments*

KB-R7943 and SEA0400 were purchased from Tocris Bioscience (Bristol, UK) and Taisho Pharmaceutical Co. Ltd (Saitama, Japan), respectively. The chemicals were dissolved in the extracellular solution to get the final concentration (5 μmol/L for KB-R7943 and 1 μmol/L for SEA0400). The chemicals were applied onto the cells when the APs were stable in normal

extracellular solution for at least 10 s, and the washout with normal extracellular solution was performed when the maximum effects on APs morphology were observed.

1.3 *Data and statistical analysis*

The data were obtained by averaging APs recorded in the last five seconds before the application of blockers (self-control) or in the last five seconds of drug presence. APD, Vdd, and MDP were obtained from the last five seconds before AP halt if halt of APs occurred. The results were presented as mean ± SEM. The statistical analysis was done with SigmaPlot using paired and unpaired student's *t*-test where applicable. *P* values of < 0.05 were considered significant.

2 RESULTS

2.1 Negative chronotropic effect of KB-R7943 and SEA0400 on EDS pacemaker-like cardiomyocytes

As listed in Table 1, both KB-R7943 and SEA0400 had negative chronotropic effects on all the tested pacemaker-like cardiomyocytes (Fig. 1). However, the KB-R7943 application arrested the spontaneous APs in E9.5 pacemaker-like cells (n = 3), while at E10.5 it only exerted a moderate negative chronotropic effect (n = 4). SEA0400 led to cessation of all tested E9.5–E10.5 pacemaker-like cells (E9.5: n = 4; E10.5: n = 2). Before the cessation of APs, Vdd was analyzed, and similar effects of SEA0400 were observed as compared to KB-R7943 (KB-R7943: Vdd was decreased by 23.6% ± 6.8%; SEA0400: Vdd was decreased by 37.2% ± 11.2%). Both KB-R7943 and SEA0400 intended to shorten APD and depolarize MDP (Table 1, Fig. 1).

2.2 Negative chronotropic effect of KB-R7943 and SEA0400 on partial EDS ventricular-like cardiomyocytes

KB-R7943 exerted a strong negative chronotropic effect on a few ventricular-like (8 out of 22) cardio-

Table 1. Negative chronotropic effects of KB-R7943 and SEA0400 on pacemaker-like cardiomyocytes

	$Control_{KB\text{-}R7943}$	KB-R7943 (n = 7)	$Control_{SEA0400}$	SEA0400 (n = 6)
Frequency (/min)	131.7 ± 25.3	77.1 ± 32.4**	133.4 ± 13.5	0**
Vdd (V/s)	0.071 ± 0.004	0.060 ± 0.008*	0.080 ± 0.009	0.055 ± 0.001*
MDP (mV)	−60.6 ± 2.7	−56.4 ± 2.7*	−59.4 ± 3.1	54.1 ± 3.0
APD50 (ms)	62.5 ± 8.0	58.6 ± 7.3	67.1 ± 12.2	50.1 ± 9.7*
APD20 (ms)	33.5 ± 5.6	30.3 ± 4.4	37.8 ± 3.4	26.0 ± 6.7*

Mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* self-control. Vdd: velocity of diastolic depolarization; MDP: maximum diastolic potential; APD50, action potential duration at 50% repolarization; APD20, action potential duration at 20% repolarization.

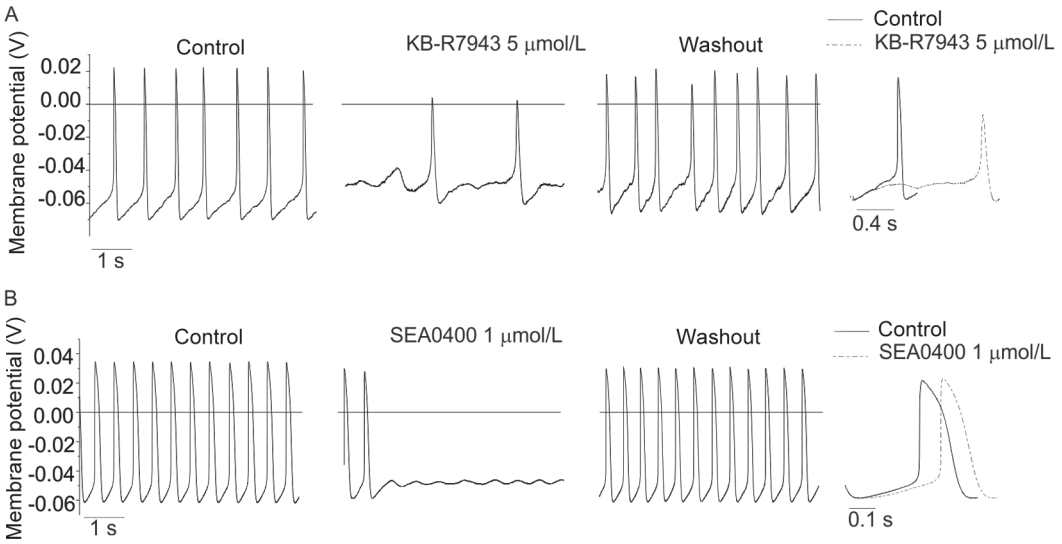

Fig. 1. Negative chronotropic effects of KB-R7943 and SEA0400 on pacemaker-like cardiomyocytes. Both 5 μmol/L KB-R7943 (*A*) and 1 μmol/L SEA0400 (*B*) had negative chronotropic effects on all the tested pacemaker-like cardiomyocytes. Figures in the last panel are the representative APs with expanded scales of control and corresponding drug-treated groups.

myocytes (Table 2, Fig. 2*A*). In these cells, the significant decrease in APD ($P < 0.05$) was observed, while Vdd was increased moderately (by 33.4% ± 3.3%, $P < 0.05$), indicating the negative chronotropic effect correlated to changes in APD, but not Vdd. The increase in Vdd might due to the depolarization in MDP (by 9.1 mV ± 2.1 mV, $P < 0.05$). In the remaining cells ($n = 14$, Fig. 3*A*), the AP frequency was increased (by 10.6% ± 2.9%, $P < 0.05$), paralleled by the increase in Vdd (by 27.8% ± 6.2%, $P < 0.01$). APD did not change significantly ($P > 0.05$). An obvious shift of effects of KB-R7943 was observed in ventricular-like cardiomyocytes with the short maturation from E9.5 to E10.5 (Fig. 4*A*). Almost all the ventricular-like cardiomyocytes ($n = 7$) listed in Table 2 were the E9.5 ones, while in the remaining cells without negative chronotropic effect, 11 ventricular-like cardiomyocytes were from the E10.5 ones.

SEA0400 persisted in a negative chronotropic effect on a higher portion of E9.5–E10.5 ventricular-like cardiomyocytes (15 out of 27, Table 2, Fig. 2*B*). The negative chronotropic effect was accompanied with a decrease in Vdd (by 64.6% ± 0.8%, $P < 0.01$) and such effects disappeared in the remaining cells (Fig. 3*B*) in which both AP frequency and Vdd were increased ($n = 12$, $P < 0.05$). APD50 and APD20 were shortened ($P < 0.05$) in cells with either KB-7943 or SEA0400 treatment. Similar to the effects of KB-R7943, with slight maturation from E9.5 toward E10.5, SEA0400 exerted negative chronotropic effects on less ventricular-like cardiomyocytes (Fig. 4*B*). The ventricular-like cardiomyocytes listed in Table 2 were mostly from the E9.5 ones ($n = 10$), while the ventricular-like cardiomyocytes without negative chronotropic effect were mostly from the E10.5 ones ($n = 10$).

Table 2. Negative chronotropic effects of KB-R7943 and SEA0400 on ventricular-like cardiomyocytes

	$Control_{KB\text{-}R7943}$	KB-R7943 ($n = 8$)	$Control_{SEA0400}$	SEA0400 ($n = 15$)
Frequency (/min)	70.7 ± 12.2	0**	58.0 ± 5.4	7.9 ± 2.7**
Vdd (V/s)	0.033 ± 0.005	0.047 ± 0.009*	0.030 ± 0.003	0.011 ± 0.003**
MDP (mV)	64.7 ± 2.2	55.6 ± 2.3**	62.8 ± 5.1	59.2 ± 1.6*
APD50 (ms)	115.5 ± 20.3	67.3 ± 9.1*	107.4 ± 11.5	76.0 ± 8.4**
APD20 (ms)	61.1 ± 13.0	33.1 ± 5.4*	58.3 ± 8.2	43.0 ± 4.7*

Mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* self-control. Vdd: velocity of diastolic depolarization; MDP: maximum diastolic potential; APD50, action potential duration at 50% repolarization; APD20, action potential duration at 20% repolarization.

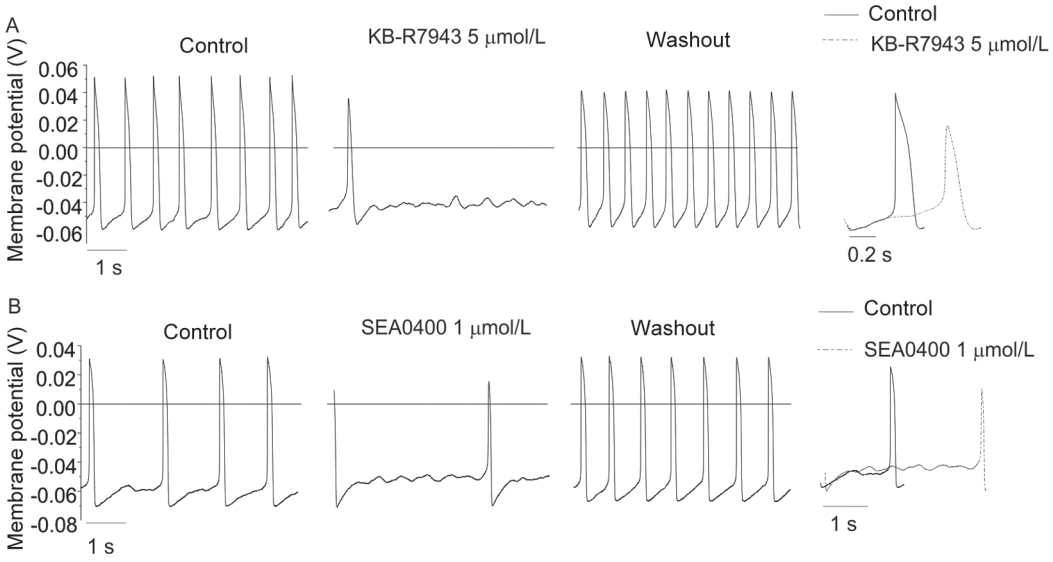

Fig. 2. Negative chronotropic effects of KB-R7943 and SEA0400 on partial ventricular-like cardiomyocytes. Both 5 μmol/L KB-R7943 (*A*) and 1 μmol/L SEA0400 (*B*) had negative chronotropic effects on some ventricular-like cardiomyocytes. Figures in the last panel are the representative APs with expanded scales for control and after the treatments of drugs.

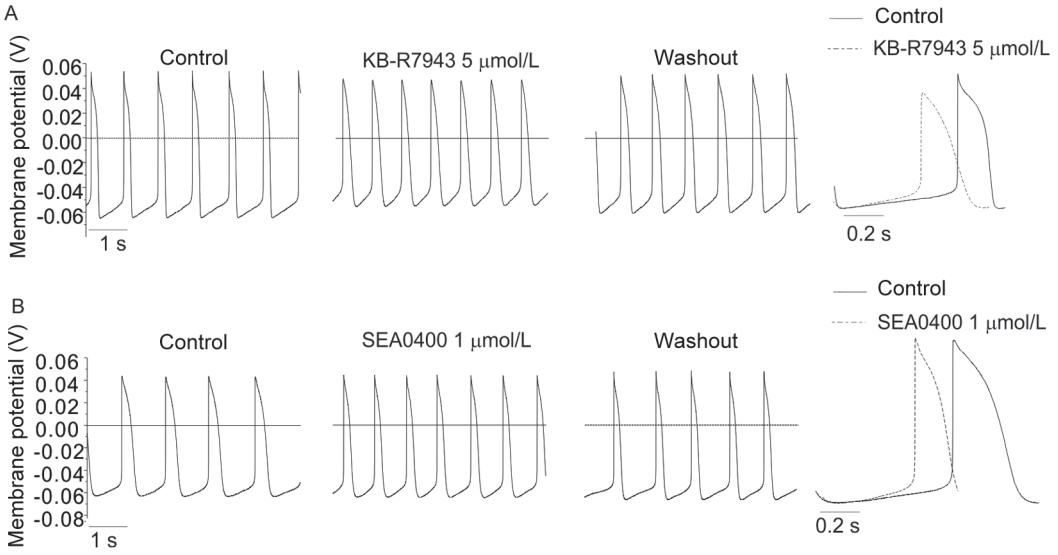

Fig. 3. Slight positive chronotropic effects of KB-R7943 and SEA0400 on partial ventricular-like cardiomyocytes. Both 5 μmol/L KB-R7943 (*A*) and 1 μmol/L SEA0400 (*B*) had positive chronotropic effects on some ventricular-like cardiomyocytes. Figures in the last panel are the representative APs with expanded scales for control and after the treatments of drugs.

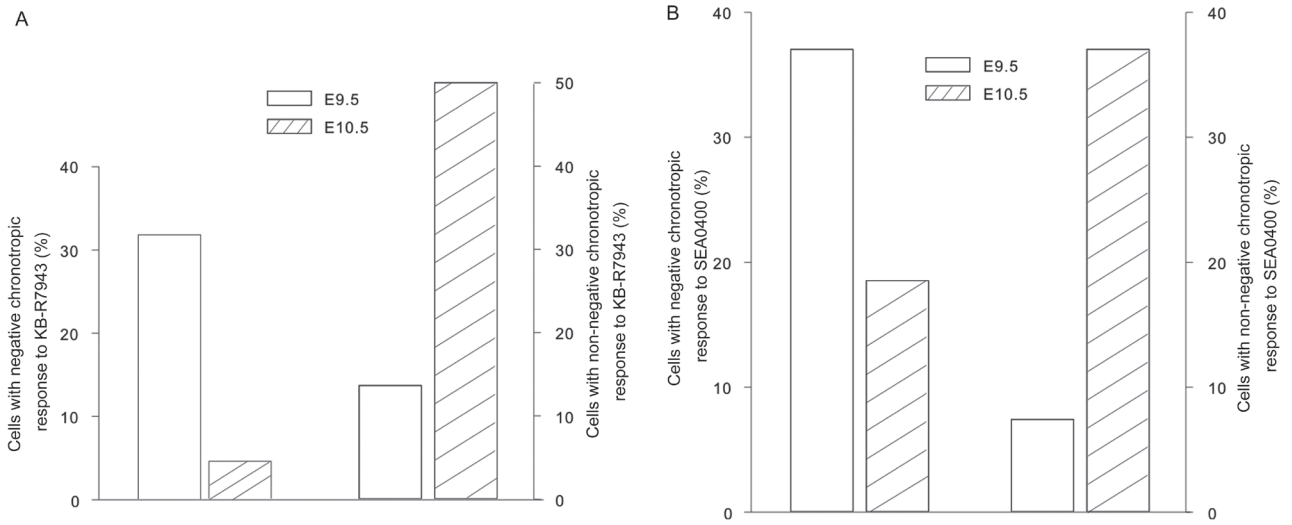

Fig. 4. Summation of the ventricular-like cardiomyocytes with different response to KB-R7943 and SEA0400. *A*: Percentages of cells with or without negative chronotropic response to KB-R7943. *B*: Percentages of cells with or without negative chronotropic response to SEA0400.

3 DISCUSSION

The functional role of NCX in pacemaking [3, 18–23] and the AP configuration [6, 10, 13–15] were investigated by many scientists, but still remained controversial. In the present study, two NCX blockers with different pharmacology were administered onto the EDS cardiomyocytes to address the functional roles of NCX in these cells. As reported, KB-R7943 could inhibit outward NCX (reverse mode) more potently (IC_{50} = 0.3 μmol/L) than inward NCX (IC_{50} = 17 μmol/L) [28–30, 34, 35]. However, KB-R7943 at 10 μmol/L, inhibited the sodium current, L-type calcium current, delayed rectifier potassium current and I_{K1} by more than 50%. SEA0400 (1 μmol/L) inhibited NCX by about 10 folds, more potently than that of KB-R7943 without inhibiting other ion channel currents [30, 35].

Many previous reports provided experimental proof of NCX activity in the diastolic depolarization [18–20], which was in accordance to the finding in the present

study that both KB-R7943 and SEA0400 had potent negative chronotropic effects on all tested pacemaker-like cells and partial ventricular-like cardiomyocytes. NCX could act as forward mode with a net inward current or reverse mode with a net outward current, but could not induce the calcium induced calcium release (CICR) from the SR which might in turn cause the myocytes depolarization [37]. The effect of KB-R7943 suggested that NCX could contribute to the diastolic depolarization in the reverse mode, as indicated by the fact that in pacemaker-like and partial ventricular-like cardiomyocytes KB-R7943 exerted negative chronotropic effects. Interestingly, KB-R7943 could exert a negative chornotropic effect only if it could shorten the APD. On the contrary, the negative effects of SEA0400 were consistently accompanied with the decrease in Vdd, regardless of changes in APD. This strongly suggested NCX activity in diastolic depolarization process in a forward mode.

It must be noted that our cell model provided two distinct cell populations. NCX functioned in the diastolic depolarization in one population which responded to the NCX blockers with negative chronotropic changes, but not in the other one. This existence of the cells without response to NCX blockers in Vdd supported the previous suspicion on NCX's contribution to pacemaker activity in drug-induced ventricular arrhythmia [23]. The contribution of NCX to partial E9.5 cardiomyocytes was similar to the findings in E8.5 cardiomyocytes that the NCX blockers treatment shortened APD and then made APs disappear [24], suggesting that E9.5 serves as a transitional stage from E8.5 to E10.5. At E10.5 KB-R7943 and SEA0400 exerted positive chronotropic effects in most ventricular-like cardiomyocytes. This implied a transition of NCX function in pacemaking from E8.5 to E10.5. At E8.5, Ca^{2+} influx through L type calcium ion channel, T type calcium ion channel and NCX was essential to generate APs which might be followed by Ca^{2+} release from SR [37]. Therefore, KB-R7943 or SEA0400 decreased the AP frequency in E8.5–E9.5 cardiomyocytes via the Ca^{2+} efflux through NCX in a forward mode. The different effects of KB-R7943 and SEA0400 at E10.5 favored the previous finding that the net inward current originated from NCX of inward mode potentially drove the diastolic depolarization [36].

Because of its $3Na^{+}$:$1Ca^{2+}$ stoichiometry, NCX activity of reverse mode potentially shortened APD [38]. Additionally, NCX activity of forward mode in phase 3 repolarization phase enhanced the repolarization [6]. It was generally accepted that the Ca^{2+} influx mode of the NCX appeared during the rapid upstroke of AP because MDP (Em) exceeds the NCX reversal potential ($E_{Na/Ca}$) in this brief period [6, 10]. Thus NCX might be inward during most of the APs under physiological condition, therefore NCX activity prolonged APD [10], as observed in the shortening effects of KB-R7943 and SEA0400 on APD20 and APD50. In some ventricular-like cardiomyocytes, KB-R7943 did not influence APD50 or APD20. This may lie in the contrary contribution of NCX of reverse and forward modes to AP configuration in the same cell, or the complicated unselective blockade of other currents [28–30, 34, 35].

In summary, during short maturation from E9.5 to E10.5, the function of NCX had experienced developmental changes as illustrated by the distinct importance of NCX activity in diastolic depolarization and APD. Since the diseased heart attempted to exhibit features of immature heart, this observation also partially displayed interesting diversities of pacemaking activity under different conditions.

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