Role of Na^+/Ca^{2+} Exchanger in the Initiation of Spontaneous Electrical Activity during Early Cardiogenesis

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Abstract Na^+/Ca^{2+} exchanger (NCX) is the earliest functional genes in the developing mouse heart. It has been proposed to contribute to intracellular Ca^{2+} homeostasis and probably excitation-contraction coupling by electrogenic exchange of 1 intracellular Ca^{2+} ion for 3 extracellular Na^+ ions. To date the functional expression of NCX and its correlation with the early spontaneous electrical activity during cardiogenesis are not thoroughly clarified. Using ES cell derived cardiomyocytes, we have found at very early development stage, NCX current ($I_{Na/Ca}$) is the major contributor of the caffeine (10 mmol/L) induced inward current at a constant holding potential of 35 mV as isomolar Li^+ replacement of external Na^+ blocked nearly 80% of the evoked inward current (n=8). NCX1 mRNA was identified in all these functionally measured cardiac cells using single-cell RT-PCR. Further functional relevance was investigated. The complete abolishment of membrane fluctuations and the intercalated action potentials (the earlier patterns of spontaneous electrical activity) by isomolar Li^+ replacing external Na^+ and Ni^{2+} (5 mmol/L) implicated the essentiality of NCX in the initiation of early membrane excitation. Thus we conclude that NCX1 is highly expressed even in very early stage cardiomycoytes and it plays a pivotal role in set-up of early spontaneous electrical activity.

Key words Na^+/Ca^{2+} exchanger; membrane fluctuations; action potentials

The Na^+/Ca^{2+} exchanger (NCX), an ion transport protein, is expressed in the plasma membrane of virtually all animal cells. It mediates Ca^{2+} entry or extrusion in combination with a reverse transportation of Na^+ under certain conditions. Up to now three genes that code for NCX have been identified in mammals: NCX1, NCX2, and NCX3[1]. In cardiac myocytes, NCX1 has been found to be the predominant isoform. It has been identified in murine embryonic heart even starting from day 7.5 in the protein level[2]. Concerning its functional implication, NCX has been proposed to play an essential role in Ca^{2+} homeostasis. It might be also involved in cardiac excitation-contraction coupling[3]. However, the functional expression of NCX in cardiomyocytes during cardiac development and its functional relevance remain unclear.

Here, using embryonic stem (ES) cell derived cardiomyocytes as a useful model, we provide evidence that NCX underlied mainly by NCX1 are strongly expressed in cardiomyocytes at very early developmental stage (VEDS, the nomenclature refer to[4]). The membrane fluctuations are mainly driven by NCX and independent from voltage-dependent Ca^{2+} channels (VDCCs). While Low voltage activated Ca^{2+} channels only inhibit the action potentials (APs), but not the membrane oscillations.

1 Materials and Methods

1.1 Isolation of single cardiomyocytes

ES cells of the line D3 were cultivated and differentiated into spontaneously beating cardiomyocytes as previously described by Wobus *et al.*[5]. Briefly, cells

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were cultivated in hanging drops (ca. 400 cells per drop) for 2 days, afterwards kept in suspension for 5 days and finally plated on gelatinized multi-well culture plates. For cardiomyocytes at VEDS (differentiation for 9 days), beating areas of 10–15 EBs were dissected and digested by using collagenase B (1 mg/ml) (Boehringer Ingelheim), as described in more detail by Wobus *et al.*[5]. Cells were plated on the gelatine-coated glass coverslips and cultivated in DMEM supplemented with 20% fetal calf serum, penicilline/streptomycine, L-glutamate, MEM (all from Gibco, Eggenstein, FRG), and β-mercaptoethanol (Sigma).

1.2 Electrophysiology

For electrophysiological recordings, perforated patch clamp technique was applied in current and voltage clamp mode using an Axopatch 200A amplifier (Axon Instruments). Data were digitized at 10 kHz, filtered at 1 kHz, stored on hard drive and off-line analyzed with Sigmaplot. Statistical analysis was performed using unpaired Student's *t* test.

Solutions used for perforated voltage and current clamp recordings are the following: 55 mmol/L KCl, 70 mmol/L K_2SO_4, 7 mmol/L $MgCl_2$, and 10 mmol/L Hepes, pH 7.3 (internal solution; pH adjusted with KOH); 140 mmol/L NaCl, 5.4 mmol/L KCl, 3.6 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4 (external solution; pH adjusted with NaOH). For perforated patch clamp, similar bath solution was used except higher $CaCl_2$ concentration (3.6 mmol/L). Nystatin stock solutions of 60 mg/ml were prepared in dimethylsulfoxide right before performing the experiments. A final concentration of 240 μg/ml was prepared with internal solution. Pipettes were submerged into nystatin-free internal solution and then back-loaded with nystatin-containing pipette solution. Series resistances below 20 MΩ were reached within 10 min after forming the giga-seals.

Test substances: Caffeine (10 mmol/L), $NiCl_2$ (30 μmol/L –5 mmol/L) nifedipine (10 μmol/L) (dissolved in DMSO to get 10 mmol/L of nifedipine stock solution, final concentration of DMSO <0.1%), All substances were purchased from Sigma, dissolved in extracellular solution. Isomolar Li^+ was applied to replace external Na^+ to block NCX.

All experiments were performed at 35–37 ℃.

1.3 Single-cell RT-PCR

Single cell RT-PCR was performed after patch clamp recording. Single cells were collected with patch pipettes, expelled into a thin-wall PCR tube, frozen on dry ice, and used as the template for RT-PCR. External solution was made with nominally RNase-free water (Milli-Q PF, Millipore, Bedford, MA) and sterile gloves were worn during the procedure to minimize RNase contamination. NCX1 and GAPDH (positive control) were amplified using one step RT-PCR kit (Qiagen) according to the manual. Briefly in details, the reaction mixture contained 6.4 μl of H_2O, 3.6 μl of 5 × buffer, 0.8 μl of dNTPs, 1.2 μl of each primer, 0.8 μl of enzyme mix, 4 μl of Q-solution, 2 μl of RNA template. The thermal cycling program for all primers sets was 50 ℃ for 30 min, 94 ℃ for 0.5 s, 56 ℃ for 90 s and 72 ℃ for 90 s, cycling was repeated 45 times and then PCR ended with 10 min final extension. The primers are the following: 5'-GTGACTCACGTGAGCGAGAG-3' (NCX, sense primer); 5'-CTCTTGAATGATCACCTCCA-3' (NCX, anti-sense primer). The splicing variants were evaluated with the primer pairs[6]. 5'-TGTCAGCAATGCATCCTGCA-3' (GAPDH, sense primer); 5'-CCGTTCAGCTCTGGGATGAC-3' (GAPDH, anti-sense primer).

2 Results

Pluripotent, murine ES cells of the cell line D3 were differentiated into cardiomyocytes (Fig.1). Spontaneously beating cardiomyocytes at VEDS were used for patch clamp experiments.

We first measured inward Na^+/Ca^{2+} exchange current ($I_{Na/Ca}$, Ca^{2+} efflux mode) by recording membrane current during rapid application of 10 mmol/L caffeine to the bath. The membrane potential was hold constantly at 35 mV prior to and during exposure to caffeine. Fig. 2A showed that a transient inward current was evoked by application of caffeine. In cardiac cells, the Ca^{2+}-activated conductance could be via nonselective cation channel and/or NCX. To clarify the ion channels mediating Ca^{2+} dependent conductance, isomolar Li^+ replacing Na^+ in the bath solution was used to inhibit NCX,

Fig.1 ES derived cardiomyocytes under the phase contrast microscope (×400)

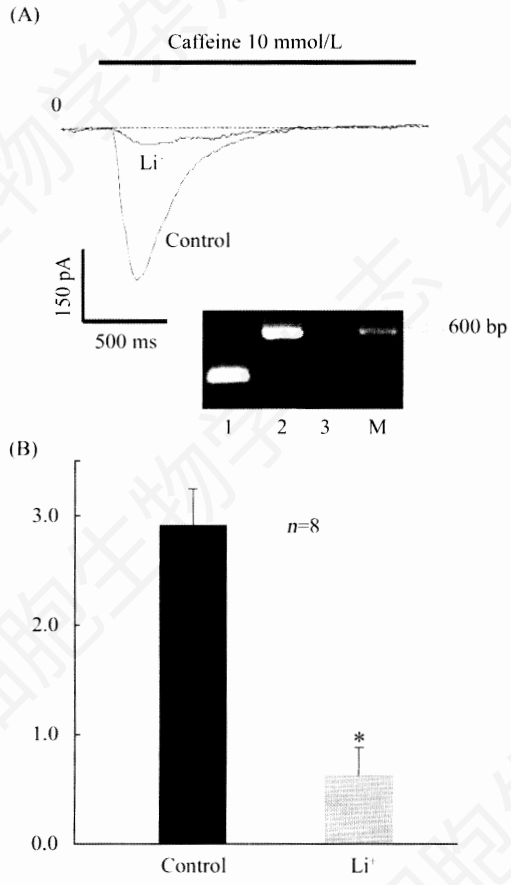

Fig.2 In ES cell derived cardiomyocytes (differentiation 8–9 days), caffeine-induced increase of $[Ca^{2+}]_i$ led to activation of inward current at a holding potential of 35 mV (A, upper) with a current density of (2.9 ± 0.6) pA/pF (n=8, B)

Isomolar replacement of Na^+ by Li^+ depressed current density to (0.7 ± 0.3) pA/pF (n=8, B, $^*P < 0.01$). Below the current trace is single-cell RT-PCR from this cell. M: 1 kb DNA size marker; 1: GAPDH (239 bp); 2: NCX amplification; 3: negative control.

while keep Na^+ channel conductive[7]. We observed that almost 80% of the inward current was blocked (n=8). To identify the molecular constituents of NCX, we combined patch-clamp recordings with single-cell RT-PCR. After measuring the current, cells were taken for RT-PCR. Fig.2A (lower panel) showed that NCX1 (520 bp) were strongly expressed in these very early stage cardiomyocytes. Thus these data indicate that cardiomyocytes during early cardiogenesis already strongly expressed $I_{Na/Ca}$, which is mainly underlied by NCX1.

To further clarify the functional relevance of NCX in the initiation of early spontaneous electrical activity, we performed current clamp experiment. Cardiomyoctes showed depolarized membrane potential (–59.3 ± 1.2 mV, n=19) and were characterized by small fluctuations of the membrane potential intercalated between the APs (Fig.3, Fig.4). These fluctuations were associated with spontaneous contractions of the cells with a frequency of (3.3 ± 0.2) Hz (n=42). As illustrated in Fig.3A, Fig.3B, Fig.3C, when isomolar Li^+ replaced external Na^+ to inhibit NCX, membrane potential fluctuations and APs were completely eliminated (n=8), while cells kept contracting. High concentration of Ni^{2+} (5 mmol/L), another known blocker of NCX, completely stopped the membrane events (Fig. 3D, Fig.3E, Fig.3F). However under this high concentration of Ni^{2+}, T- and L-type Ca^{2+} channels were also blocked. Therefore, the relevance of these two VDCCs was investigated by pharmacological tools. We found that nifedipine (10 μmol/L), a selective blocker of $I_{Ca\text{-}L}$, did not exert any influence on the membrane events (n=4, Fig.4D, Fig. 4E). In 5 out of 7 cells, addition of low concentration of Ni^{2+} (30 μmol/L), a widely applied T-type Ca^{2+} channel blocker [8], interrupted the generation of APs but not the membrane potential fluctuations and the accompanying contractions (n=15, Fig.4A, Fig.4B, Fig.4C). Thus, we had following observations: (1) NCX was the major channel responsible for the membrane potential fluctuations; the fluctuations were independent of the opening of VDCCs. (2) Based on the membrane fluctuations the APs were triggered and $I_{Ca\text{-}T}$ participates in the generation of the AP spikes.

3 Discussion

In our study, we clearly demonstrates that $I_{Na/Ca}$ is

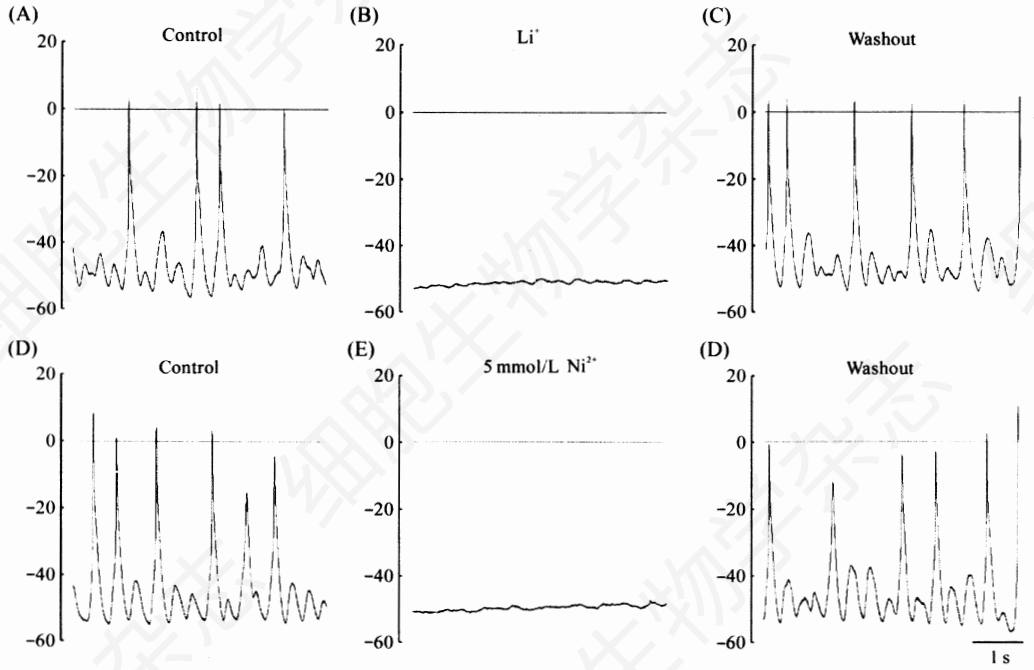

Fig.3 Early spontaneous electrical activity was characterized by membrane oscillations and irregular AP spikes (A, C, D, F). Replacement of external Na^+ by isomolar Li^+ diminished the membrane fluctuations and APs (A–C). Similarly application of Ni^{2+} (5 mmol/L) abolished the membrane events

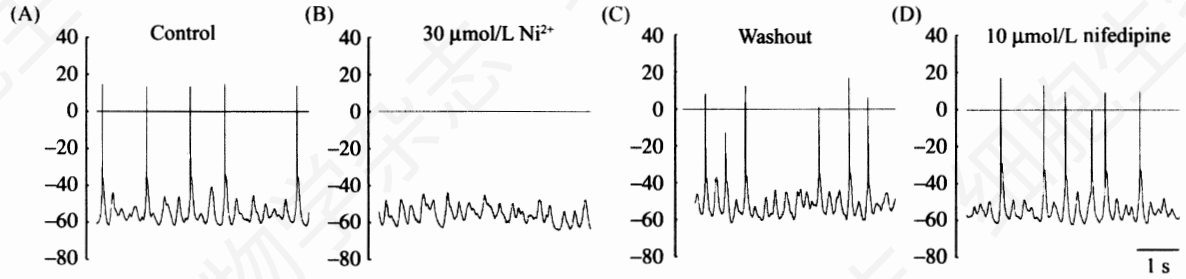

Fig.4 Early spontaneous electrical activity was characterized by membrane oscillations and irregular AP spikes (A, C, D). Low concentration of Ni^{2+} (30 μmol/L) abolished the spikes while membrane oscillations sustained (A–C). Blockade of L-type Ca^{2+} channel by nifedipine (10 μmol/L) has no effect on the early spontaneous electrical activity (C, D)

strongly expressed in cardiomyocytes at very early develop stage. It plays a pivotal role in the initiation of early spontaneous electrical activity.

Concerning spontaneous electrical activity, it has been well accepted that the heart contraction is due to the electromechanical coupling. APs are formed by the successive opening and closure of Na^+ channel, voltage-dependent Ca^{2+} channels (VDCCs) and K^+ channels *et al.* driving fast changes of membrane potential. In this procedure, Ca^{2+} influx via VDCCs triggered Ca^{2+} release from internal Ca^{2+} store (CICR), leading to a marked increase of the free cytosolic Ca^{2+} concentration[9,10], which results in myosin-actin interaction and subsequently contraction of cardiac cells. In pacemaker cells, AP is self-regenerated because of the diastolic depolarization based on activation of I_f[11], and VDCC[12]. This hypothesis has been confirmed by the finding of Ca^{2+} sparks[13].

While different from the regular rhythmic APs, we observed that the spontaneous electrical activity in very early stage cardiomyoctes were characterized by small fluctuations of the membrane potential intercalated between the APs, which is similar to the finding from Viatchenko-Karpinski *et al.*[14]. In his study, the initiation

of the spontaneous electrical activity during early stages of cardiomyogenesis followed a different mechanism: The spontaneous contractions occurring in early stage cardiomyocytes is induced by intracellular Ca^{2+} oscillations. This oscillation in the meanwhile translated into membrane fluctuations, triggering early irregular APs. Therefore it is tempting that the Ca^{2+}-activated conductance (nonselective cation current and/or NCX) might be responsible for intracellular Ca oscillation induced membrane events. However, the ion channels mediating Ca^{2+}-activated conductance has not been clarified. Our data clearly exhibited that the NCX, a transporter protein, were activated by the elevation of intracellular Ca^{2+} concentration. It was the pivotal contributor for the initiation of membrane potential fluctuations ——the earlier pattern of spontaneous electrical activity. Activated NCX pumped Ca^{2+} out to maintain intracellular Ca^{2+} homeostasis, as an exchange, Na^+ were moved in. An inward net current driving membrane depolarization was thus induced by this reverse transportation of three Na^+ and one Ca^{2+} via NCX, membrane potential fluctuation was formed. The elimination of early APs together membrane fluctuations when NCX was blocked indicated that the early APs were triggered by the fluctuations. Further pharmacological test proved that T-type Ca^{2+} currents were involved in the AP formation since low concentration of Ni^{2+} (30 μmol/L) eliminated AP spikes.

It is well known that under pathological conditions early developmental programs are reuptaken. Our observation of $I_{Na/Ca}$ playing an important role in the initiation of spontaneous electrical activity thus could be linked to the finding of an upregulated expression of NCX in hypertrophied heart[15,16].

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钠钙交换在心脏发育早期自主膜电活动发生中的作用

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摘要 钠钙交换是小鼠心脏发育中最早有功能性表达的通道基因。它的功能主要是通过泵出 1 个钙，泵入 3 个钠位置细胞内的钙稳态，此外可能参与兴奋收缩偶联。但是，至今钠钙交换在心脏发育过程中的功能性表达及其在细胞早期兴奋形成中的作用还不是很清楚。采用胚胎干细胞分化的心肌细胞为研究对象，发现在发育极早期，电压钳制在 35 mV 的条件下，10 mmol/L 咖啡因诱导的内向电流的 80% 能被灌流液中 Na^+ 被等浓度的 Li^+ 取代（n=8）。此为钠钙交换电流。所有钳制的细胞单细胞 RT-PCR 都检测到了 NCX1 亚型的 mRNA 表达。进一步研究了钠钙交换的功能，发现等浓度 Li^+ 取代灌流液中 Na^+ 及应用高浓度 Ni^{2+} 阻断了膜电位震荡及与震荡相间的动作电位(早期膜兴奋形式)。因此认为钠钙交换(NCX1 亚型)在心脏发育极早期的心肌细胞中已有大量功能性表达，它对于早期自主性兴奋活动的发生起着关键性的作用。

关键词 钠钙交换；膜振荡；动作电位

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