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Research Article

Differences in Expression and Function in the Atrium versus Ventricle of the Sodium-Calcium Exchanger in the Embryonic Chicken Heart

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Heart function is well known to be dependent on intrinsic electrical activity. This electrical activity is primarily mediated by a combination of interactions among various ionic channels and transporters. In this study, we demonstrate that the Na^+-Ca^{2+} exchanger (NCX) is equally present in both atrial and ventricular cells at early stages of development (st. 13). However, ventricular cells exhibit an increase in NCX messenger ribonucleic acid (mRNA) levels during later stages of development, while levels in atrial cells remain constant. We demonstrate that the current density of the NCX increases with development in the ventricle but remains constant in the atrial cells. Furthermore we demonstrate that the NCX has a major role in shaping the cardiac action potential at early stages mainly in ventricular cells (st. 14) than later mainly in the atrial cells (st. 30).

1. Introduction

The heart is one of the most important organs and its proper function is critical for normal complex organism development and survival. Its role during the development of the embryo has been widely studied for many years [1, 2]. The heart, in the simplest terms, is a tissue pump with the signal for its pumping action provided by spontaneous intrinsic electrical activity. The heart generates electrical activity throughout its lifetime and any irregularity in its generation and/or conduction can have lethal physiological implications [3, 4].

It is well recognized that the Na^+-Ca^{2+} exchanger (NCX) is an integral component of the excitation-contraction coupling cycle in the adult cardiac muscle. The plasma membrane NCX is a bidirectional electrogenic ($3Na^+ : 1Ca^{2+}$) and voltage-sensitive ion transport mechanism, which is mainly responsible for the Ca^{2+} extrusion which follows excitation. For Ca^{2+} extrusion, energy is provided by the Na^+ gradient which is established by the Na^+ pump (for review see [5]). However, the NCX can also work in reverse mode. In this mode internal Na^+ can be exchanged for external calcium. Thus, if the Na^+-pump is inhibited, the elevated levels of Na^+ inside the cell may increase the influx of Ca^{2+} via the NCX [5].

Cardiac contraction is initiated by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, which triggers a release of Ca^{2+} from the sarcoplasmic reticulum (SR) by a Ca^{2+}-induced Ca^{2+} release mechanism. Relaxation is accomplished by the extrusion of Ca^{2+} from the cell by NCX and by re-uptake of Ca^{2+} into the SR (for review see [6, 7]). Thus, the NCX is the dominant cellular Ca^{2+} efflux mechanism in the myocardium in many species. Another pathway for

Ca^{2+} extrusion is the ATP-dependent Ca^{2+} pump, present in the cardiac sarcolemma [8–10]. In cardiac cells the SR is the main regulator of intracellular calcium levels [11]. However the SR is not well developed in embryonic hearts [8]. In the adult heart Ca^{2+} fluxes cross the sarcolemma with each contraction and therefore, it is anticipated that the excitation-contraction coupling would not function in the absence of the NCX. Furthermore, the exchanger, under special circumstances, may reverse direction and transport Ca^{2+} into cardiomyocytes. The importance of the "reverse mode" has been controversial, but it may play an important role during heart failure and during early cardiac development [12, 13]. In both fetal and neonatal hearts, NCX activity is upregulated while SR is relatively sparse, increasing the role of transsarcolemmal Ca^{2+} fluxes in the excitation-contraction coupling [12, 13].

The importance of NCX during development has been previously described in the NCX homozygous knockout (KO) mice which are embryonic lethal by about 11 days post coitum (dpc). The lethality among these mice has been associated with the lack of a beating heart [14–16]. However, surprisingly, embryos from 9.5 dpc exhibit apparently normal Ca^{2+} transients elicited by direct electrical stimulation of the heart tubes from the $NCX^{-/-}$ embryos [15, 16]. In contrast, cardiac-specific NCX knockout mice are viable to adulthood with almost normal cardiac performance [17, 18]. A recent study on the cardiac-specific NCX-KO mice shows a shortened ventricular action potential (AP) duration, together with an increase in transient outward K^+ current and decrease in the L-type Ca^{2+} current [19]. This increase in outward K^+ current is probably responsible for the AP shortening and thus reduced Ca^{2+} influx, thereby preventing Ca^{2+} overload. The reduction of L-type Ca^{2+} current, which is characteristic in this model, seems to be due to inactivation of some Ca^{2+} channels and not to a lower expression of the channels [20]. Furthermore, homozygous overexpression of NCX results in mild cardiac hypertrophy [18] and decline of the Ca^{2+} transient. Relaxation of contraction is increased and the reverse mode of NCX is augmented. The overexpression also leads to higher susceptibility to ischemia-reperfusion injury and to a greater ability of NCX to trigger Ca^{2+}-induced Ca^{2+} release (for review see [18]).

Little is known about the expression/function of NCX in the action potential shape of the different regions of the embryonic chick heart. Previous studies have focus only in chick embryonic ventricular cells [21] or from mouse [22] and rat embryos [23]. In this study, we further investigated the presence and effect of NCX on the spontaneous electrical activity in the atria and ventricles cells during the early stages of heart development. Our results indicated that blocking NCX induces effects in amplitude, duration, and maximum rate of rise of AP in both atrial and ventricular cells similar to those previously described in adult ventricular cells [24, 25]. We also found that the NCX played a degree of difference in atrial versus ventricular cells during same early stages. However, these differences receded in later stages of development.

2. Material and Methods

All animal procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by the Animal Care and Use committee at the University of Missouri-Columbia. Chick embryos were obtained from fertilized White Leghorn eggs (Hy-Line W-36, Hy-Line North America, LLC, West Des Moines, IA) and incubated for a period of up to 6 days at 38°C in an atmosphere of 80% humidity. The embryos were then removed from the egg and placed in modified Tyrode solution as described by Arguello et al. [26–28].

2.1. RNA Isolation and qRT-PCR. Chick embryos cultured for different stages were removed and processed for single cell qRT-PCR. Various stages from the entire heart were anatomically divided into the specific regions (atria, AV-C, ventricle, or outflow tract) and each tissue dissociated into single cell cultures, as shown before [28]. Two hours after plating, intracellular recordings were performed on individual cells which exhibited spontaneous beating. Cells that exhibited AP properties (amplitude, duration, and maximal rate of rise) similar to those previously described for each of the four selected regions [26–29] were collected by a second suction microtube and deposited in a 0.5 mL centrifuge tube. Five to ten spontaneous beating cells were collected and processed together to extract mRNA using SuperScript III cells direct or cells direct kit (Invitrogen). First strand cDNA from total RNA was made by using BD Advantage RT to PCR kit (BD Mountain View, CA). β-actin and GAPDH were used as expression controls. The primers were designed and synthesized in collaboration with a commercial supplier (http://realtimeprimers.com/) and had the following sequences: Forward Primer 5′-TTG GTG GCT TCA CAA TCA C-3′ and Reverse Primer 5′-TTC TTC CTC CTC CTT GCT G-3′; this set of primers produced a product size of 152 bp with TM 58°C that was obtained from Region 1922–2073 (NM_001079473); β-actin forward primer TGA GTT CCA CTT CAC CCA CCT CAT-3′ and reverse primer 5′-GCA CCA TCT CAT ACA GCG CAT AGT-3′ produced a product size of 164 bp obtained from region 704–867 (NM_205518); GAPDH forward primer 5′-GCA TTG TGG AGG GTC TTA TC-3′ and reverse primer 5′-TCA GCT CAG GGA TGA CTT TC-3′ produced a 194 bp product size from the region 266–460 (NM_204305).

β-Actin and/or GAPDH were amplified as a control to verify RNA integrity and to estimate the amount of RNA used in each PCR reaction. PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide, and photographed with a Polaroid camera (Kodak, Rochester, NY).

qRT-PCR was performed by triplicate and Cycle Thresholds (CT) from 5 different experiments were pooled together. Fold increase (Fold increase $= (2 \times \text{Efficiency})^{\Delta\Delta C_T}$ where, $\Delta\Delta C_T = C_T[(Ct_{G.O.I\text{-}Ctrl} - CT_{HK.G.Ctrl}) - (Ct_{G.O.I\text{-}Stim} - Ct_{HK.G.\text{-}Stim})]$ as shown as mean ± SE [30]. (G.O.I.: gene of

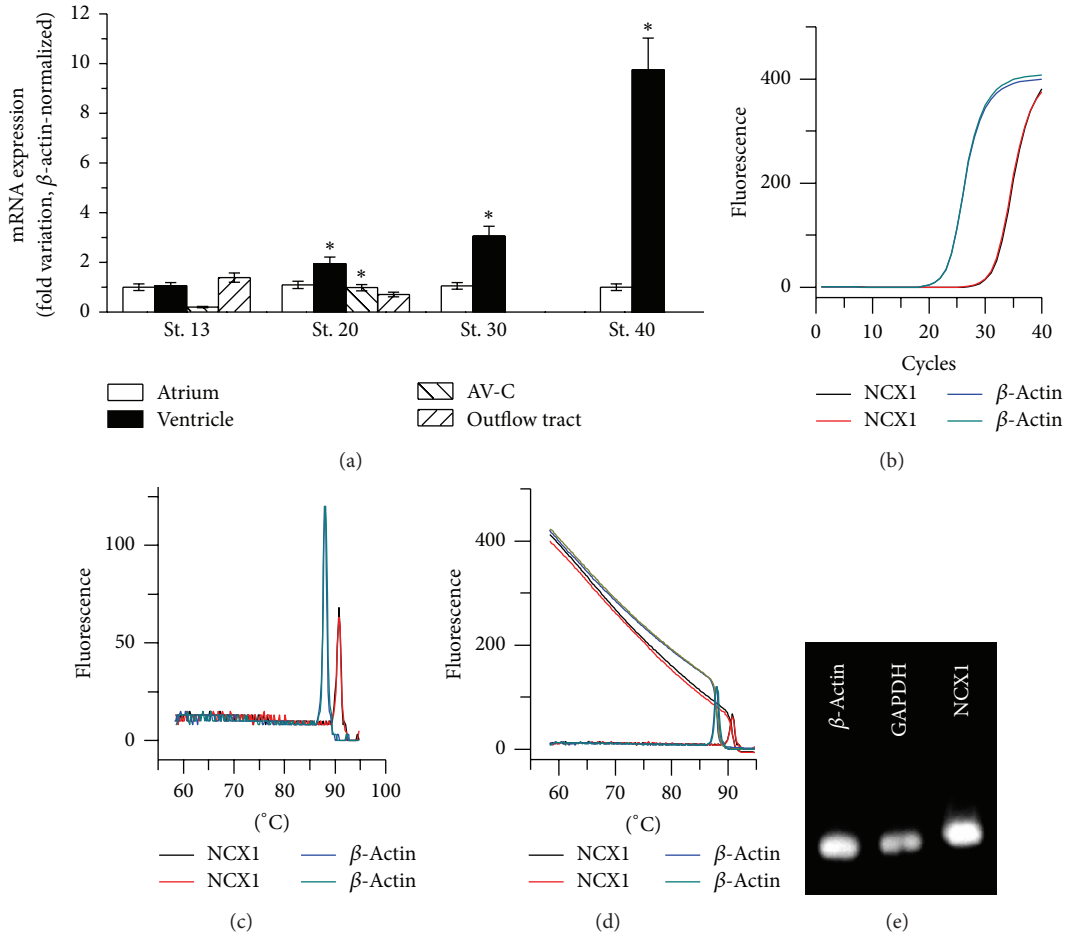

FIGURE 1: Changes in NCX1 mRNA levels in the chick heart at different stages of development. (a) Five to 10 spontaneous beating single cells from each heart region (atria, ventricles, AV-C, and outflow tract) were identified by the AP characteristics. Each tissue sample was analyzed three separate times. All the data were combined together. $^{*}P < 0.05$. For each condition the cells were collected and processed to obtain mRNA and transformed to cDNA. QRT-PCR was performed by triplicate and Cycle Thresholds (CT) from 5 different experiments were pulled together. Fold increase (Fold increase = $(2 \times \text{Efficiency})^{\Delta\Delta C_T}$ where $\Delta\Delta C_T = C_T[(Ct_{G.O.I\text{-}Ctrl} - CT_{HK.G.Ctrl}) - (Ct_{G.O.I\text{-}Stim} - Ct_{HK.G.\text{-}Stim})]$ as shown as mean ± SE [30] (G.O.I.: gene of interest, Ctrl: control, HK. G.: housekeep-ing gene, and Stim: stimulated). (b-d) Examples of real time PCR reactions. PCR reactions for β-actin (similar response was observed when GAPDH was used) and NCX1 from atrial cels at stage 13 (HH). Cycle threshold was determined from fluorescence curves (b), the first derivate (c) and melt curves (d) indicates a single peak at appropriate temperature for the amplicon. Each trace represents the mean of three different reactions from the same sample (e). Real time PCR products on an agarose gel. Each PCR reaction was performed in triplicate (figure shows only one sample of each). Predominant bands of the appropriate size are evident for amplicons of the reference gene (see materials and methods for details).

interest, Ctrl: control, HK.G.: housekeeping gene, and Stim: stimulated).

2.2. Intracellular Recordings. Chick embryos from stages (st.) 13, 21, 26, and 29 were killed by decapitation and hearts were quickly removed and transferred to Tyrode's solution as previously described [26, 27]. The heart was then transferred immediately to a custom made temperature controlled chamber (37°C) which was perfused with oxygenated (95% O_2, 5% CO_2) Tyrode's solution. The spontaneous electrical activity was recorded by using sharp microelectrodes (Sutter instruments, Novato, CA) filled with 3 M KCl. The intracellular recordings were performed in intact hearts to measure spontaneous electrical activity before and after administration of the NCX inhibitor KB-R7943 (0.1 nM to 10 μM) with glass microelectrodes connected to Amplifier BA-1S (NPI Electronic Instruments, Germany). The data from the amplifier was acquired and fed through a computer using A/D converter Digidata 1440 (Axon instruments, California, USA). Recordings were simultaneously displayed and recorded using Axoscope 10.0 (Axon instruments, California, USA).

2.3. Action Potential Classification. APs were classified based on the properties of the AP as measured by the maximum rate of rise of the AP (dV/dt_{max}), the AP duration (APD-APD50 and APD90—duration measured at 50% or 90% repolarization), AP amplitude (APA), and prominence of the

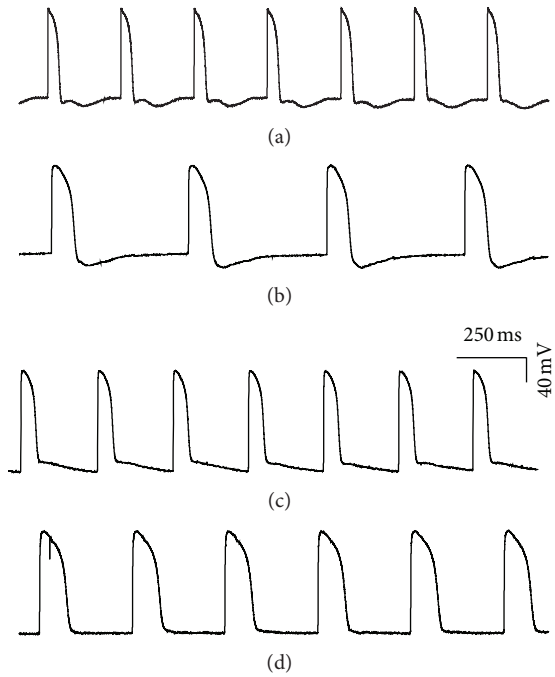

Figure 2: Changes in spontaneous beating frequency induced by blocking the reverse mode activity of the NCX by KB-R7943 (1 μM). Blocking the reverse mode of NCX1 decreased the frequency in both the atrial cells ((a), control, (b) + drug) and ventricular cells ((c), control, (d) after drug addition). Recordings from atria and ventricle were made from two different spontaneous beating hearts of similar age (st. 26).

phase 4 depolarization, maximal diastolic potential (MDP), and resting membrane potential (RMP). We consider a difference of $P < 0.05$ statistically significant.

2.4. Statistical Analysis. All values presented as means ± SE with n values representing the number of recordings in the data set. Statistical significance was evaluated by the Student's paired or unpaired *t*-test (two-tail) as required. One-way ANOVA analysis followed by a Newman-Keuls test or Tukey-Kramer test was used for multiple comparisons. Differences of $P < 0.05$ were considered statistically significant. Statistical analysis was performed using Origin 8.5 (OriginLab, Northampton, Massachusetts).

2.5. Patch Clamp. I_{NCX} was determined using the whole cell patch-clamp technique as described previously [22, 31–34]. To avoid measurement of I_{NCX} in noncardiomyocytes, individual cardiomyocytes were selected accordingly to their typical morphology and spontaneous beating activity. $[Ca^{2+}]_i$ was buffered with 150 or 500 nM BAPTA. The external solution (standard NCX solution) was K^+-free and contained (in mmol/l) Na^+ 135, Ca^{2+} 2, MgCl 1, Glucose 10, Hepes 10, CsCl 10 (to block the inward rectifier K^+ current, I_{K1}, and the Na^+/K^+ pump), Ouabain 0.010 (Na^+/K^+ pump inhibitor), and verapamil 0.010 (dihydropyridine antagonist) adjusted to pH 7.4 with CsOH. The internal solution contained (in mmol/l) CsCl 136, NaCl 10, Aspartic Acid 42, MgCl 3, HEPES 5, tetraethylammonium (TEA) 20, MgATP 10, and 150 nM or 500 nM free $[Ca^{2+}]_i$, adjusted to pH 7.4 with CsOH as previously described [21]. The holding potential was set to −30 mV to block T-type Ca^{2+} and Na^+ channels. Slow-ramp pulses were applied (+60 to −120 mV; 0.09 V/s) at 5 or 10 sec intervals and current-voltage (*I-V*) relationships were constructed. As previously described I_{NCX} was measured as the bidirectional Ni^{2+} (5 mM) sensitive current. I_{NCX} density was normalized with respect to the cell size (pA/pF).

2.6. Peptide Transfection. XIP, a 20-amino acid peptide inhibitor of NCX, with sequence RRLLFYKYVYKRYRA-GKQRGT, was a gift from Dr. Mark A. Milanick of the University of Missouri. The peptide was synthesized and sequenced to assure purity, by the Peptide Synthesis Laboratory at the University of Kentucky. Transfection was carried out by using Chariot Protein Delivery Reagent (Active Motif, Carlsbad, CA) following manufacturing instructions as previously described [35]. The heart from stage 25 was removed and bathed in Tyrode's solution containing 10 μM of XIP peptide and the Chariot delivery reagent for 30 min. After transfection the heart was transferred to the recording chamber with superfusion of fresh Tyrodes solution (bubbled with 95% O_2-5% CO_2) at 37°C. Normal heart, as evidenced by spontaneous contraction. Hearts undergoing the transfection protocol without the peptide did not show differences from hearts not transfected and thus data were pooled to provide a control group.

3. Results

3.1. Changes in NCX1 mRNA Levels in the Chick Heart at Different Stages of Development. Previous studies of NCX in hearts have been mainly limited to adult and embryonic ventricular cells [21–23, 36–40]. However, there is no information regarding at which stage of development NCX is present and functional in the atria. Thus, as a first approach, we conducted a qRT-PCR study of the presence of NCX mRNA levels in the different morphological regions of the heart (i.e., atria, ventricle, atrioventricular canal, and outflow tract) at four representative stages of heart development (st. 13, 20, 30, and 40 in accordance with [41]—see methods for details).

Our results show almost equal amounts of mRNA expression in atria and ventricles at early stages of development (st. 13, Figure 1). These levels remained almost constant for atria during all stages of development until close to birth (up to st. 40). However, the ventricles showed a consistent increase in mRNA expression levels of NCX across all the stages tested (from st. 13 to 40). The two transient structures in the early heart, the AV-C and the outflow tract, show interesting changes in NCX mRNA expression levels. The AV-C showed an increase in NCX mRNA levels from stage 13 to 20, in contrast with the outflow tract that shows a decrease in the same period. We also observed that NCX mRNA labels were similar in the atria and outflow tract at stage 13 (Figure 1).

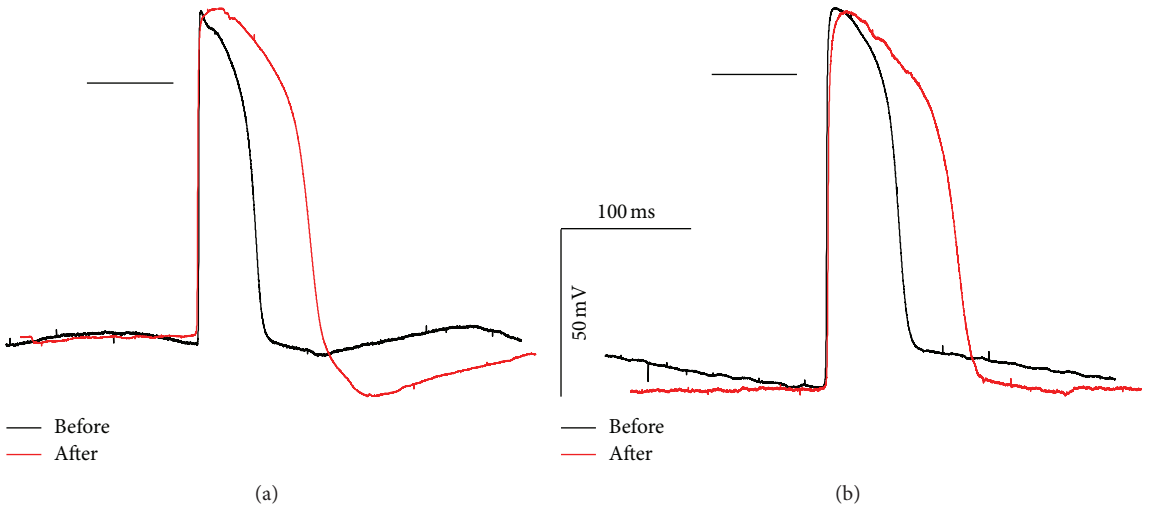

Figure 3: Changes in the action potential morphology induced by blocking the reverse mode activity of the NCX in spontaneous beating hearts. Blocking the reverse mode activity of NCX increased the AP time in atrial (a) and ventricular (b) cells. Two superimposed traces from intracellular recordings of the same cell from an intact, spontaneously beating heart of a stage 26 embryo: before (black line) and after (red line) after 1 minute of the acute application of NCX blocker (KB-R7943 1 μM). (a) Atrial, (b) ventricular. APs were displayed independently of their time of activation. (n = 16).

Although gene expression is a good indicator of the potential presence of a given protein, it does not necessarily follow that the protein is synthesized and becomes functional [42]. Furthermore, evaluation of NCX protein expression in chicken is not possible due to the lack of antibodies cross-reactivity. Thus to confirm protein expression and functionality, we performed a series of electrophysiological studies, in which we evaluated the presence of NCX and functions in the atria and ventricular cells.

3.2. Changes in Spontaneous Beating Frequency by Blocking the Reverse Mode of NCX. To test the presence and functionality of the NCX in the developing heart, studies were performed in the presence of KB-R7943 [43–45]. KB-R7943 has been extensively used in many preparations [24, 25, 43–46]. Intracellular recordings of a single cardiomyocyte from a spontaneously beating heart (from a st. 26 embryo) were recorded before and after superfusion with 1 μM KB-R7943. We observed that the frequency of heart beats decreased ~40% in the first 30 seconds after perfusion. In parallel, with the decrease in AP frequency, we observed a gradual increase in AP duration in both atrial and ventricular areas which became steady after 45–60 seconds (see Figures 2 and 3). However, these effects appeared to gradually dissipate after ~15–20 minutes in the continued presence of the inhibitor. Similar results were observed with different concentrations of the drug (from nM to μM). In spite of this problem, we found, unexpectedly, that the drug effects remained steady up to several hours in the same preparation when similar experiments were performed under conditions of reduced ambient light. Thus, the rest of our experiments were conducted with minimal illumination and drug solutions were protected from light.

Concentration-response relationships were examined to evaluate the effects of blocking NCX on AP morphology from both atria and ventricle at different concentrations of KB-R7943 in stage 26 embryos. We compare AP morphology only from stable recordings in which we were able to keep stable recording from the same cell/heart before (control) and one minute of the addition of the different concentrations of KB-R7943 (after). We found frequently that inclusive after 30 min of washing out the solution containing the KB-R7943 solution a residual change in AP morphology was present. Some other times were not possible to maintain the recording stables from the same tested cell for such periods of time. Thus we use only one record\embryo\dose tested from the atria or ventricle. Our results indicate that the minimum amount required to produce change in AP morphology on either atria or ventricular tissues was 100 nM (Figure 4).

Changes in AP amplitude and maximum rate of rise were not consistent between atria and ventricular tissues and seem independent of the concentration of the drug. Hearts were sensitive to a drug concentration of 1 μM and above for changes in duration of AP. The changes produced were maximized at 10 μM and response tended to saturate at higher concentration above that (data not shown). There was a steep increase in duration (rise time and decay time included) of AP from 1 μM to 3 μM and continued for 10 μM as well (Figure 4).

The APs generated by the different regions of the hearts vary significantly during different stages of development in terms of their resting membrane potential, amplitude, duration, and maximal rate of rise [26–29, 47, 48]. Thus, we analyzed the effects of NCX on atria and ventricular cells during different stages of development. Intracellular recordings were performed in atria and ventricular cells from spontaneously beating hearts from stages 14, 21, 26, and

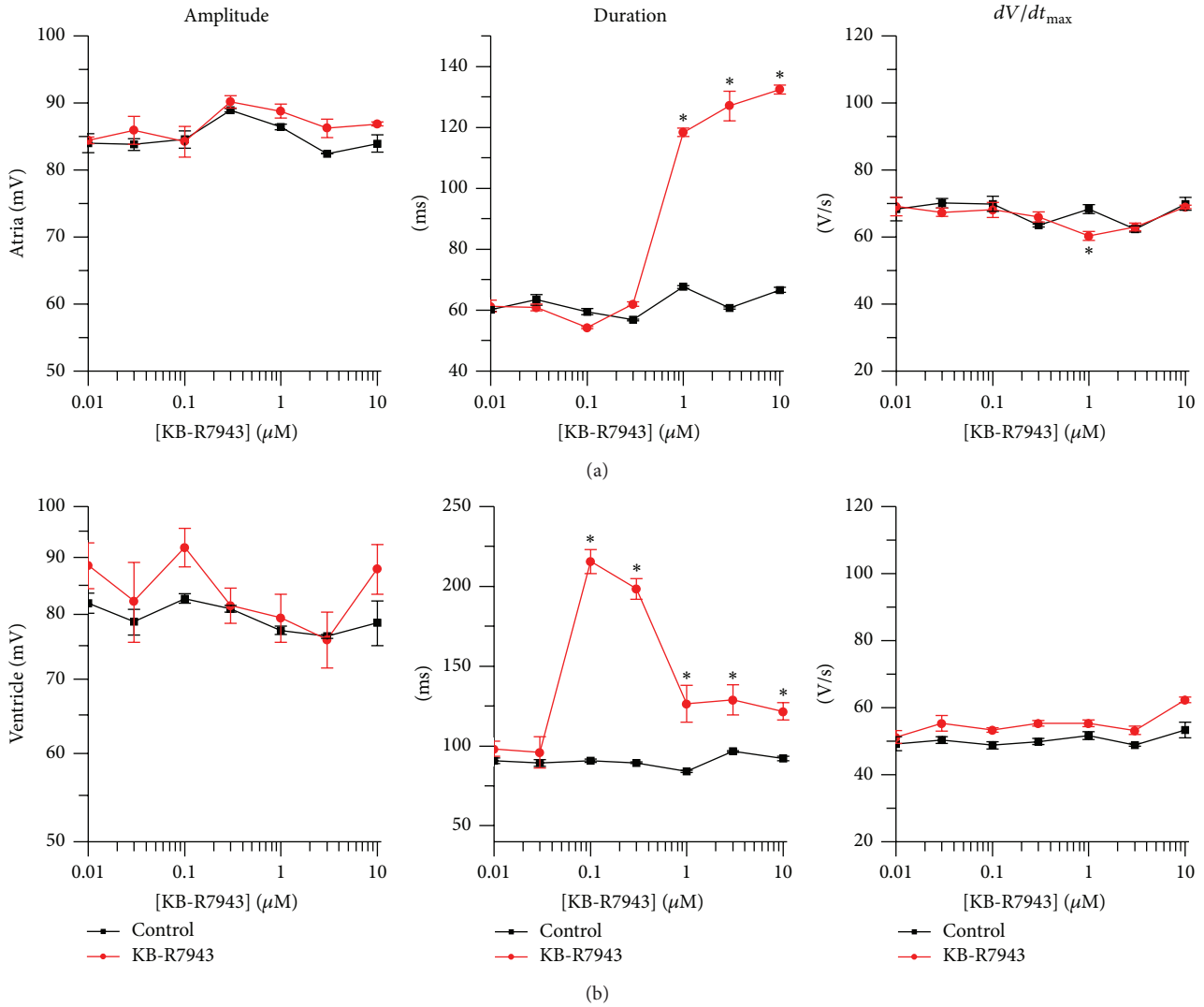

Figure 4: Effects on cardiac AP electrophysiology by blocking the reverse activity of the NCX by different concentrations of KB-R7943 on amplitude (left column), duration (middle column), and maximal rate of rise (right column) on atria (a) and ventricles (b) from a stage 26 embryo. Intracellular recordings from spontaneous single beating cells were considered only when we were able to record at least 1 min before and 1 min after the addition of the drug. ($n = 18$. Mean ± SE, $^*P < 0.05$).

29 and we evaluate the AP characteristics from the same recording cell before and after the addition of 1 μM KB-R7943 (Figure 5).

We observed that the duration of the AP of atrial cells from stage 14 was not sensitive to the drug, whereas the ventricular cells from the same stage 14 show the largest increase in AP duration. In the atria, the changes in AP duration increase over time to become maximal approximately stage 30. In contrast, the ventricle displays an opposite behavior, by decreasing the changes in AP duration under the effect of KB-R7943. At all the stages tested, atria cells show an increase in AP duration under the effects of NCX blockage. In both atrial and ventricular cells, the maximal rate of rise was reduced at all of the stages tested. However atrial cells exhibited the maximal reduction of MRR at the oldest stages tested (st. 30), whereas the amplitude and the resting membrane potential (data not shown) remain unchanged (Figure 5).

Several reports in the literature suggest that KB-R943 may not be a specific inhibitor of the reverse mode of the NCX [43, 45]. Such effects, however, seem to be tissue- and species-related [24, 49, 50]. Hence, we tested a well-characterized, highly specific inhibitor peptide (XIP) [51–58]. XIP contains primarily basic and hydrophobic residues; it is noncompetitive with both Na^+ and Ca^{2+} and acts at the intracellular surface of the exchanger [51]. Thus, XIP must be injected or transfected into cardiac cells.

Importantly, intracellular recordings from stage 26 hearts transfected with 1 μM of the XIP peptide showed similar changes to those in the presence of KB-R7943 (Figure 6), that is, a negligible change in amplitude in both atria and

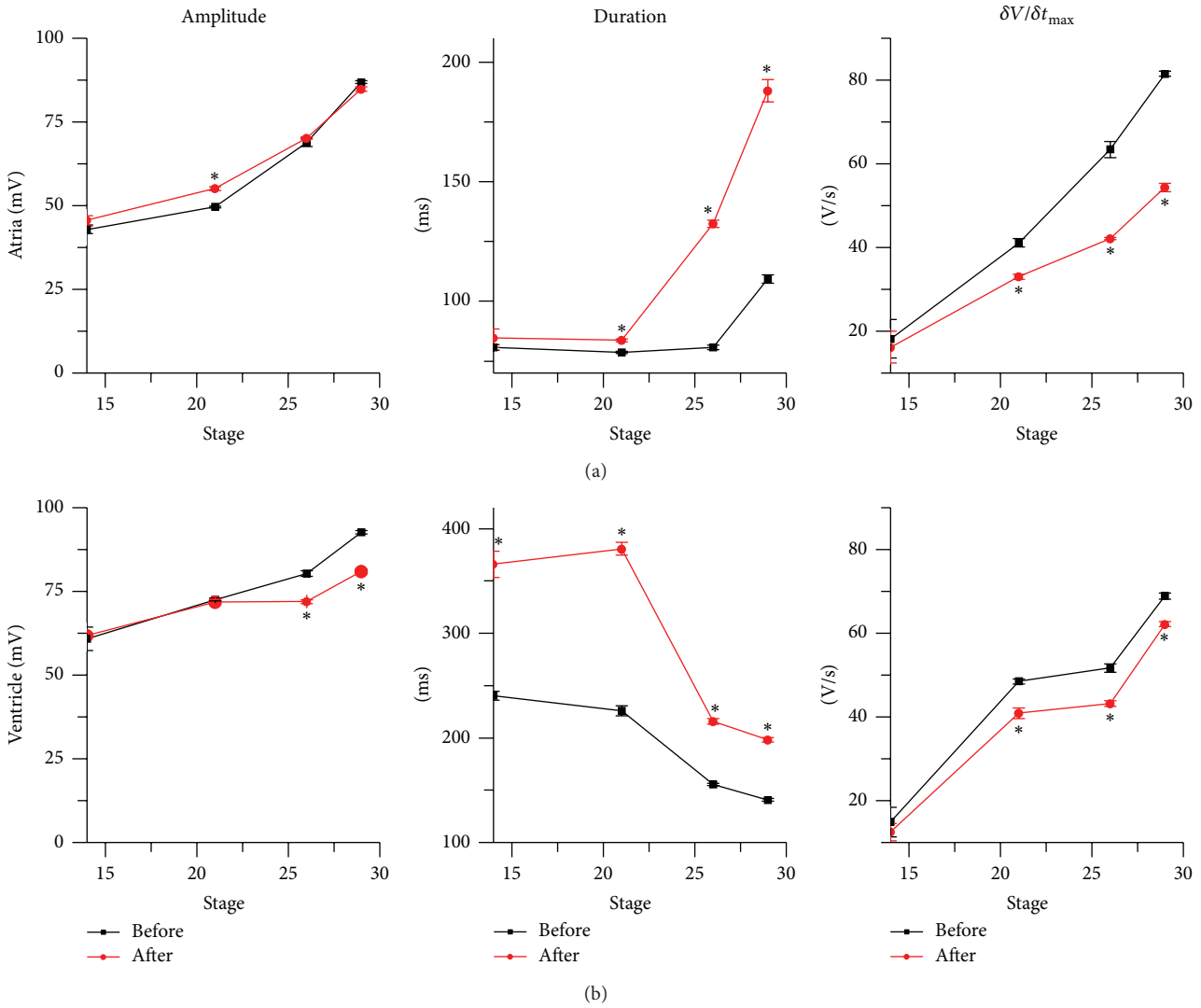

Figure 5: Effects on action potential characteristics at different stages of development by blocking the reverse activity of the NCX. KB-R7943 (1 μM) an inhibitor of the NCX reverse activity was applied to spontaneous beating hearts. AP characteristics; amplitude (left column), duration (middle column), and maximal rate of rise (right column) on atria (a) and ventricles (b) from st. 14 (n = 9), 21 (n = 12), 26 (n = 19), and 29 (n = 14) from a single cell before (control) and 1 min after the perfusion of KB-R7943, were evaluated. (Mean ± SE, $^*P < 0.05$).

ventricular cells, an increase in duration, and decrease in maximal rate of rise. However XIP blocks the forward mode of the NCX.

3.3. Age and Chamber Changes of I_{NCX} Density during Development. To corroborate our findings we measured the I_{NCX} current density in both the atria and ventricle cells in the embryonic chick heart cells from st. 13 to 40. We use protocols and solutions as those that previously described the elicited I_{NCX} [21, 59, 60]. We found that as the heart develops, there is a gradual increase in the magnitude of the I_{NCX} in the ventricular cells, where in the atria cells it remains almost constant (Figure 7) from st. 14 to 29.

4. Discussion

The electrical activity of the heart is critical for embryonic development and as such it is important to identify underlying cellular mechanisms. Candidate mechanisms include the different ion channels and membrane transporters that are responsible for shaping the electrical activity of heart and also play important roles during development. Further, transport of calcium, both influx and efflux, is critical to pathophysiological conditions including ischemia [61]. Among several known calcium pathways, the NCX has been identified as one of the most important transporters for maintaining calcium balance in cardiomyocytes. Though it has been identified in adult hearts, its functional role

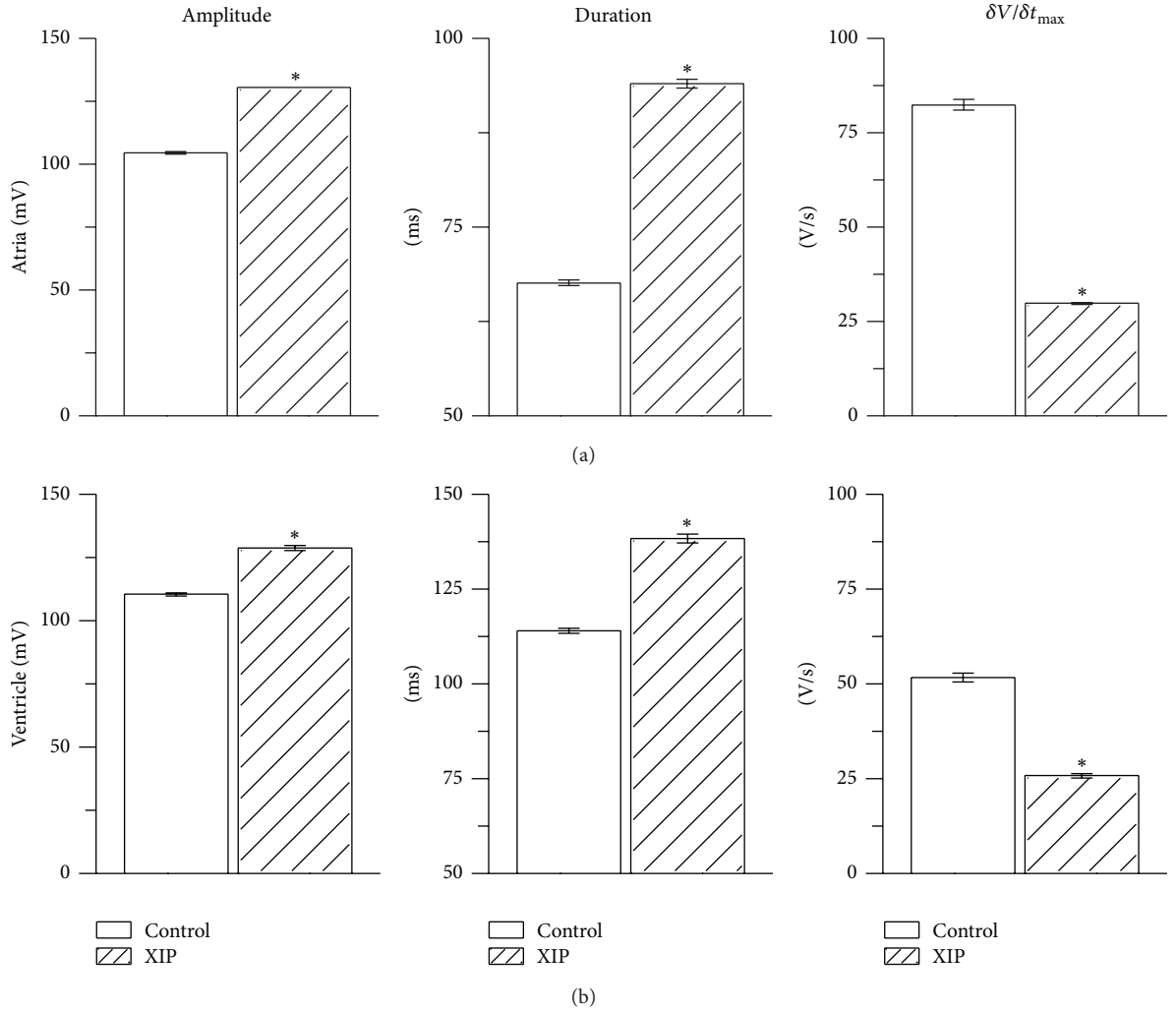

Figure 6: Effects of blocking NCX-1 by transfecting chicken heart st. 25 with NCX-1 blocker peptide XIP. (a) Atria, (b) ventricle on AP amplitude (left), duration (middle), and maximal rate of rise (right) ($n = 6$, mean ± SE, $^*P < 0.05$).

during embryonic development, however, remains unclear. Our results demonstrate, for the first time, the functional role of NCX in avian hearts during various early stages of development in both atria and ventricular cells. We also show that NCX plays an important role in maintaining the duration of APs, and hence, shaping the electrical activity of heart.

The reversal potential for the exchanger can be mathematically expressed as $E_{Na/Ca} = (nE_{Na} - 2E_{Ca})/(n-2)$ [62], where n is the coupling ratio of the exchanger. When E_m (membrane potential) is more than the value of reversal potential value for NCX, it favors the outward I_{NCX}. Hence, it is reasonable to believe that NCX operates in both modes during one action potential cycle. It acts as a calcium entry mode for a brief period of time and then extrudes calcium through forward mode during the rest of the cycle.

In our results, we show for the first time that NCX is present as early as stage 13 [63] of embryonic chick hearts in the different regions of the heart: atria and ventricles and, further, it affects the electrical activity of the heart during early development. Interestingly, the effects of blocking of NCX in atria and ventricles differ significantly. In atria, the increase in duration of APs is minimal during stage 14 to 21 and almost doubles at stage 29. In contrast, ventricles exhibit large changes during stage 21 with less-significant changes during later stages of development. The changes in duration were caused mainly by changes in decay time of the APs while not being markedly associated with rise time of APs in either atria or ventricles. Hence it is clear that NCX affects the plateau phase of action potentials which is characterized by the calcium entry into the cell.

4.1. Previous Studies in Mouse and Rat. Development of the mechanisms which underlie Ca^{2+} movement in embryonic hearts is still not completely understood; there have been several studies relating to calcium influx through voltage-gated calcium channels and other membrane transporters including NCX. Here we show that NCX can be an important mode of Ca^{+2} entry during development of the embryonic heart. It is important to point out that our studies are in intact heart, while previous studies have examined NCX in

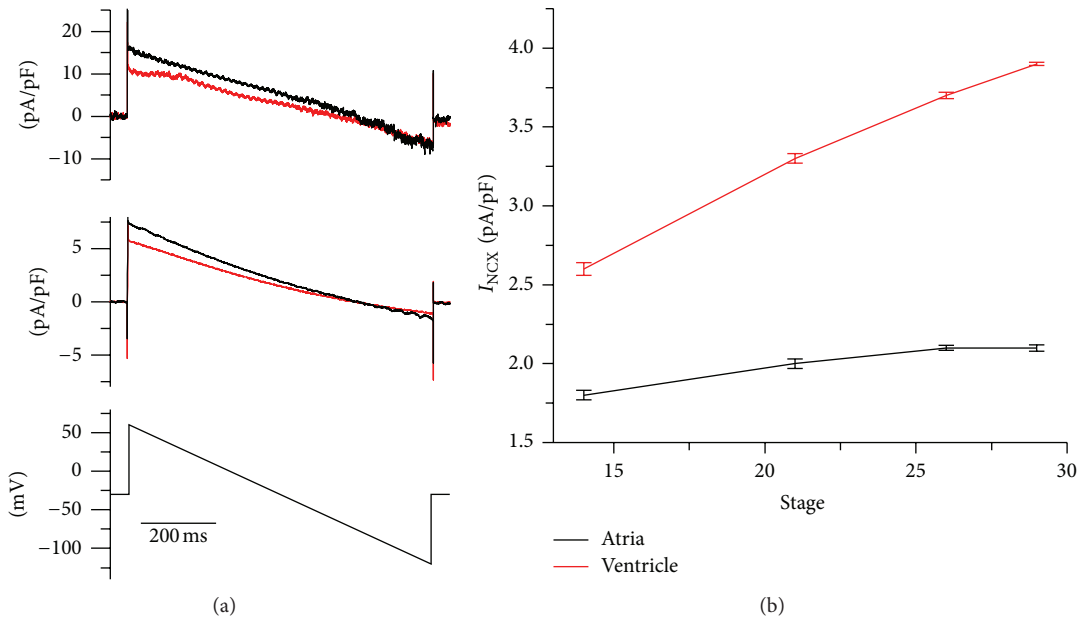

FIGURE 7: Age dependence of the I_{NCX} density current in freshly isolated single atria and ventricular cells. (a) I_{NCX} was measured as the bidirectional Ni^{2+} (5 mM) sensitive current from a freshly isolated single cells in culture (see methods for details). Top representative traces from an atria and middle ventricle cells (st. 26). Black line control current, red line Ni^{2+} sensitive current. Bottom Slow-ramp pulses (+60 mV to −120 mV; 0.09 V/sec; holding potential −30 mV) at 0.25 Hz were applied. (b) Changes on I_{NCX} in atria (black line) and ventricles (red line) st. 14 (n = 11), 21 (n = 22), 26 (n = 16), and 29 (n = 17) (Mean ± SE).

isolated cells [64]. Isolation of cells is likely to disrupt cell signaling and in many cases it significantly alters membrane protein expression. The role of calcium mobilization via the SR is also very important for contractile force generated by ventricles and for EC coupling. The contractile force of pulmonary veins of adult rabbit hearts decreases when treated with NCX inhibitor [65]. The SR is poorly developed in embryonic hearts when compared to adult hearts and hence, during development there is likely not sufficient calcium influx through SR for effective EC coupling [66, 67]. Therefore, NCX may supplement the calcium influx necessary for EC coupling in embryonic hearts, and hence inhibition of NCX shows significant decreases in spontaneous heart activity although not being completely blocked even at higher inhibitor concentrations.

KB-R7943 has been reported to be capable of blocking voltage-gated Na^+ channels when administered in high dosage in adult hearts [50]. We observed that the AP maximum rate of rise increases during stage 21 and decreases during later stages of development in both atrium and ventricles. This may be attributed to the sensitivity of voltage-gated Na^+ channel to KB-R7943 during early stages of development. Importantly, our results appear physiologically relevant as using intracellular recordings in the intact preparation; these effects can be observed simultaneously as opposed to studies of isolated cells.

Liu et al, 2001 [13] showed that calcium influx is dependent on L-type Ca^{2+} channels and that there is considerably less dependence on NCX in embryonic ventricular cells derived from rat. Further, in chicken hearts, T-type channels are not present in early ventricle cells and inhibition of L-type calcium channels completely abolishes the current implicating L-type channels [68] as the main source of calcium influx.

Our drug response graphs (Figure 4) show that the minimum amount of drug required to produce AP changes was 0.1 μM while 10 μM of the response is saturated. This is consistent when compared to previous studies [65, 69]. To further investigate the different effects observed in atrium and ventricles, we performed RT-PCR studies. Surprisingly, mRNA levels were found to be equal in both atrium and ventricles during the early stages of development, while the later stages showed a rapid increase in ventricular mRNA in the face of near constant atrial levels. This differential expression level can be one of the possible reasons for different AP effects in atrium and ventricles.

Apart from normal heart development, NCX is also important in heart failure, ischemia-induced arrhythmias, and arrhythmogenesis during myocardial reperfusion [22, 70]. In such cases, NCX mediates calcium influx through its reverse mode which triggers SR Ca^{2+} release an ultimately an uncontrolled calcium overload. Our results show that it is possible to reduce calcium influx by selectively blocking the exchanger through KB-R7943 or other more specific suitable inhibitors. It also strengthens the call to use NCX inhibitors as potential antiarrhythmic agents.

However, we should point out that the use of KB-R7943 blocks mainly the inward (reverse mode) of the NCX where the XIP peptide blocks the outward modes. These differences could account for the unique changes observed in the AP

morphology observed by the two compounds (Figures 4 and 5 versus 6). Also these differences should be taken in to consideration in the design of antiarrhythmic agents.

There is a general agreement that the NCX is upregulated whereas the Na^+ pump is downregulated [71, 72] in several modes of congestive heart failure. If the NCX is working in normal mode (Ca^{2+} extrusion) during most of the cardiac cycle, upregulation of the exchanger may result in SR Ca^{2+} depletion and further impairment in contractility. It has been proposed that, in end-stages of human heart failure, the reverse mode of the NCX is activated during the cardiac AP by bringing in Ca^{2+}, thereby compensating the impaired SR Ca^{2+} function and producing positive inotropy. However, the normal mode of the NCX also increases, leading to a generation of an inward current due to the stoichiometry of the exchanger (3 : 1) leading to an arrhythmogenesis [73].

In summary, we have demonstrated a functional role of NCX during development of the avian heart. These results clearly indicate that NCX is an important calcium-influx pathway along with voltage-gated calcium channels.

Authors' Contribution

Luis Polo Parada and Amol A. Modgi contributed equally to this work.

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