THE ROLE OF SODIUM-CALCIUM EXCHANGER IN THE ELECTRICAL ACTIVITY OF EMBRYONIC CHICKEN HEART

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By
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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TABLE OF CONTENTS

LIST OF FIGURES

THE ROLE OF SODIUM-CALCIUM EXCHANGER IN THE ELECTRICAL ACTIVITY OF EMBRYONIC CHICKEN HEART

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ABSTRACT

Heart is one of the most important organs of the body and also it is one of the first organs during to the development. The electrical activity of heart is essential for its function. Hence it is very important to understand the role of different ion channels and transporters which shape the electrical activity of heart. It has been shown that the sodium-calcium exchanger (NCX) is an important exchanger for calcium homeostasis. We hypothesize that the NCX may be present during development. To test this hypothesis we recorded the spontaneous electrical activity of heart from the embryonic chicken heart before and after blocking NCX during different stages of development with different concentration of KB-R7943. We analyzed the data for changes in amplitude, duration, and maximum rate of rise, rise time and decay time of AP. We found that the blocking of this exchanger does have significant effect on the amplitude and the rise time of AP. We found out that changes in duration of AP were less in atrium in early stages of development and increased during later stages of development. Interestingly these results were opposite in ventricles where increase in duration of AP was more during early stages of development and decreased during later stages of development. Similarly there was an increase in decay time of atrium and ventricular action potential at different stages

of development. There were relatively few very significant changes in rise time of AP. Hence it is quite apparent that the blocking of NCX affects the plateau phase of AP or the calcium extrusion phase. Also the different changes in atrium and ventricular duration effects can not be understood completely. We carried out RT-PCR experiments to evaluate the presence of mRNA expression of NCX and we found that it is almost constant in atrium from early to elder stages of development where as there was increase in amount of mRNA expression in ventricles. It is also certain that NCX is one of the exchangers present in early embryonic hearts to actively play role in shaping the electrical activity.

CHAPTER 1

LITERATURE REVIEW

Embryonic heart development is an extremely complex process and involves a rapidly changing environment. This chapter has been organized in three different sections and they include calcium homeostasis, introduction to sodium-calcium exchanger and inhibitors of sodium calcium exchanger – (NCX). The objective of this chapter was to gather more information available about calcium regulation and specifically identify the role of a calcium transport mechanism like NCX in it. Also, there were different studies that used different techniques to study NCX. We reviewed the work done by many other research groups and their publications to build a model for our work.

1.1 Calcium homeostasis in embryonic hearts

Apart from sodium, calcium is important in signaling and other biological mechanisms. Calcium is required in almost all types of cells for different reasons. Though calcium is an important aspect in cellular communication it can also be extremely dangerous for cells. Hence, it is very important to control the amount of calcium going in and out of the cell based on the type of cell and also the mechanism by which it can be regulated. In adult cardiomyocytes Ca^{+2} enters the cell during the rapid upstroke through voltage gated Ca^{+2} channels and causes calcium induced calcium release from SR. This sudden influx of calcium generates contractile force necessary for circulation. This calcium is removed by SR uptake and NCX extrusion (Kitchens et al., 2003). Considering this fact, it is reasonable to believe that the different mechanisms

regulating calcium are very important (Davies et al., 1996). The Ca^{+2} channel in the heart consists mainly of 2 types of Ca^{+2} currents and they are high threshold slow (L-type) and low threshold early transient (T-type) currents. The L-type currents are expressed in entire heart of all species. T-type current is not observed in the entire heart, but it is observed in some specialized areas like SA node, purkinje cells. Also, its expression is dependent on tissue growth and is species specific. The expression of T-type current can be related to growth and hypertrophy in some species. T-type currents are not found in adult ventricles but they play a significant role in embryonic cardiomyocytes (Kitchens et al., 2003). Apart from these two currents there is also another type of current called F-type (fetal type) present in the rat ventricle only during the fetal stages of development. It was identified by using a specific blocker for DHP sensitive L-type and T-type blockers. Although the 2 types of calcium currents described above are major pathways for Ca^{+2} influxes, it is not limited to them. It is thought that the influx coming through these channels may not be enough for EC coupling and hence there can be more pathways like NCX playing major role in fetal/embryonic cardiomyocytes (Tohse et al., 2004). These channels can be classified based on their properties like change in permeability or their activation and inactivation types. Risso et al. (1993) showed that whole cell L-type current disappears with nifedipine, a L-type calcium current blocker on day-7 chick ventricular myocytes. Similar results were obtained by Kitchen et al. (2003) for studying the contribution of L-type and T-type to calcium transients. There is more contribution from the L-type current than from the T-type in later stages of development. The influence of the T-type current on calcium transients is reduced in later stages of development and there is also a decrease in the calcium transients in general. This

decrease can be associated with the maturation of SR and SR induced Ca^{+2} release. Also, after the blocking of the L-type current and caffeine induced SR Ca^{+2} releases, there was significant amount of calcium transient measured in the experiment. Apart from the role of T-type currents it is believed that the reverse mode of NCX can play a major part in contributing to this current (Kitchens et al., 2003).

Ionic movement of the heart should be translated into the mechanical force of contraction and it requires a reasonably large amount of calcium influx. This influx is achieved through calcium induced calcium release through SR and is essential for excitation contraction coupling. This mechanism is handled differently in mature and immature cardiomyocytes. In adult heart the CICR is facilitated through L-type calcium channels in T-tubules and junctional SR ryanodine receptors (RyRs). It is believed that L-type calcium channels play an important role in SR calcium release (Schroder et al., 2006). Structurally, RyRs is dependent on influx through voltage gated calcium channels. The calcium binds with RyRs and causes a major trigger mechanism for calcium release (Takeshima, 2002). This well developed mechanism is not present in embryonic ventricular myocytes. The SR is not well developed in embryonic hearts and hence the calcium release through SR. Therefore, EC coupling is primarily dependent on the influx through L-type calcium channels and through reverse mode of sodium-calcium exchanger. There is lot of information available on the presence of the L-type calcium channel in embryonic heart and the calcium influx through it, but there has been very less information available about the role of NCX in EC coupling of embryonic heart. Schroder et al. (2006) showed some very interesting results about the role of calcium channels in maintaining calcium transients in embryonic rat ventricular myocytes. They

showed that selectively blocking certain calcium channels with a specific blocker did not suppress the calcium transients completely, but there was definitely some lag in it. Also the amplitude of calcium transients was reduced at a higher concentration of the blocker. Similarly, they investigated the role of RyRs in calcium transients. The blocking of these RyRs did not abolish calcium transients where as in adult hearts it significantly affected the EC coupling, It was clear from their studies that internal calcium stores and Ca^{+2} channels are not the only mechanism for EC coupling but there could be some other mechanism by which supporting the EC coupling and calcium transients in embryonic ventricular myocytes.

1.2 Sodium-Calcium exchanger

We reviewed the role of sodium and calcium for generating action potential in embryonic and adult cardiomyocytes. Also, there was clear indication that these ions should be transported across the cell membrane. NCX is one of the important membrane transporters for transporting these two ions. The mechanism for this transport was not exactly known and there were different speculations surrounding it. Initially it was thought that there is a competition among these two ions for its transport across the plasma membrane. But later it was discovered that there exist counter coupled transport mechanisms for sodium and calcium rather than just a competition between these two ions. Also, there was no clear information available about the details for this mechanism. Blaustein and Lederer (1999) reviewed these developments in detail for discussing the role of NCX and its physiological implications. The amount of sodium and calcium transported across the plasma membrane was dependent on extracellular and intracellular sodium and calcium concentration. It was also dependent on the membrane potential of

the cell. All these factors were studied by different investigators over a period of time and we will review the important aspects of this exchanger (Blaustein and Lederer, 1999). The main concern for NCX was to know more about the possible stoichoimetry and regulatory mechanism. Researchers reviewed different studies pertaining to the stoichoimetry and regulation of NCX in different species by different investigators. The most accepted stoichoimetry is 3:1 for the exchanger indicating that there are three sodium ions transported across the membrane for one calcium ion, there have been a few species which show the 4:1 ratio with the exchanger and it mainly involved the Na/Ca exchange in rat brain. Adult cardiac muscles in almost all species have the ratios of 3:1 which include most of the mammalian species like guinea pig, rat and bovine hearts in both atrium and ventricles. In this review it is important to note that most of these studies were performed with the help of patch clamp techniques to individually monitor the NCX current or the sodium/calcium current. Also, it is important to note that there is very little information available about its role in embryonic heart development. Since NCX moves positively charged ions across the cell membrane in a definite direction, it is electrogenic and also sensitive to voltage changes. This was discovered by a very interesting property of exchanger and it is called a dual mode of the exchanger. These modes are known as forward mode and reverse mode and it is dependent on the membrane potential. The reversal potential for the exchanger can be mathematically expressed as $E_{Na/Ca} = (nE_{Na} - 2E_{Ca})/(n-2)$ where is n is the coupling ratio of the exchanger. Hence, when E_m is more than the value of reversal potential value for NCX, it favors the outward I_{NCX} . In other words, it allows calcium to flow in and sodium flow out of the cell. This is very important in understanding the ionic influx during AP. For a typical stoichoimetry of 3:1

for Na^{+1} and Ca^{+2}, respectively and assuming the typical values for E_{Na} is 70mV and for E_{ca} is 125mV. Therefore, in resting cardiomyocytes, NCX works mainly as calcium extrusion mechanism and for a brief period where $E_m > E_{Na/Ca}$ is, it works as a calcium intrusion mechanism. This mode is particularly of our interest since KB-R7943 block the Ca^{+2} entry mode of the exchanger (Bers et al., 2003).

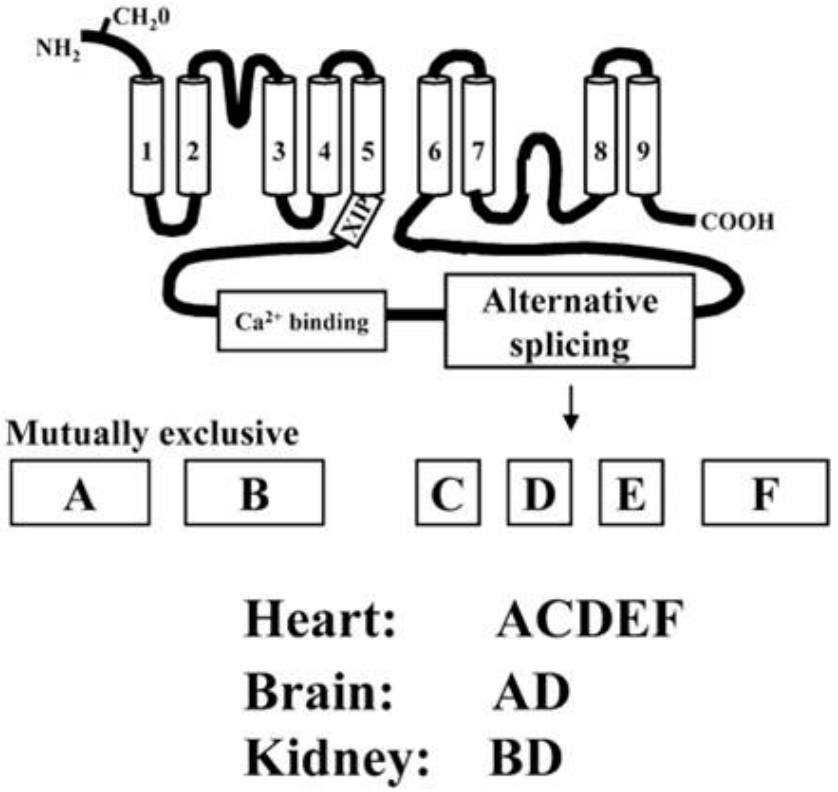

Figure 1. Schematics of NCX structure

The role of NCX is also important in a different region of the heart. It has been shown that there is a wide variety in the electrical activity of the heart found in the atrium and ventricles. Also, this activity goes under a change during different stages of development. Hence, it is important to identify the effects on NCX in atrium and ventricles. The available literature mainly studies the role of NCX in ventricles and there is very little information available about its role in atrium. The reasons behind this can

not be known exactly through literature review. The I_{NCX} has been studied under different experimental conditions which include the following characteristics and experimental conditions: (A) extracellular calcium triggers the outward current at all membrane potential; (B) the current is not time dependent, the I-V relationship of the current is approximately exponential and the outward current requires the presence of intracellular calcium ions. Many studies showed the I_{NCX} by application of extracellular calcium at different concentrations during different stages of development. Their results primarily indicate that there was no increase in the density of sarcolemmal NCX between embryonic stages. Also, the increase in the NCX current was due to the increased affinity of intracellular calcium at the regulatory site. This in turn means that NCX is less dependent on intracellular calcium for its activation during older stages of development.

There have been a number of issues associated with this exchanger and it will be appropriate for us to briefly review those factors which affect the direct measurement of I_{NCX} through different types of cells. It has been found that the exchanger is asymmetric and changes its binding affinity for sodium on the internal and external faces of the exchanger. This asymmetry changes the fluxes based on the intracellular and extracellular sodium concentration and transport different amount of calcium fluxes. Since the exchanger is voltage dependent, it changes the net movement of the charges according to membrane potential. Hence, I_{NCX} needs to be separated from all other currents for its accurate measurements since a specific inhibitor could not be designed for NCX and was a major hurdle in analyzing the effects of NCX. Also, more information about NCX can not be derived based on the changes in intracellular calcium because of the number of

factors contributing to intracellular calcium levels through calcium channels, intracellular calcium channels and few others (Blaustein and Lederer, 1999).

Wongcharoen et al. (2006) studied the effect of NCX in the pulmonary vein of adult rabbit hearts. Blocking of the exchanger in the pulmonary vein of rabbit ventricles with a specific inhibitor produces increase in the action potential duration and decrease in action potential amplitude. The concentration used was of 10 and 30 µM. They also found that there was remarkable decrease in the calcium transients. This is indicative that NCX is also present and important in the electrical activity of pulmonary vein of adult rabbit hearts (Wongcharoen et al., 2006). Also the NCX has found to have important function in regulating excitation contraction (EC) in the heart. There have been different opinions about its role in EC coupling. There is also very less information available about the developmental changes in calcium handling. It is known that in the adult heart, the Ca^{+2} entering through calcium channels triggers the SR and rapid influx through these SR stores produces the contractile force. Hence, this mechanism is extremely sensitive and very important in maintaining EC coupling. In embryonic hearts this mechanism is not very well developed. We reviewed the role of NCX in EC coupling to understand its importance in its forward and reverse modes. Some of these studies include the over expression of NCX in mice hearts. This overexpression leads to an enhanced outward current and changes the action potential from single cardiomyocytes. It results in lowering the peak and increased plateau phase and increased duration. Also, this over expression of NCX can compensate for a reduced SERCA function. Similarly to NCX overexpression, the effects of NCX were also studied by genetic manipulation and reduced function and expression. The knock out NCX was not lethal and knocked out

mice could demonstrate normal heart function with reduced contractile forces. Also there was decrease in the NCX current under voltage clamp conditions (Reuter et al., 2005). The development in molecular biology techniques helped in understanding the structure of NCX and understanding its stoichoimetry. There have been several studies for identifying the exact structure for NCX. We reviewed few detailed studies about the structure of NCX for better understanding of the molecular detail of the transporter. This would provide more rationale for understanding sodium and calcium regulation in greater details. These structural details help in understanding the activation and inactivation sites for the exchanger. A study by Hilge et al. (2007) shows remarkable details about NCX and the binding domains for calcium. They predict nine transmembrane segments and large cytosolic loop of 500 residues. The activation is based on Ca^{2+} binding and inactivation is caused due to Na^{1+} binding. The Ca^{2+} binding is facilitated by 2 transmembrane domains commonly referred to as calcium binding domains (CBD) for CBD1 and CBD2. These domains show some sequence identity in their structure. Also, the studies go in greater details for identifying calcium affinity based on structural properties. They identified that structural variation and orientation differ in the exchanger and these binding sites act as calcium sensor. Binding affinity varies significantly for these two domains for different intracellular calcium levels (Hilge et al., 2007). These details can also be studied with the help of optical techniques. One of the studies used the optical technique based on the FRET. This optical technique has an advantage of revealing more information about the calcium dynamic based on the structural binding of flurophores. This technique can be considered as an alternative approach for studying the exchanger and calcium regulation. The study shows that there are conformational

changes in NCX after calcium binding. Since the technique is based on FRET, the increase in fluorescence intensity can be linked with the change in distance between flurophores used to bind NCX. These intensity changes were more at elevated calcium levels. This was included in the literature review to provide an introduction to a different approach for studying NCX.

1.3 Studying NCX with inhibitors

The study of NCX was limited initially because of lack of availability of a specific inhibitor. It was difficult to identify I_{NCX} under different experimental conditions and rapid changes in calcium levels. This was overcome by the discovery of different chemical compounds to block NCX. In this section we review these inhibitors for NCX. These inhibitors are very important for studying NCX irrespective of the technique used for studying NCX. These inhibitors have been developed in the last few years. Researchers reviewed the role of different inhibitors and were very useful in understanding their properties. These inhibitors can be classified as non selective and selective inhibitors.

Early non selective NCX inhibitors include natural divalent and trivalent cations like Cd^{+}, Ni^{+2}, and La^{+3}. These ions have been used for many calcium dependent processes as inhibitors. There have been many studies we found that used Ni^{+2} as a blocker in patch clamp studies. Similarly, authors also review different benzyloxyphenyl NCX inhibitors which include KB-R7943, SN-6, SEA0400, YM244769, and YM281956. All these compounds differ in terms of specificity and the mode they block. All these benzyloxyphenyl compounds block the reverse mode of the exchangers and may block

forward mode under unidirectional ionic conditions. Apart from this, Blaustin and Lederer (1999) also reviewed some NCX inhibitors used in experiments. They mentioned that amiloride analogs, antiarrythmic agents, peptide inhibitors, and several other organic molecules can be used as NCX inhibitors. Amiloride is not so popular a choice because of its low specificity and solubility. Different peptide inhibitors can also be used as an NCX blocker. The exchanger inhibitory peptide (XIP) is among the most popular peptides for NCX studies because of its high specificity and ability to reveal more information about the modulatory role of large intracellular loop based on calmodulin binding site.

After reviewing different types of inhibitors, we reviewed different studies which use these inhibitors for studying NCX inhibitory properties of KB-R7943 on different modes of the exchanger. They clearly see the inhibition of the reverse mode of the exchanger and the forward mode is not affected at all. Their results indicate that the outward current completely abolishes at 20μM and reduces significantly at 3 μM from control experiments where inward currents are activated at 10 μM. concentration.

Wongcharoen et al. (2006) studied the role of the NCX inhibitor KB-R7943 on the electrical activity of the pulmonary vein from adult rabbit cardiomyocytes and also on oubain induced arrhythmias. These studies found the decrease in action potential duration and contractile force at a different concentration of KB-R7943. Also, they found a decrease in I_{NCX} and calcium transients when treated with KB-R7943. Interestingly, they also found a decrease in the $I_{Ca\text{-}L}$ currents at higher concentrations. These were very interesting observations regarding the specificity of KB-R7943. Similarly Reppel et al. (2007) used the function expression of NCX in the embryonic mouse heart and used Ni^{+1}

as an NCX inhibitor for their electrophysiological studies and observed changes in I_{NCX} studies. Amran et al. (2004) used KB-R7943 and SEA0400 to study effects in guinea pigs *in vivo, in vitro* and using computer simulation studies. Their recording from guinea pig ventricular myocytes show increase in action potential duration when treated with KB-R7943 (10µM) and do not show any significant change in action potential duration when treated with SEA0400 (10µM). Similarly there have been a number of studies that used several other studies and other inhibitors but we found that KB-R7943 is the most widely used inhibitor.

From our literature review, we identified the importance of calcium homeostasis in cells and specifically for cardiac cells. Cardiac cells specifically undergo rapid changes in calcium for generating action potentials and we identified different factors affecting these influx-efflux mechanisms. This mechanism is crucially important in embryonic stages and there is very little information available about calcium regulation during cardiac development. Through this literature we identified that NCX is one of the important calcium transporters. This transporter plays an important role in the calcium influx and efflux mechanism. NCX has been widely studied in adult heart but there is no information available about its active role during development and its effect on the electrical activity of the heart. Hence, we decided to study this exchanger in intact embryonic chick hearts using intracellular recoding using its inhibitor KB-R7943. This study will also be supplemented by RT-PCR studies to identify its expression levels during development.

CHAPTER 2

MATERIALS AND METHODS

2.1 Intracellular recordings

Chick embryos from stages 21, 26 and 29 were killed by decapitation and the hearts were quickly removed and stages transferred to Tyrode's solution as previously described to retain its electrical activity (Arguello et al., 1986; 1988). The heart was then transferred immediately to a homemade recording chamber with a heating and perfusion unit attached to it to maintain a constant temperature at 37.1°C. and supply oxygen and Tyrode, respectively. The spontaneous electrical activity was recorded by a machine pulled (Sutter Instruments, Novato, CA) glass capillary microelectrode and filled with 3M KCl. The intracellular recordings were performed in intact hearts to measure spontaneous electrical activity before and after administration of the compound KB-R7943 with glass microelectrodes connected to an Amplifier 1440 (Axon Instruments, CA). The data from the amplifier was acquired and fed to the computer through Digidata 1440 (Axon instruments, CA). Recordings were simultaneously displayed and recorded using Axoscope (Axon Instruments, CA). The electrical activity of the heart was recorded during stages 21, 26 and 29 at 1µM. KB-R7943 in the dark and several hundred action potential were recorded from each stage. Also, the dose response was calculated for .1 µM- 10 µM concentrations of the same compound.

2.2 Immuno-staining and imaging

Chick embryos from stage 20 to stage 30 were killed by decapitation and the heart was quickly removed and fixed with 3.7% formaldehyde (Sigma, St. Louis, MO) for 30 minutes, washed 5 times over 5 minutes with a phosphate buffer solution (PBS), washed for 30 minutes with a 5% sucrose solution in PBS, and cryoprotected with a 20% sucrose solution overnight at 4°C. Cross sections were cut and treated with Xylene, 70%, 80%, and 95% ethyl alcohol each for 20 minutes followed by 3 steps of 100% ethyl alcohol for 20 minutes each. Sections were then air-dried and stored at 4^{o}C. Immunostaining was performed by blocking the sections with 2% BSA in PBS and incubating them with the Anti-Rabbit primary antibody for NCX at 4^{o}C for 24 hours, followed by incubation with fluorochrome-conjugated secondary antibodies (Zymed, San Francisco, CA) for 2 hours at room temperature, washing in PBS, and finally coverslipping the slides with Prolog Antifade (Molecular Probes). Images were digitally photographed (10x or 40x) with an upright BX51WI Olympus (Olympus America Inc. Center Valley, PA) microscope equipped with a Retiga EXi Fast1394 (Qimaging, Surrey, BC Canada) digital camera. Some pictures were taken using a confocal microscope Fluoview FV100 (Olympus America Inc, Center Valley, PA). The images were processed with Imaris 4.2.0 (BitPlane AG, Saint Paul, MN), a 3-D rendering program using MIP (maximal intensity pixel) to generate the pictures.

2.3 RNA isolation and RT-PCR

Cultured chick embryos for different stages were removed and heart RNA obtained using a MELT total RNA Isolation System (Ambion, Austin, TX). For single cells RT-PCR, the entire heart or specific regions of the heart (atria, AV-C, Ventricle or outflow tract) were dissociated in single cells. Two hours after plating the cells, we

performed intracellular recordings of spontaneously beating individual cells. Cells that exhibited similar action potential properties to those previously described were collected by a second suction micro tube and deposited in a 0.5 ml centrifuge tube. Five to ten 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 were made by using BD Advantage RT to PCR kit (BD Mountain View, CA). PCR was carried out by mixing l cDNA with five l 10x buffer, one l 10mM DNTPS, two l primer for each (100 ng/ l), 5 l BSA (2.5 g/ l), 0.5 l Taq polymerase and 32.5 l d H20. PCR reactions were carried out for 35 cycles. PCR product (10 l) was run on 1.4% agarose gel in 1X TAE. GAPDH (330 bp) was used as a control. The primers were prepared as needed. RT-PCR product was subsequently subcloned into a TOPO system (Invitrogen), and sense and antisense riboprobes were synthesized and labeled with digoxigenin (Boehringer Mannheim, Indianapolis, IN). Both sense and anti-sense riboprobes were sequenced to confirm faithfulness of PCR amplification by in-house facilities (Molecular Biology Program Core Research Facilities, University of Missouri-Columbia).

RNA isolation and PCR: After 24 hrs of treatment of cultured hearts with antisense and mixed base ODNs (8 µM) or control, the hearts were processed to obtained total RNA using Melt-Total Nucleic Acid Isolation System (AMBION) according to manufacture's specifications. Total RNA was reverse transcribed into cDNA by using the RT to PCR kit Advantage (Clontech-BD) in accordance with the manufacturers' instructions. The step cycle program for the PCR was set to denaturing at 94°C for 30 sec, annealing at 58°C for 60 sec, and extension at 72°C for 105 sec for 35 cycles. The primers were obtained from a commercial supplier (RealTimePrimers.com) and had the

following sequences: NCX 1-specific primers (from position 2714 to 2899) NCX 5'-TCT CCG CCA TCA CTC TCT TCA CC-3' forward and 5'-TTA TGT GGC AGT AGG CTT CC -3' reverse. This All PCRs were analyzed on 1.5% agarose gels, stained with ethidium bromide, and photographed with a Polaroid camera (Kodak, Rochester, NY).

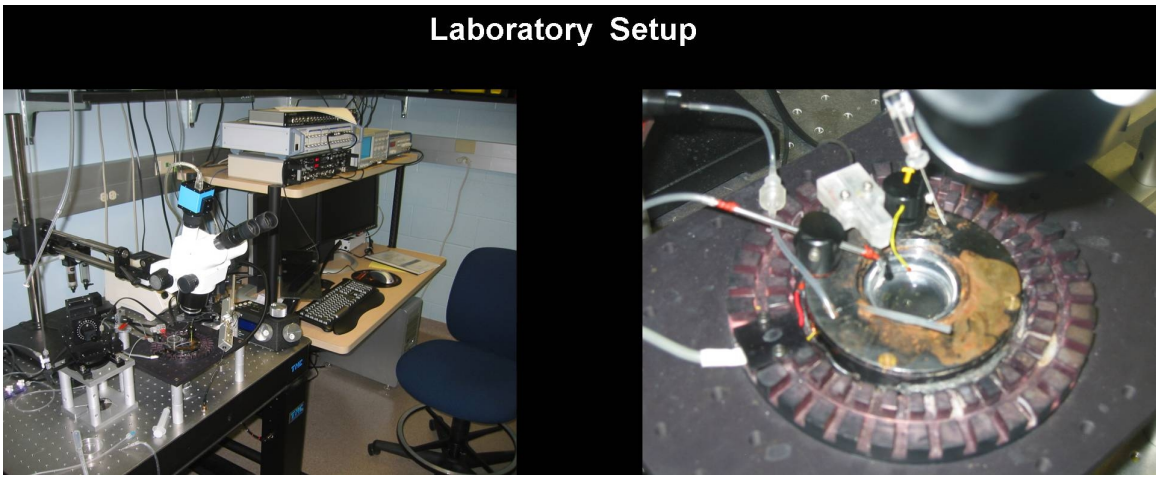

Figure 2. Experimental setup for intracellular recording

CHAPTER 3

RESULTS

Electrical activity of the heart is its most important function and it is one of the important steps during embryonic development. Spontaneous electrical activity of the heart develops between stages 7-8 (Hamburger and Hamilton, 1992). This electrical activity is specific to different regions in the heart and shows characteristic behavior e.g. smaller duration of APs in atrium than in ventricles (Sperelakis and Pappano, 1983). The exact mechanisms for this variation during development are still not completely known. Many studies indicate the importance of these variations and the factor influencing the electrical activity of heart during adulthood as well (Blaustein and Lederer, 1999; Liu et al., 2002).

We hypothesized that NCX is present during the early stage of development and play an important role in the electrical activity of heart. We tested this hypothesis by blocking the exchanger with KB-R7943 (1µM) during stage 26 and recording the spontaneous electrical activity before and after introduction of the drug. These data were then analyzed for changes in electrical activity of heart. These experiments indicated a significant decrease in the frequency of APs in both atrium and ventricles of stage 26 (Fig. 4). This primary data was then analyzed for finding the specific effects on properties of AP. These experiments were performed under daylight and we observed that effects disappeared 15-60 min after perfusion. We found that the KB-R7943 was light sensitive. Therefore the rest of our experiments were performed in the dark so as to have a consistent effect of the blocker for the entire duration of our experiments. Experiments

were performed in stages 21, 26 and 29 of development based on their developmental characteristics during these stages (Sperelakis and Pappano, 1983). All the experiments were performed with 1µM concentration and later we used different concentrations to characterize the drug response.

The action potentials generated by heart very significantly during different stages of development in terms of their amplitude, duration and maximal rate of rise (Figs. 4, 5, 6) and blocking of NCX may have different effects during different stages of development. We did not observe consistent changes in the amplitude of action potential in both atrium and ventricles. Similarly, the changes in the maximum rate of rise were not consistent. Atrium amplitude remained almost the same for stages 21 and 26 and increased ±10mVs in stage 29. The maximum rate of rise increased for stage 21 in both atrium and ventricles and decreased for stage 26 and 29. Atriums show prominent decrease in maxim rate of rise when compared to ventricles during later stages of development. The most significant effects were found in the duration of APs (Fig. 7) in both atrium and ventricles during all stages of development**.** Duration was further divided into the rise time and decay time of action potential.

The duration of ventricular action potentials was more sensitive during the early stages (stage 21) of development and it increased from 200 mSec to 400 mSec approximately. The increase in duration was reduced during later stages of the development in ventricles. The action potential has a very fast rise time and comparatively longer decay time. The rise time increased during stage 21 and 26 and did not have any change during stages 29. Also decay time changes in same proportions as of total duration during all stages of development.

Although the NCX has been studied in adult hearts, there is very little information available about its function in the atrium during development. Our studies involved the analysis of the electrical signal recorded from the atrium before and after blocking of the exchanger. In contrast with the ventricles, the atrium does not show any increase during stage 21 of development in action potential duration, but it increases for stages 26 and 29. The maximum increase is for stage 29 from approximately 110 msec to 190 msec. Rise time of atrium action potential did not change very significantly during all stages. Also the decay time did not change during stage 21 and changed significantly during stage 26 and 29 with maximum change for stage 29 from approximately 60 msec to 140 msec.

We also calculated the effects of blocking the exchanger at different concentrations of KB-R7943. Some of the studies in the past indicate a very high concentration in the mM range for blocking of the exchanger, whereas our results indicate that the minimum amount required to produce change is 1 µM in both the atrium and ventricles. The experiments were performed on stage 26 as being an intermediate stage of our selection. The changes in amplitude and maximum rate of rise were not consistent and seem independent of the concentration of the drug. Hearts were sensitive for a drug concentration of 1 µM and above for changes in duration of action potential. The changes produced were highest at 10 µM and response tended to saturate at a higher concentration above that. There was a steep increase in duration (rise time and decay time included) of action potential from 1 µM to 3 µM and continued for 10 µM as well. Also, the available literature suggests that KB-R943 is not a very specific inhibitor of NCX. Hence, we tested the effect of NCX with XIP which was a more specific inhibitor of the exchanger. The XIP was delivered through a newly developed system based on

shockwave. The XIP and KB-R7943 both produced a similar change with some variation (Fig. 25). Hence, it is evident that KB-R7943 is specific enough to block NCX selectively.

The effects produced by the atrium and ventricles were different in both the atrium and ventricles and needed further investigation of the exchanger. We carried out the RT-PCR experiments to test the mRNA levels in the atrium and ventricles. The results show almost the equal amount of mRNA expression in the atrium and ventricles during early stages of development. These levels remain almost constant for the atrium during later stages of development and increases in multifold in the ventricles. It remains almost constant in other regions of heart.

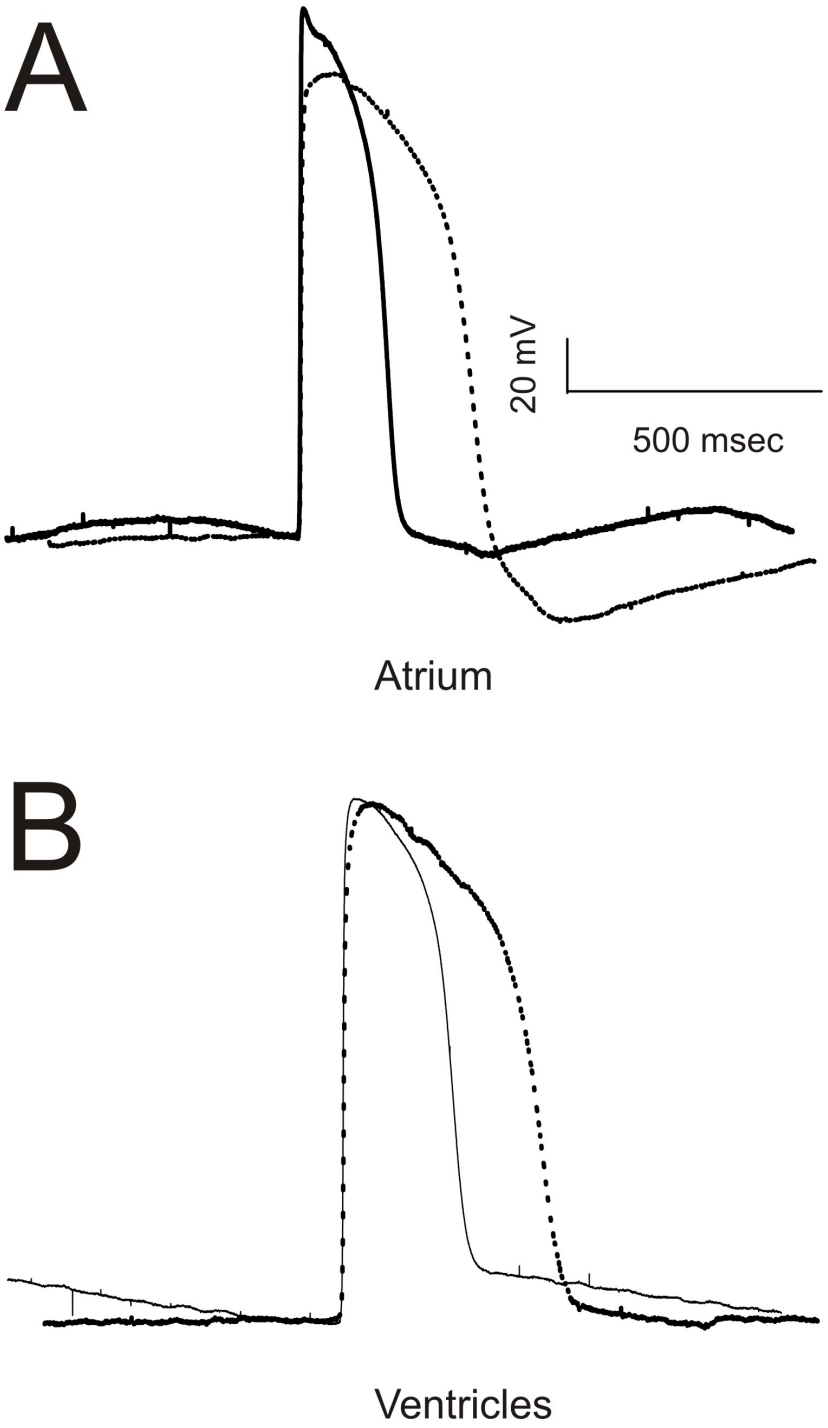

Figure 3. Changes in single AP after blocking NCX

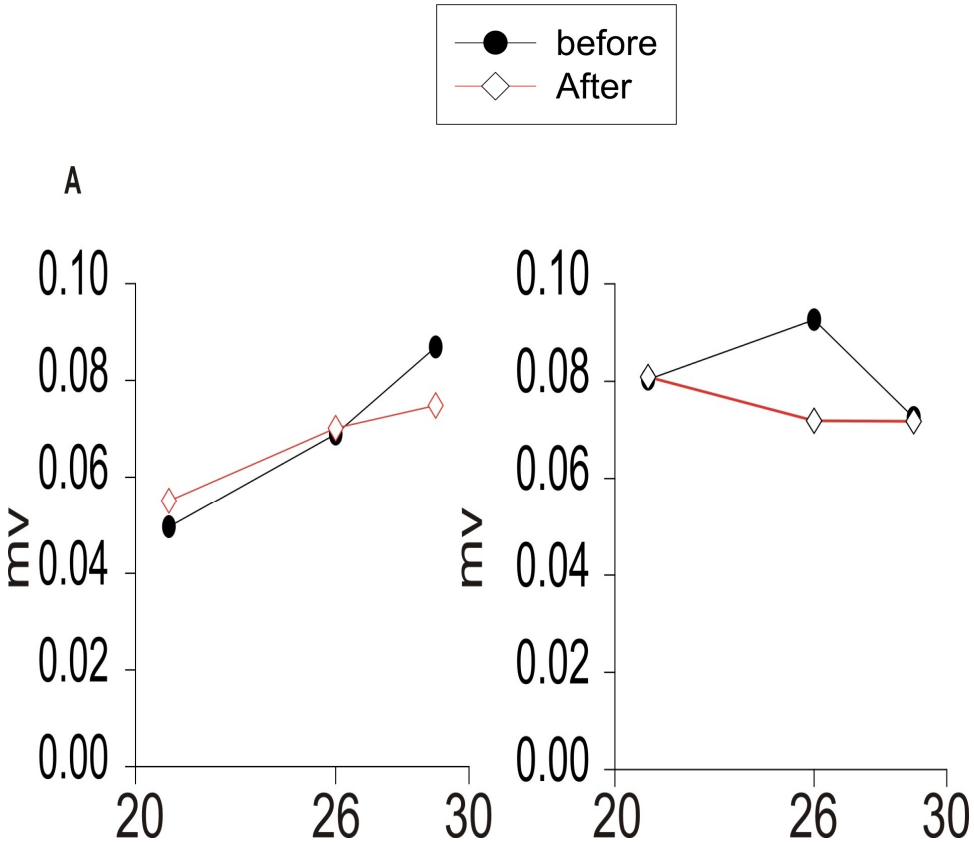

Figure 4. Changes produced in the amplitude

The amplitude increased for both atrium and ventricles for stage 21 and decreased for stages 26 and 29 at 1µM KB-R7943 concentration. The decrease in atrium is relatively more than the ventricles in later stages of development

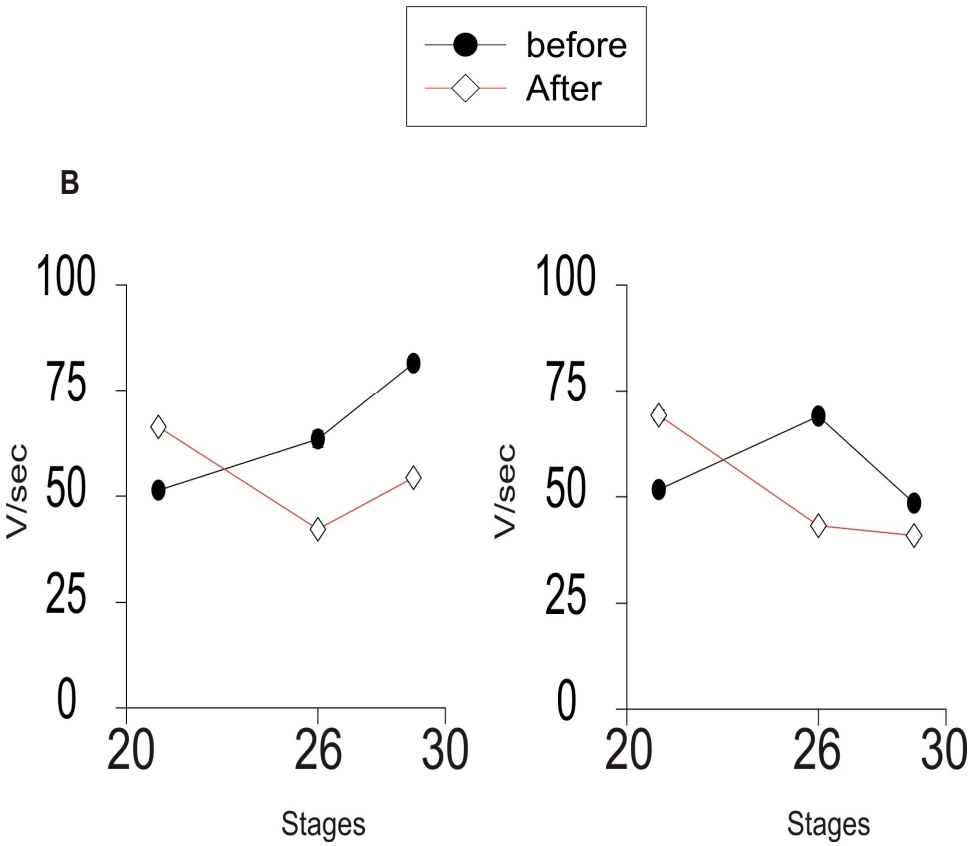

Figure 5.Changes produced in the maximum rate of rise

Changes produced in the maximum rate of rise (Max. dv/dt) in the atrium (left) and ventricles (right). The max dv/dt increased for both the atrium and ventricles for stage 21, and decreased for stages 26 and 29 at 1µM. KB-R7943 concentration. The decrease in the atrium is relatively more than the ventricles in later stages of development.

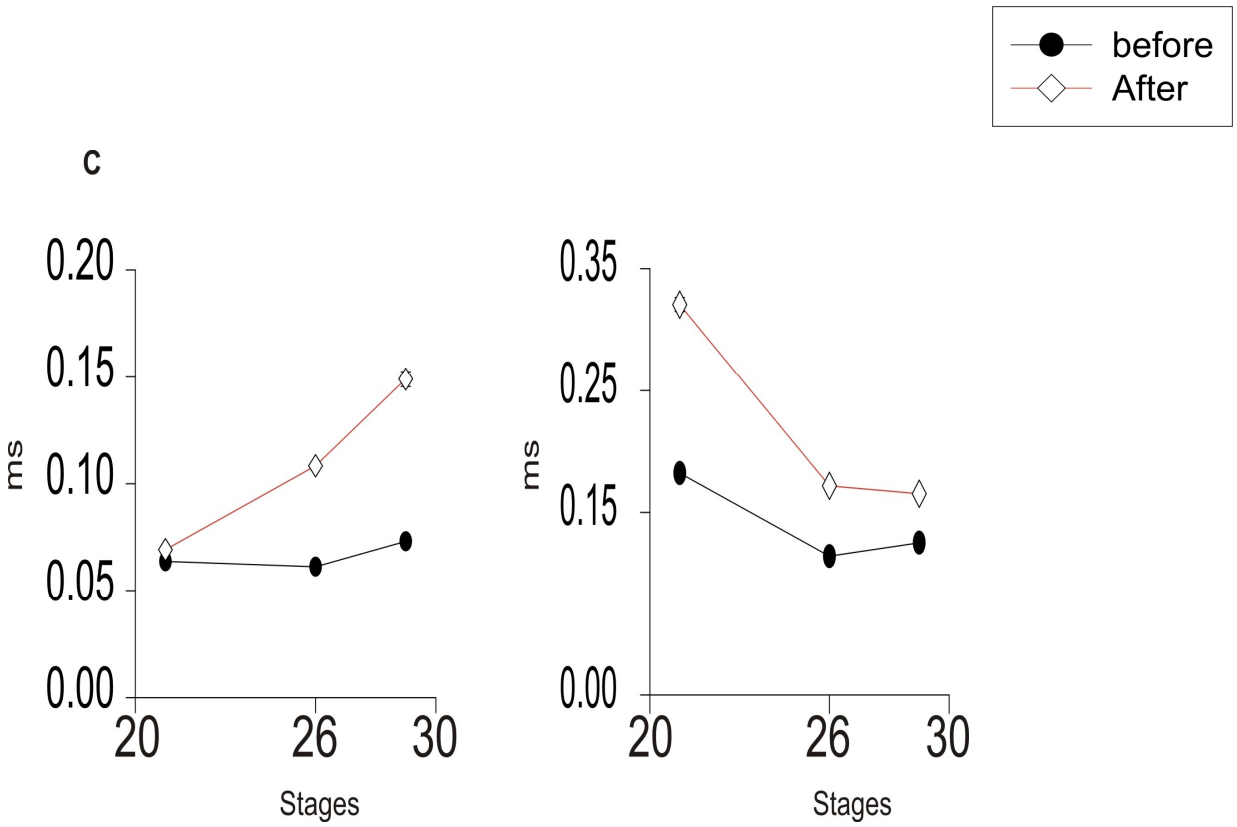

Figure 6. Changes produced in the duration

Changes produced in the duration of APs in atrium (left) and ventricles (right). Atrium action potential did not increase at stage 21 but increased significantly at stage 26 and 29 after blocking NCX where as ventricular action potential duration showed reverse trend. It increased at stages 21 and showed relatively lesser increase during stages 26 and 29 after blocking of the exchanger.

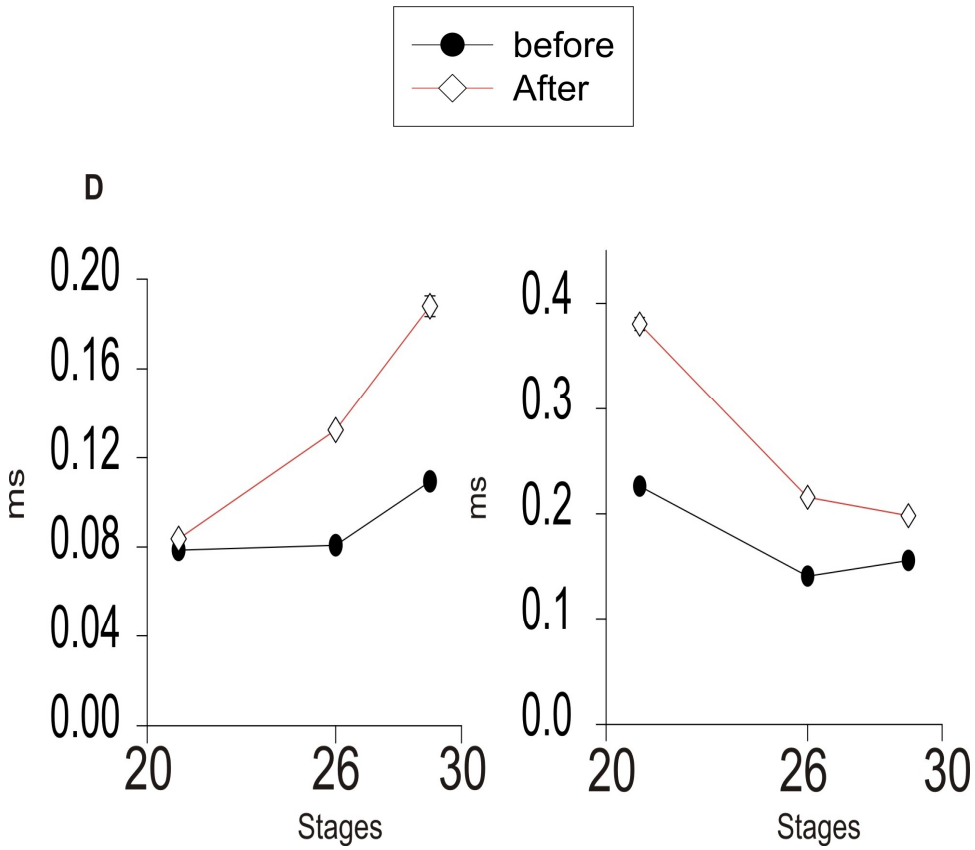

Figure 7. Changes produced in the decay time

Changes produced in the decay time of APs in the atrium (left) and ventricles (right). Decay time of the Atrium action potential did not increase at stage 21 but increased significantly at stages 26 and 29 after blocking NCX, where as ventricular action potential decay time showed reverse trend. It increased at stage 21 and showed a relatively smaller increase during stages 26 and 29 after blocking of the exchanger.

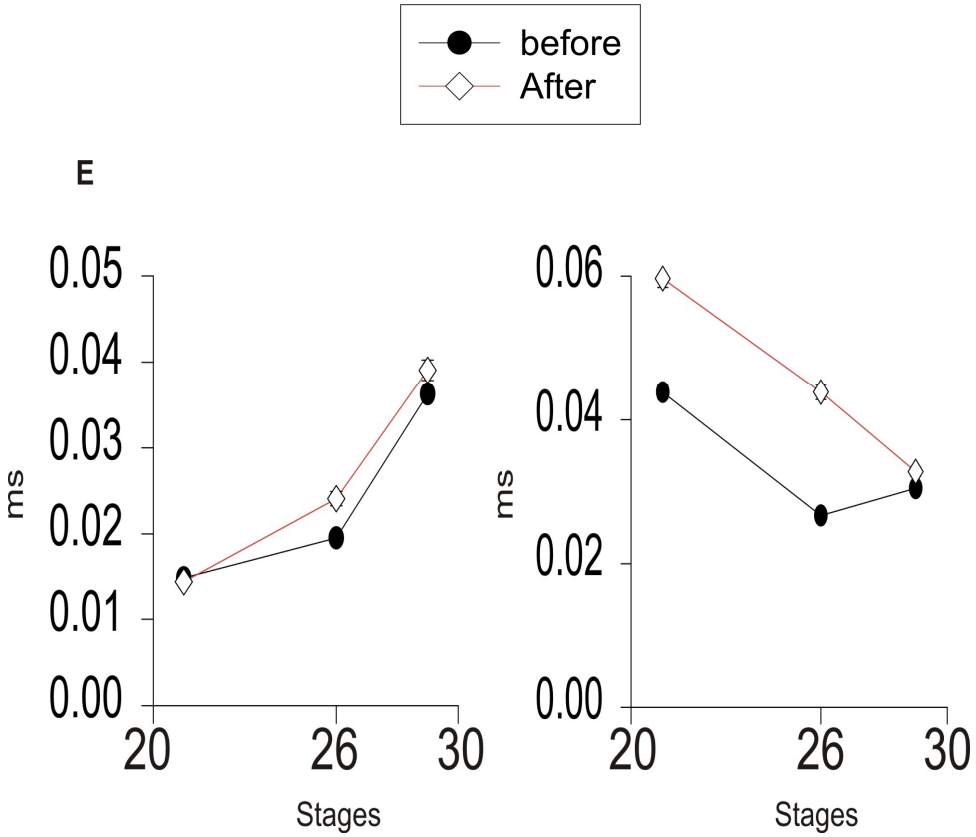

Figure 8. Changes produced in rise time

Changes produced in the rise time of APs in the atrium (left) and ventricles (right). Atrium action potential did not increase significantly at all stages after blocking NCX, where as ventricular action potential duration showed different effects. It increased at stage 21 and showed a relatively smaller increase during stages 26 and 29 after blocking of the exchanger.

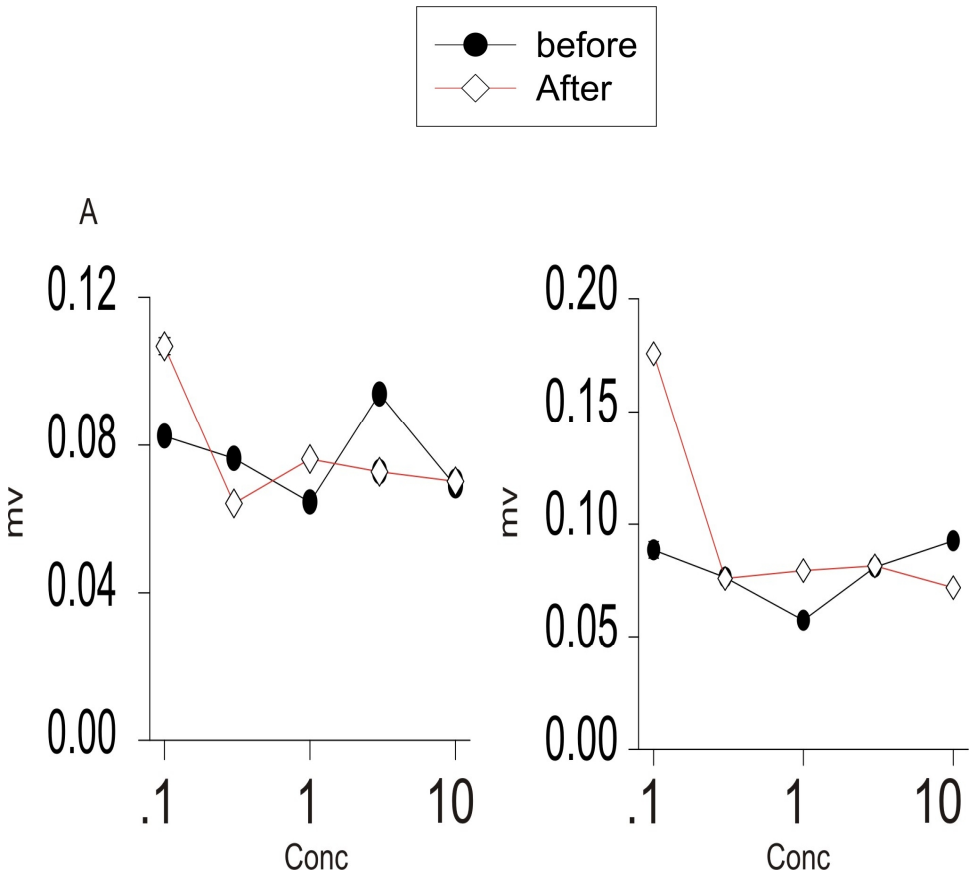

Figure 9. Change in amplitude as a drug response

Changes in amplitude of the atrium (left) and ventricles (right) of stage 26 at different levels of concentration of KB-R7943. We did not find any consistent changes in amplitudes in both the atrium and ventricles.

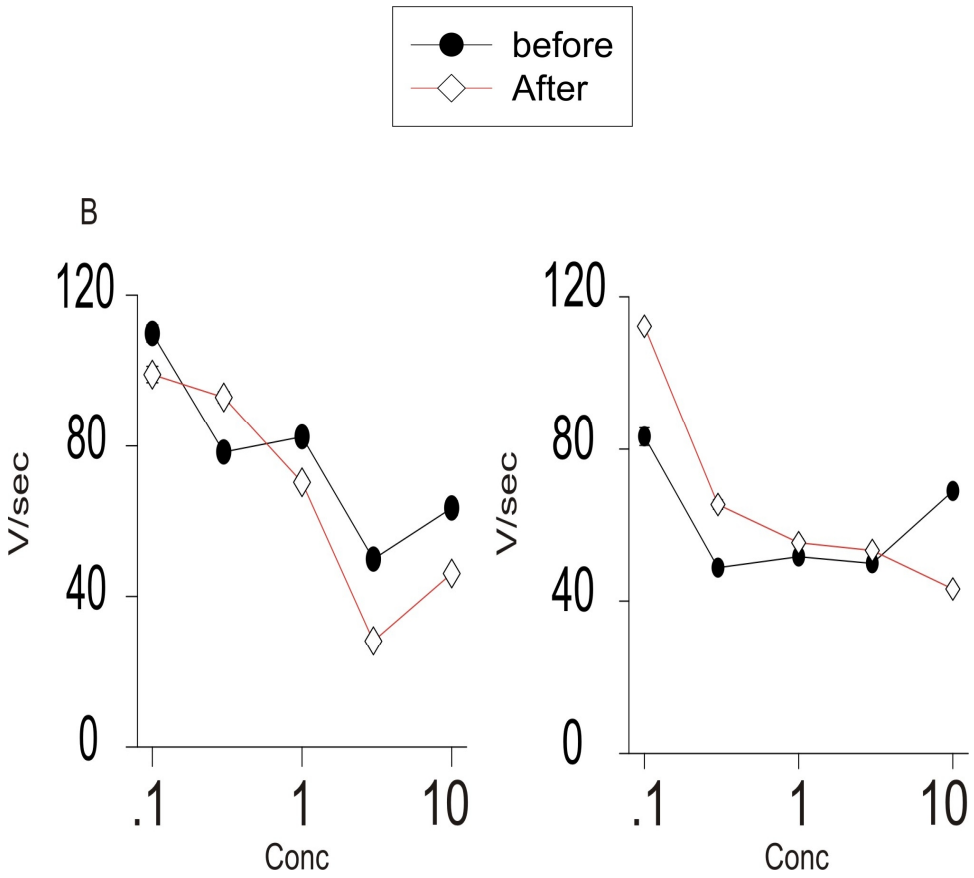

Figure 10. Change in rate of rise as a drug response

Changes in maximum rate of rise of atrium (left) and ventricles (right) of stage 26 at different levels of concentration of KB-R7943. The changes in the atrium were more consistent at higher concentrations whereas ventricular action potentials showed consistent changes at lower concentrations.

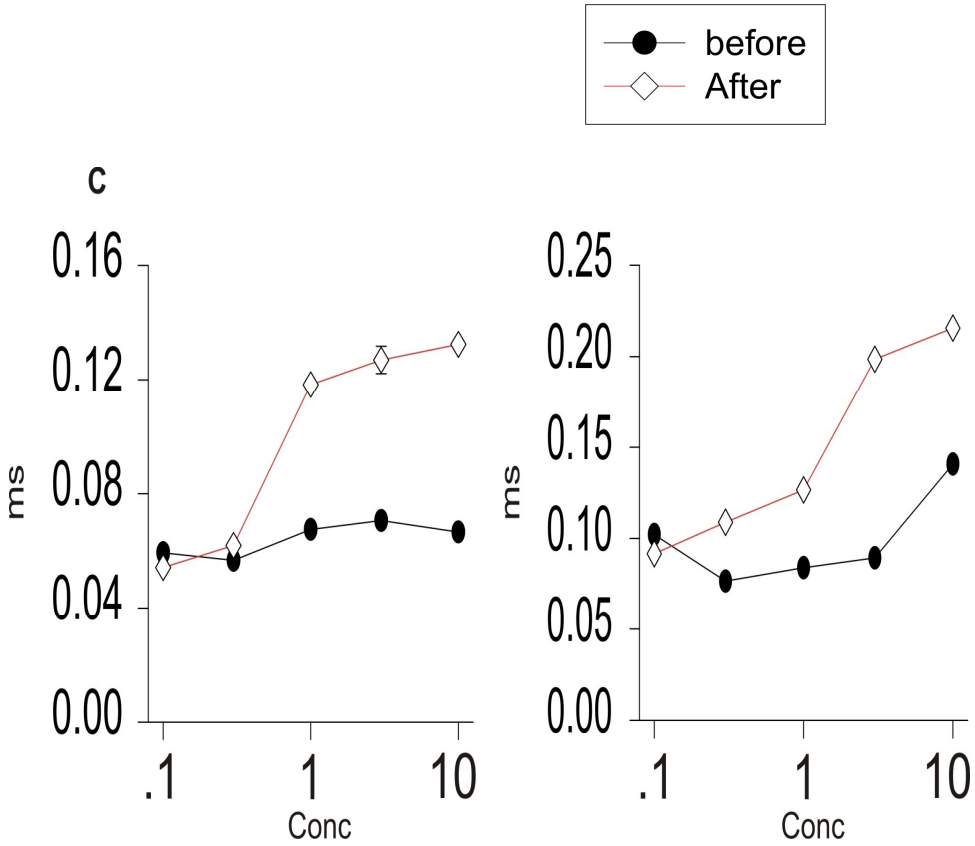

Figure 11. Change in duration as a drug response

Changes in duration of the atrium (left) and ventricles (right) action potential recorded from stage 26 at different levels of concentration of KB-R7943. The changes in the atrium were more consistent at higher concentrations whereas ventricular action potentials showed consistent changes at lower concentrations.

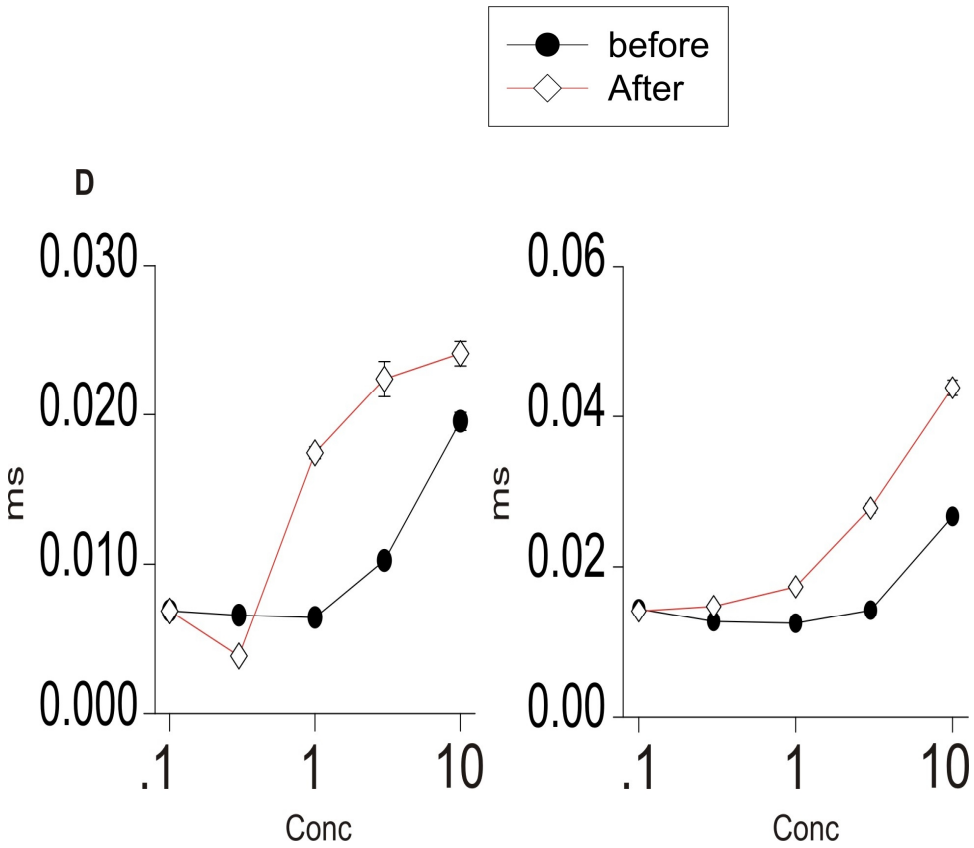

Figure 12. Change in rise time as a drug response

Changes in rise time of the atrium (left) and ventricles (right) action potential recorded from stage 26 at different levels of concentration of KB-R7943. The changes in ventricles were more consistent at higher concentrations. The rise time remained almost unchanged at lower concentrations in both the atrium and ventricles and increased sharply at higher concentrations. Also, the response tended to saturate at higher concentrations in the atrium but not in ventricles.

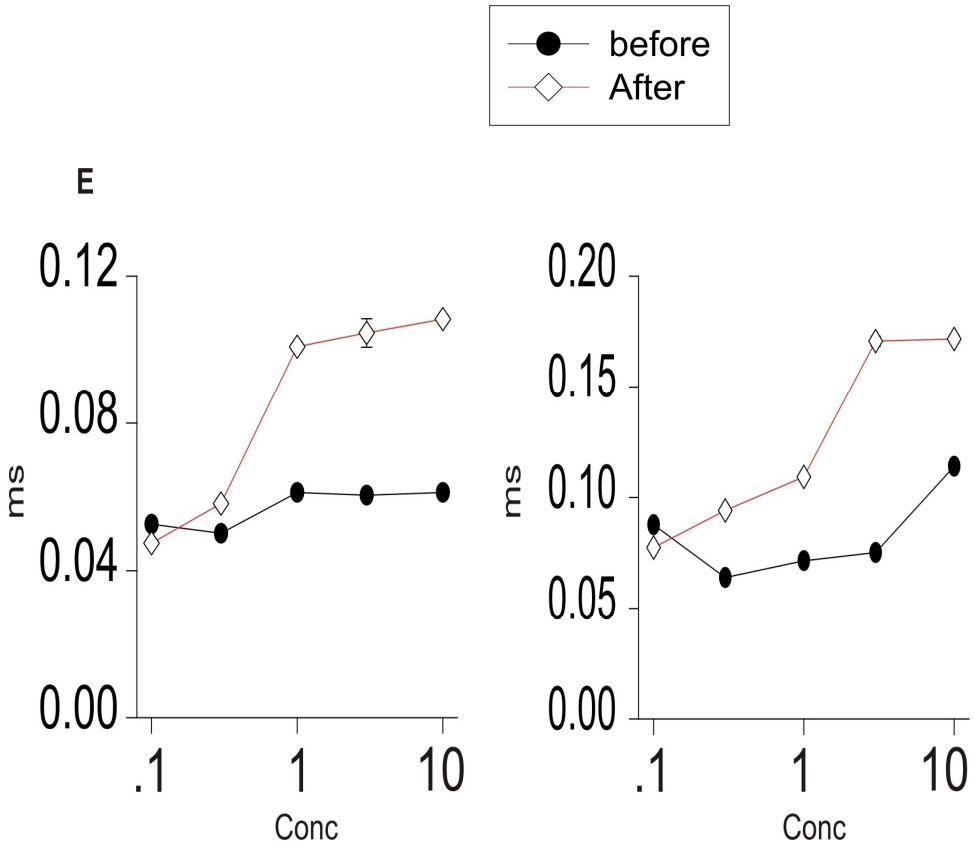

Figure 13. Change in decay time as a drug response

Changes in decay time of atrium (left) and ventricles (right) action potential recorded from stage 26 at different levels of concentration of KB-R7943. The changes in atrium and ventricles were equally consistent at higher concentrations as well as lower concentrations. In both atrium and ventricles the response tended to saturate at higher concentrations.

Immunostaining images from stage 26 and stage 29

Note - There will be a couple of images from each stage 26 and 29, respectively to show more details.

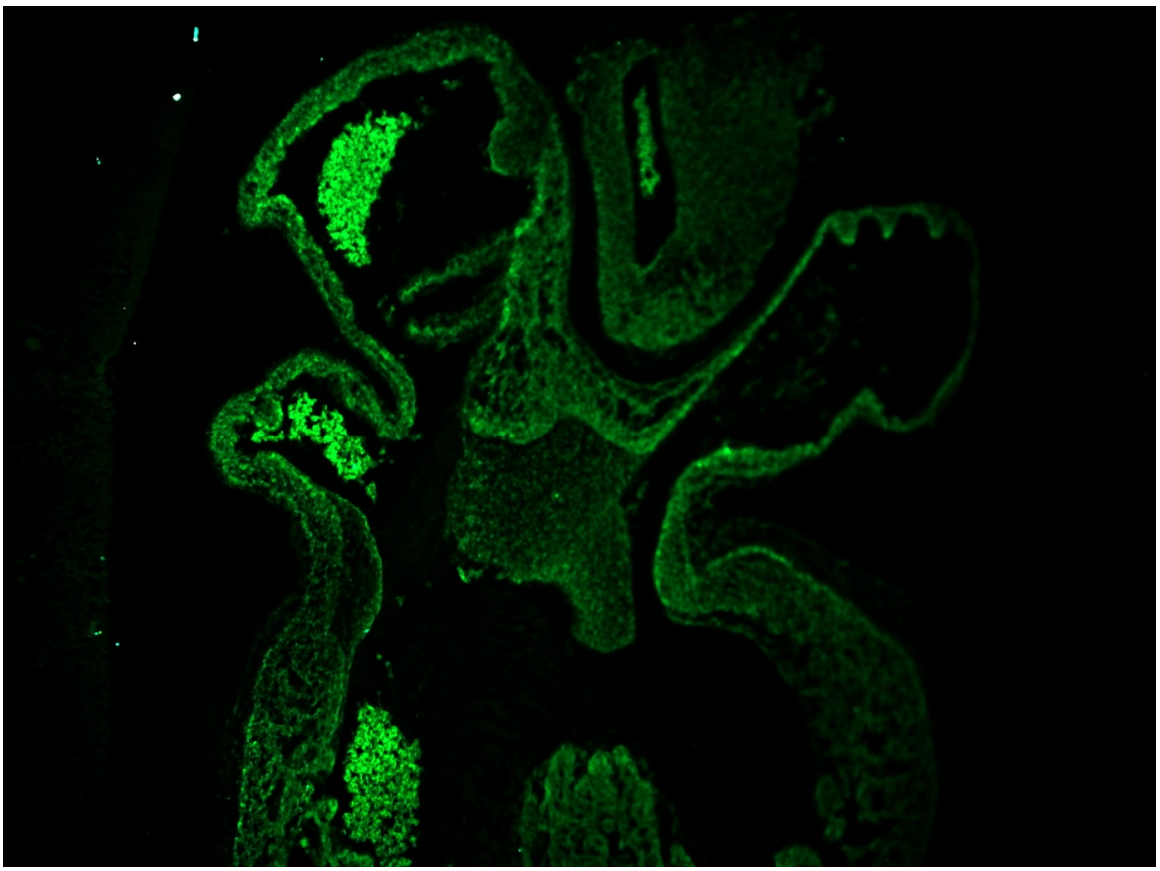

Figure 14. Stage 26 (A) Immunostaining

Partial section of an embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of the atrium and ventricles.

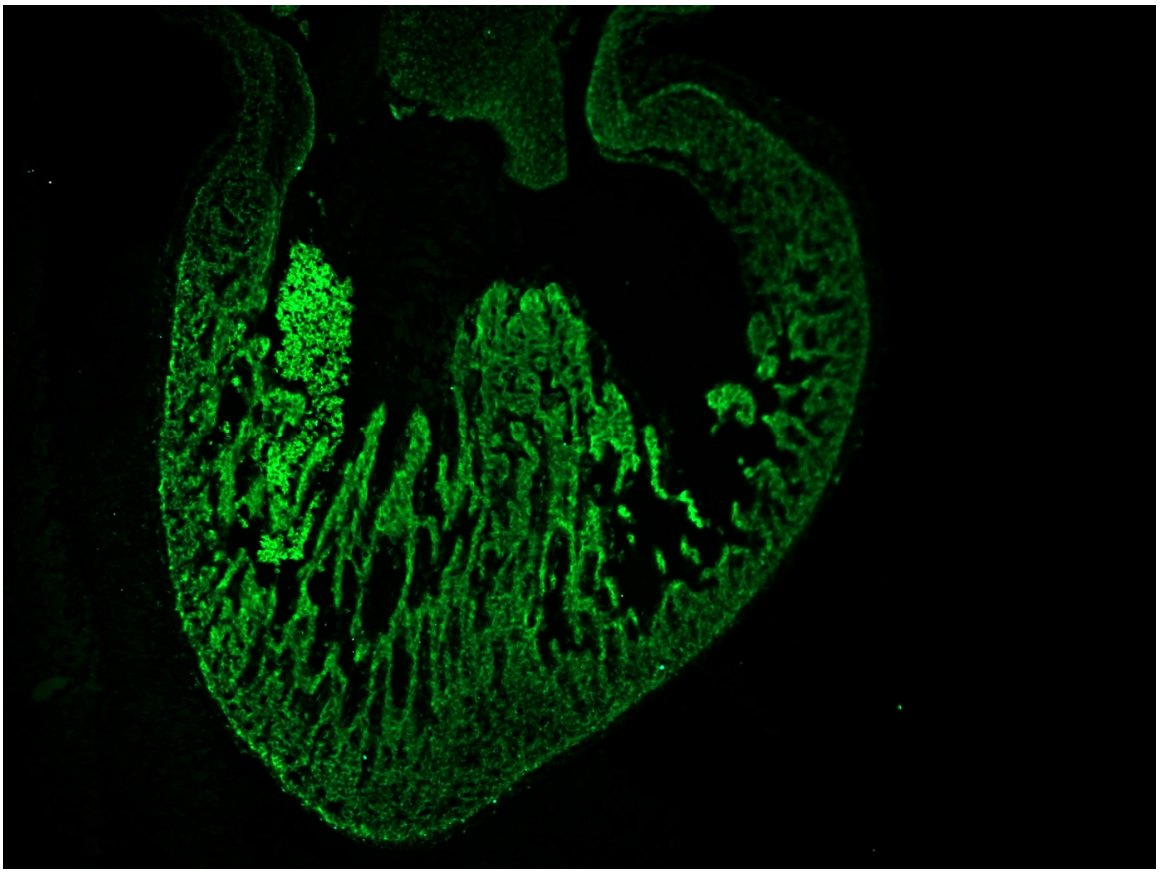

Figure 15. Stage 26 (B) Immunostaining

Partial section of a embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of atrium and ventricles.

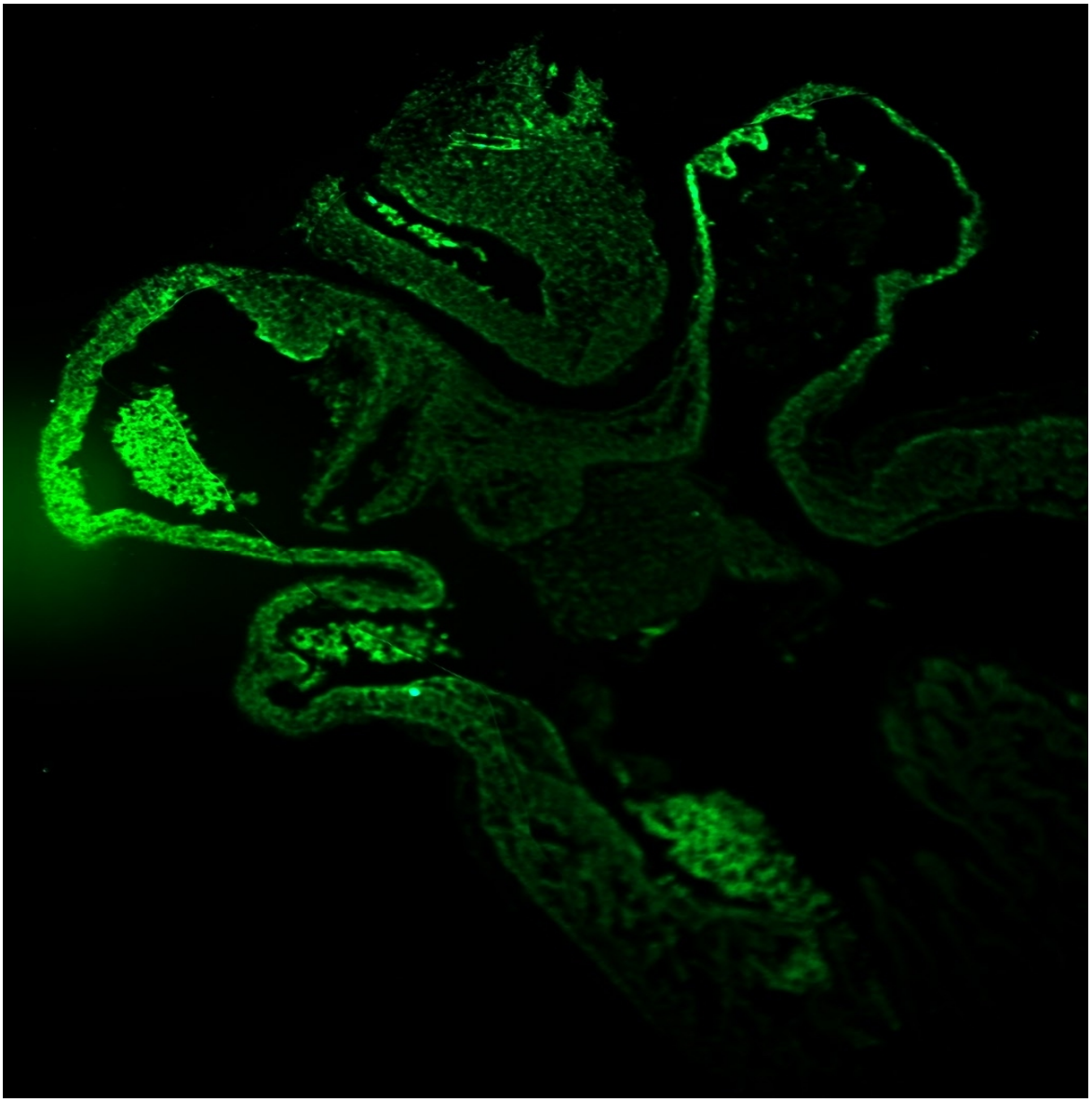

Figure 16. Stage 26(C) Immunostaining

Immunostaining of stage 26 – Partial section of an embryonic heart Immunostaining Showing the localization of the protein mainly in the wall of atrium and ventricles.

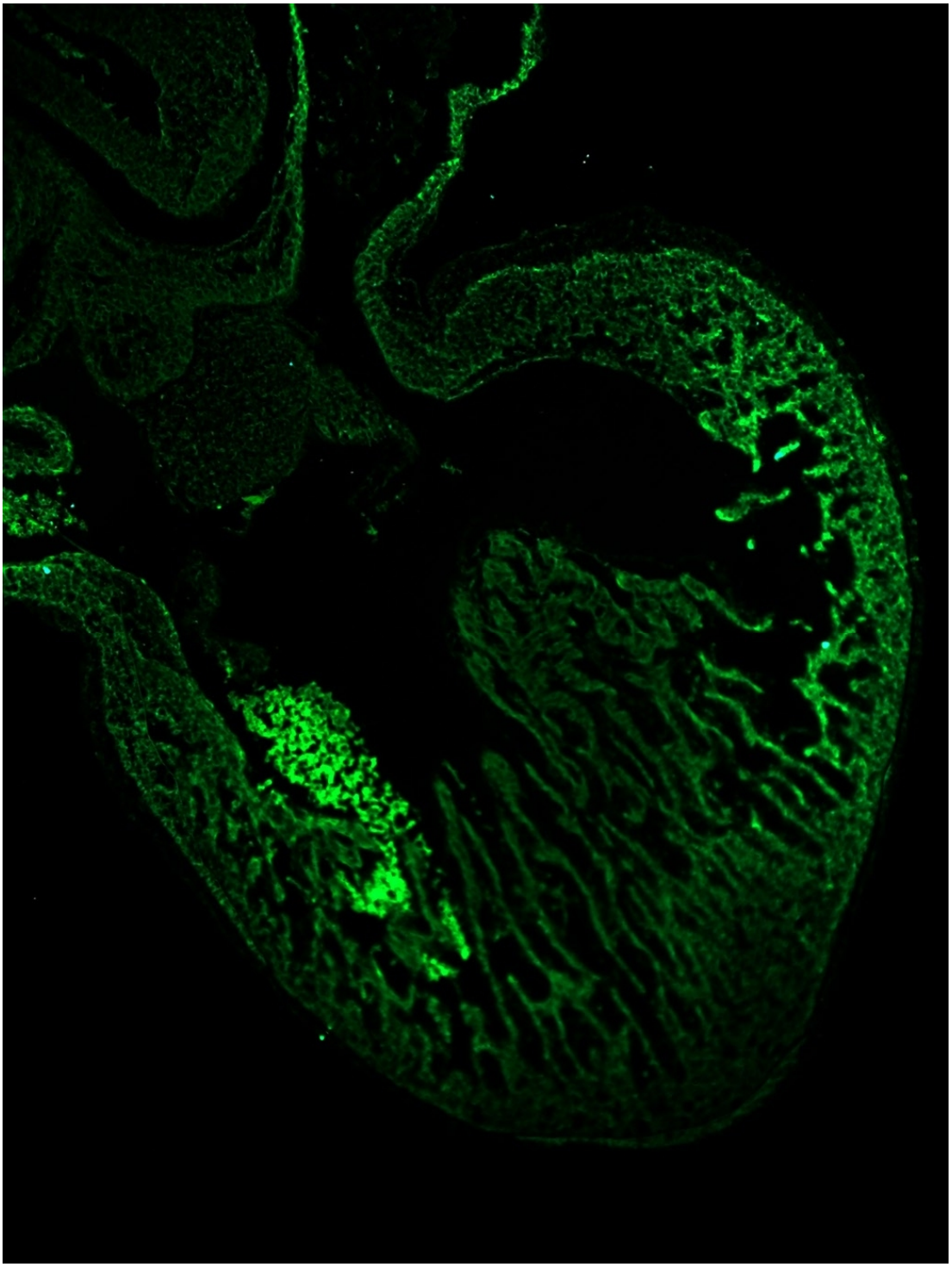

Figure 17. Stage 26(D) Immunostaining

Partial section of embryonic heart. Immunostaining shows the localization of the protein mainly in the wall of atrium and ventricles.

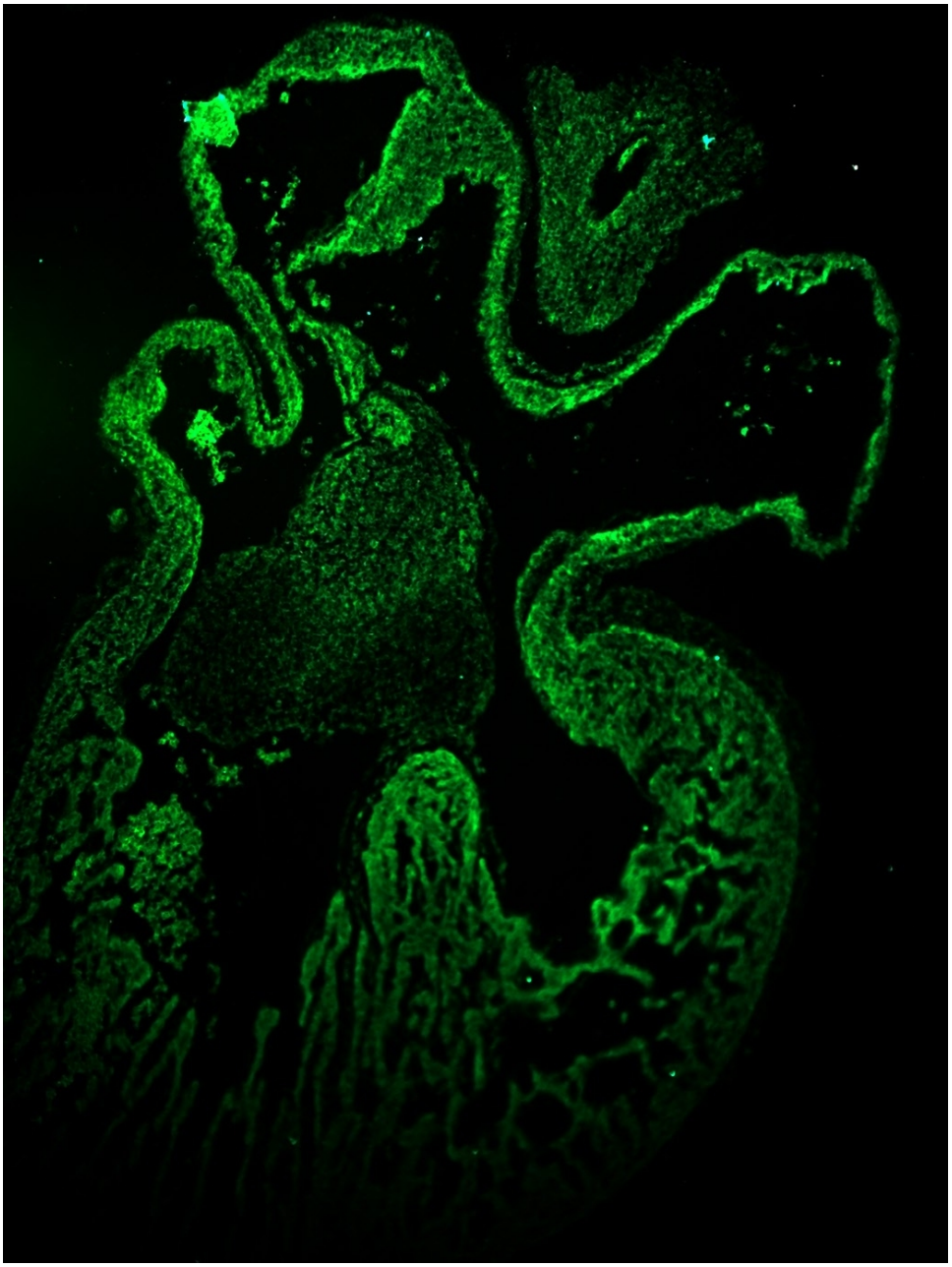

Figure 18. Stage 26(E) Immunostaining

Partial section of an embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of atrium and ventricles.

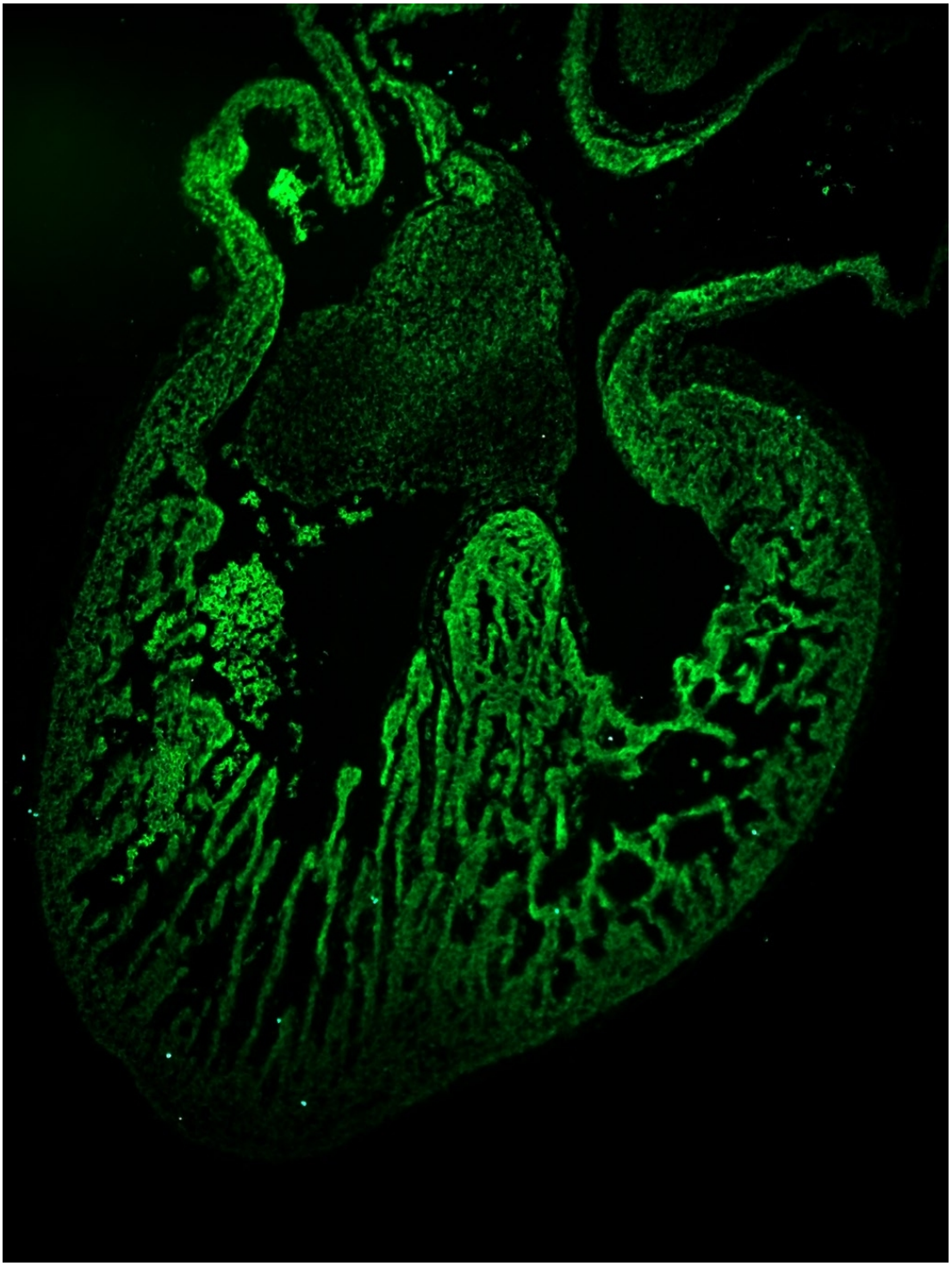

Figure 19. Stage 26(E) Immunostaining

Partial section of embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of the atrium and ventricles.

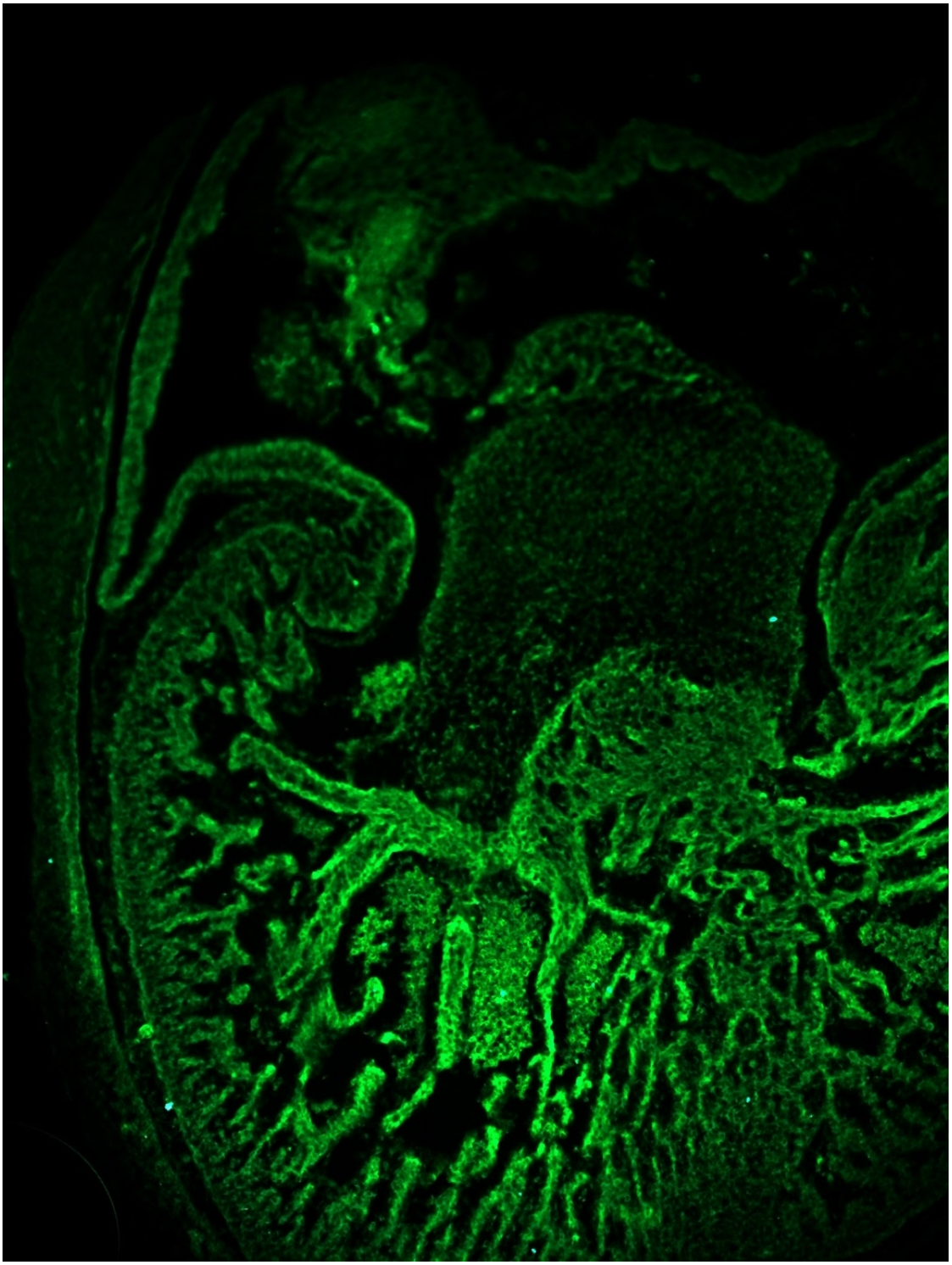

Figure 20. Stage 29 (A) Immunostaining

Partial section of embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of the atrium sand ventricles

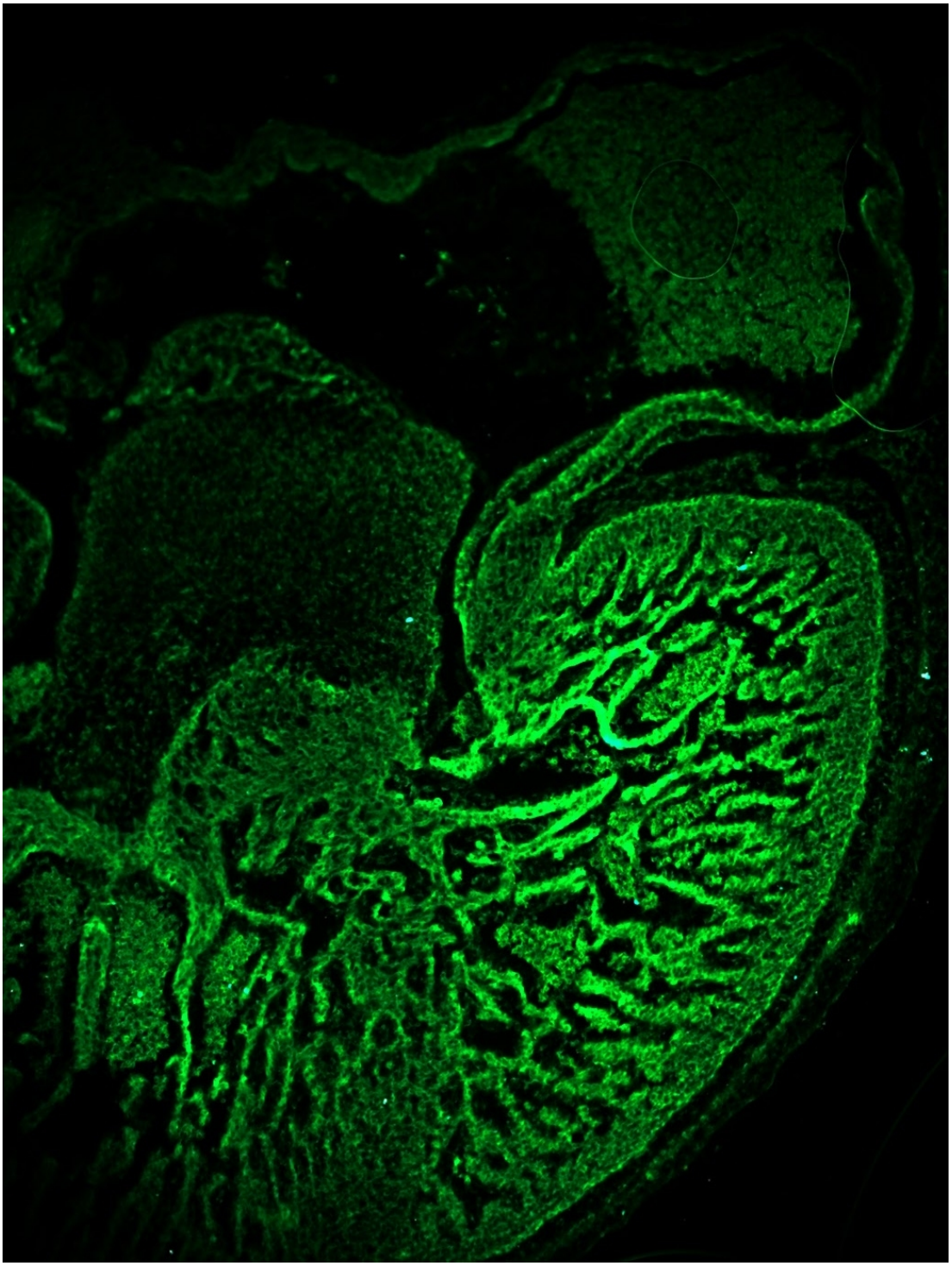

Figure 21. Stage 26 (B) Immunostaining

Partial section of embryonic heart. Immunostaining showing the localization of the protein mainly in the wall of the atrium and ventricles.

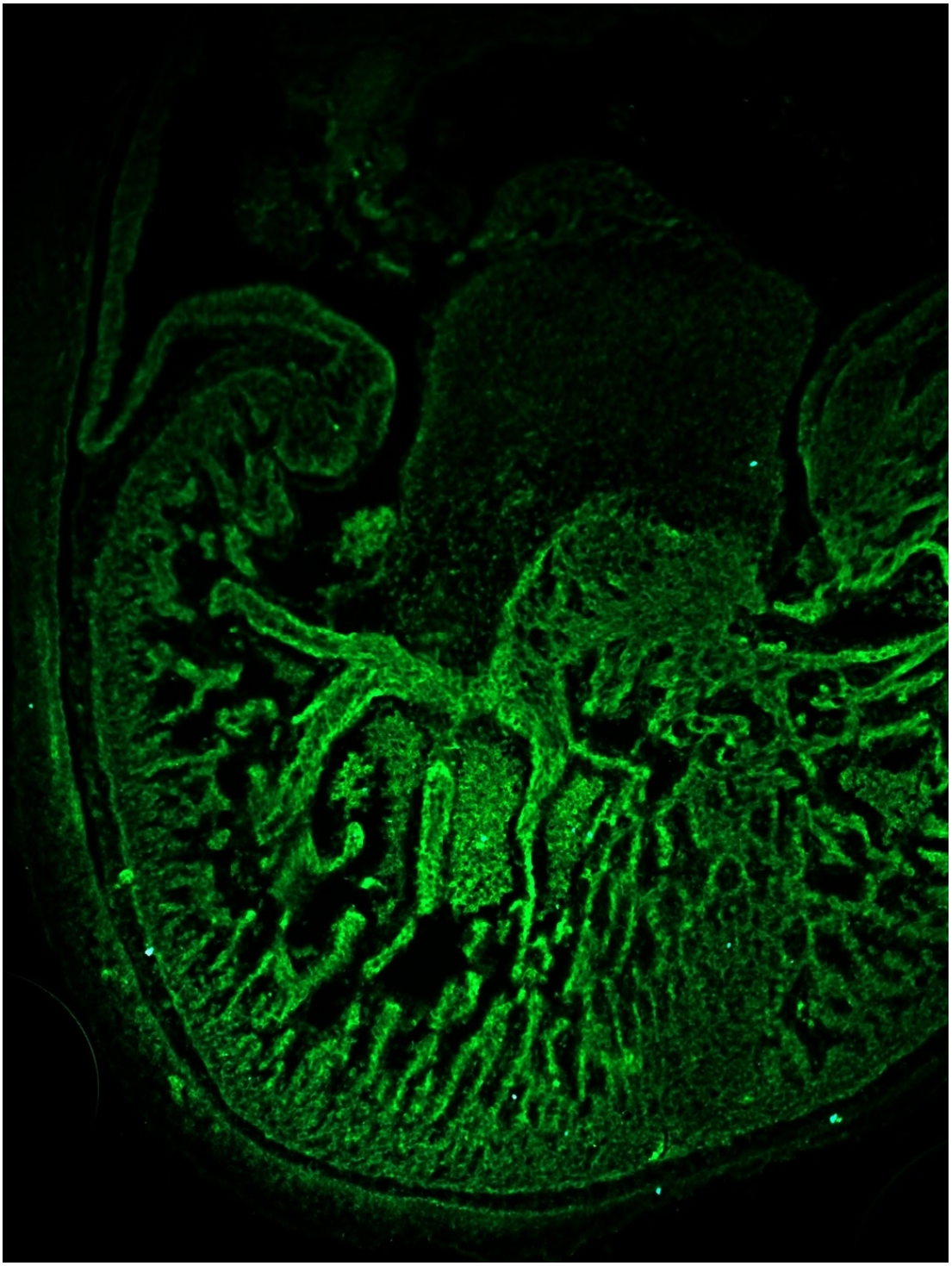

Figure 22. Stage 29 (C) Immunostaining

Partial section of an embryonic heart. Immunostaining showing the localization of the protein mainly in the walls of the atrium and ventricles.

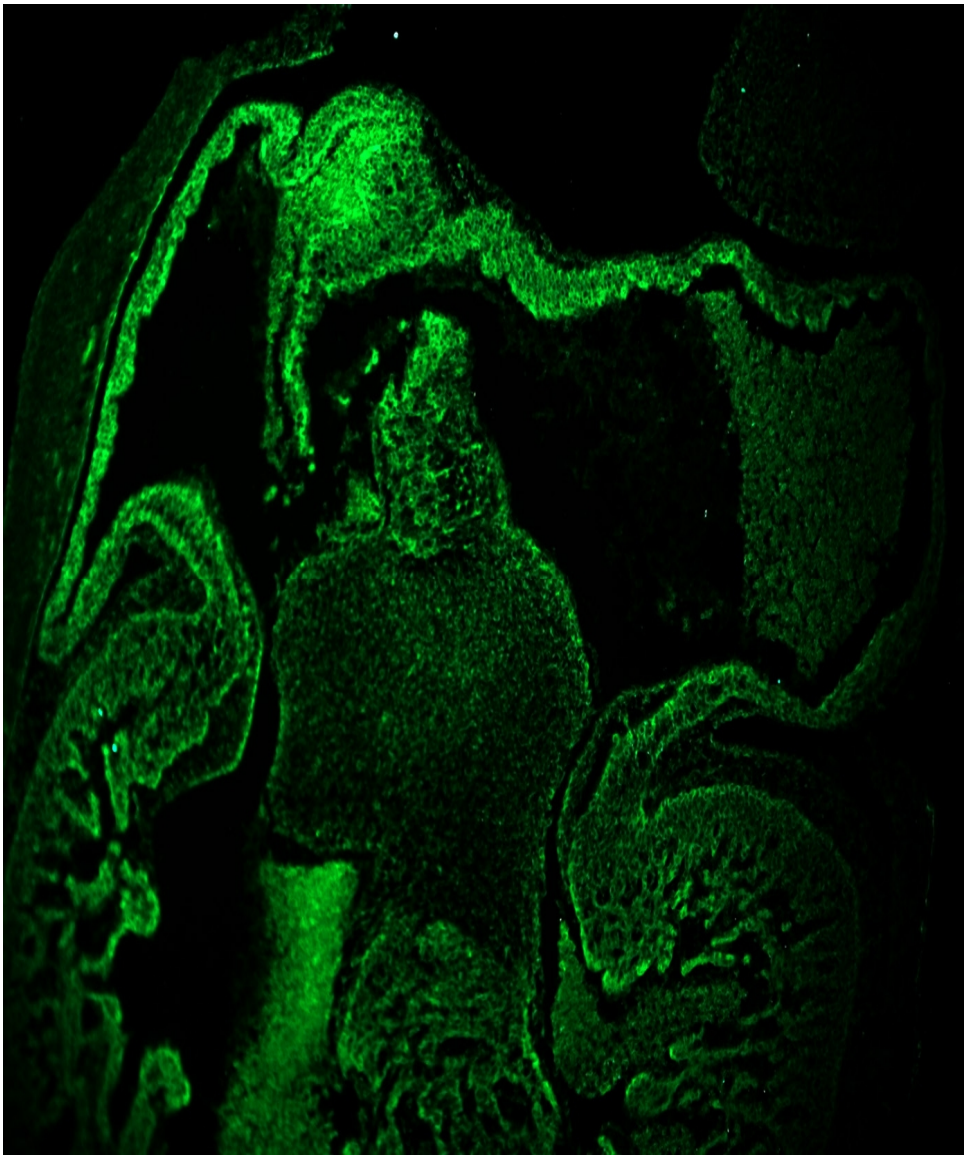

Figure 23. Stage 29 (D) Immunostaining

Partial section of an embryonic heart. Immunostaining showing the localization of the protein mainly in the walls of the atrium and ventricles.

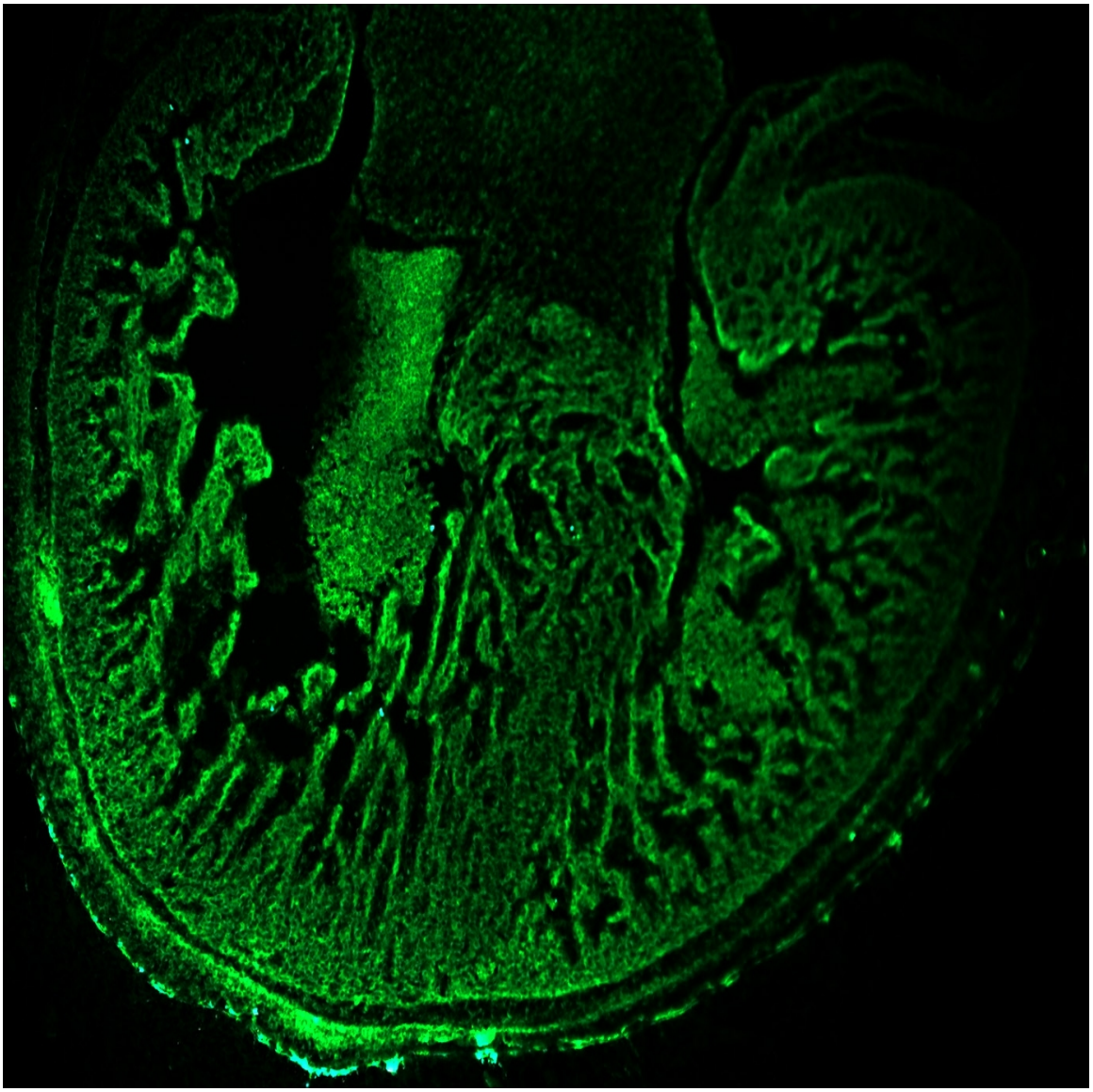

Figure 24. Stage 26 (E) Immunostaining

Partial section of an embryonic heart. Immunostaining showing the localization of the protein mainly in the walls of the atrium and ventricles.

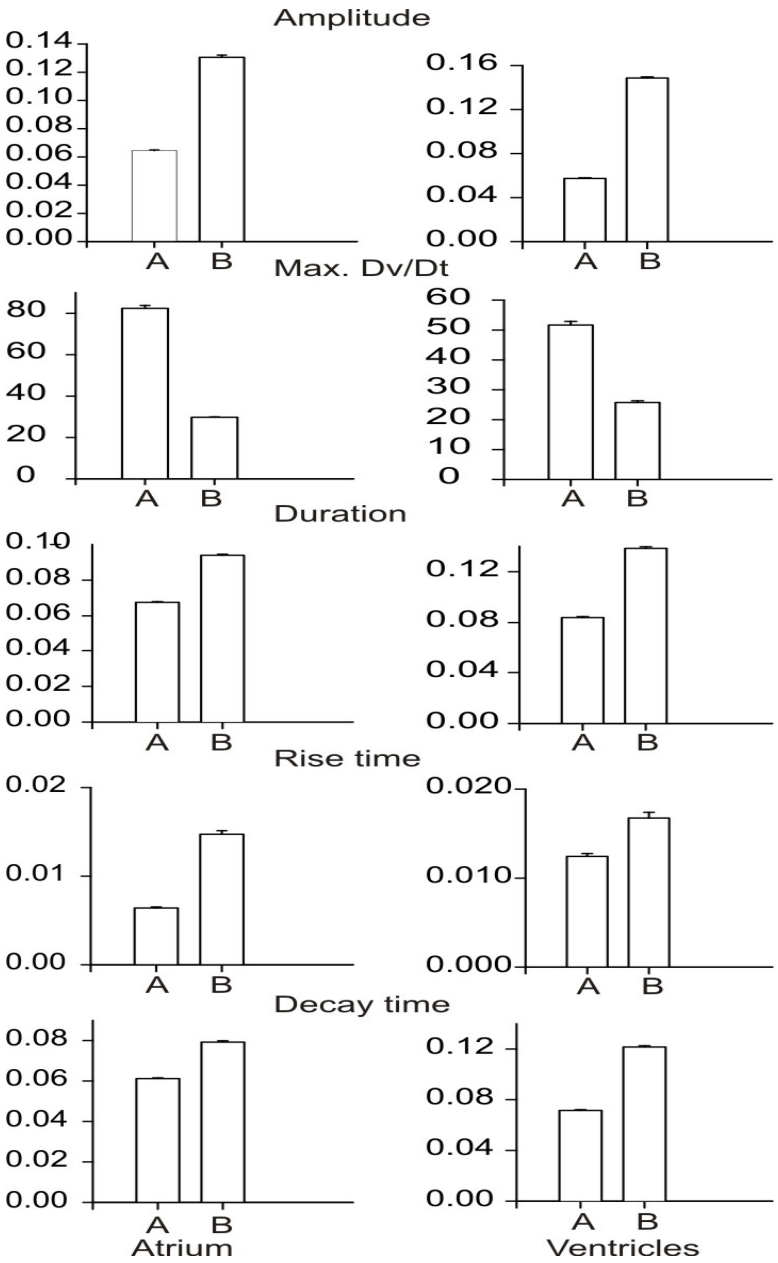

Figure 25. Change in AP parameters after treating with XIP

Effect of shockwave induced XIP on the electrical activity of the heart at stage 26 of development. XIP is a known specific blocker of the exchanger than KB-R7943. We found similar results using XIP as with KB-R7943.

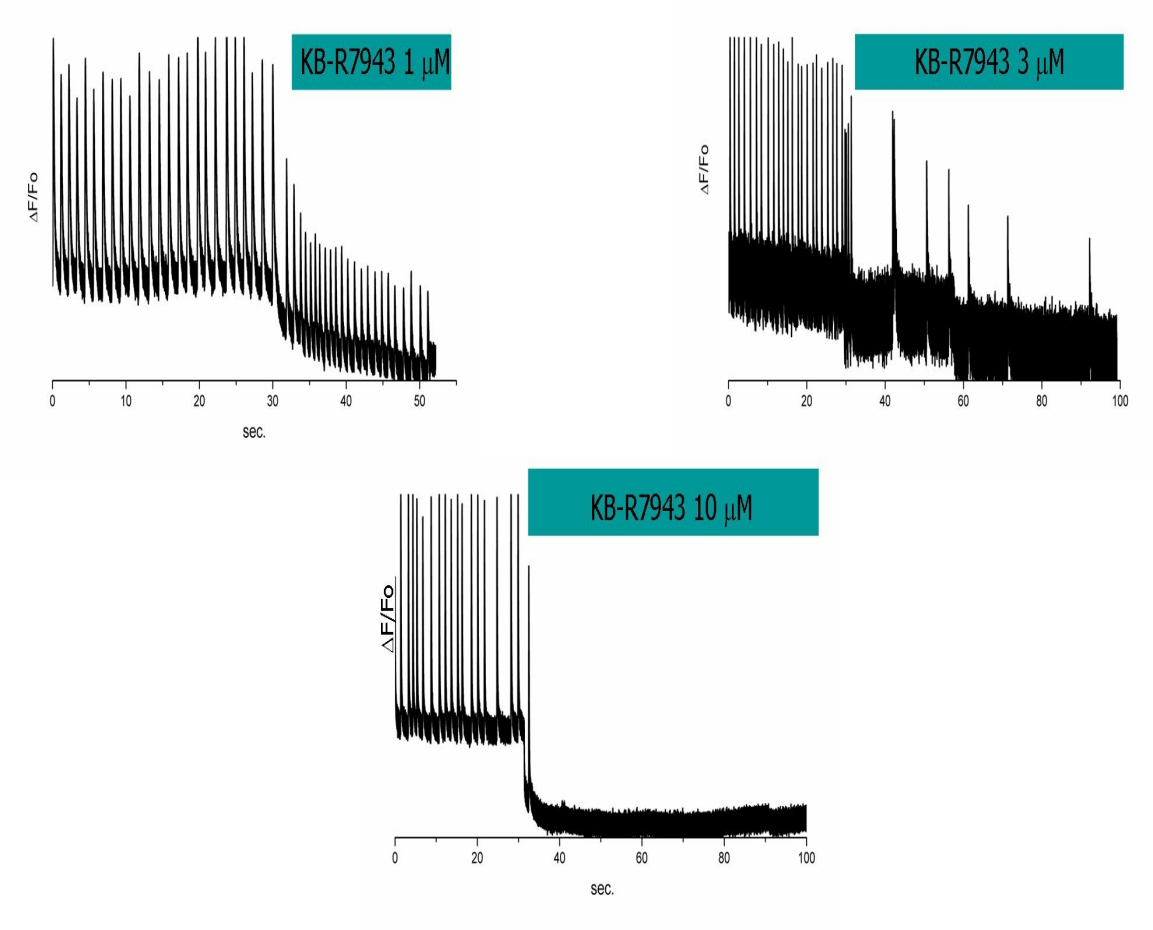

Figure 26. Optical measurement of intracellular calcium after blocking NCX

Effect of blocking NCX on the intracellular calcium in single cells using Flou-4 dye at different concentrations of KB-R7943. Decrease in fluorescent intensity indicates the decrease in intracellular calcium after the blocking of exchanger.

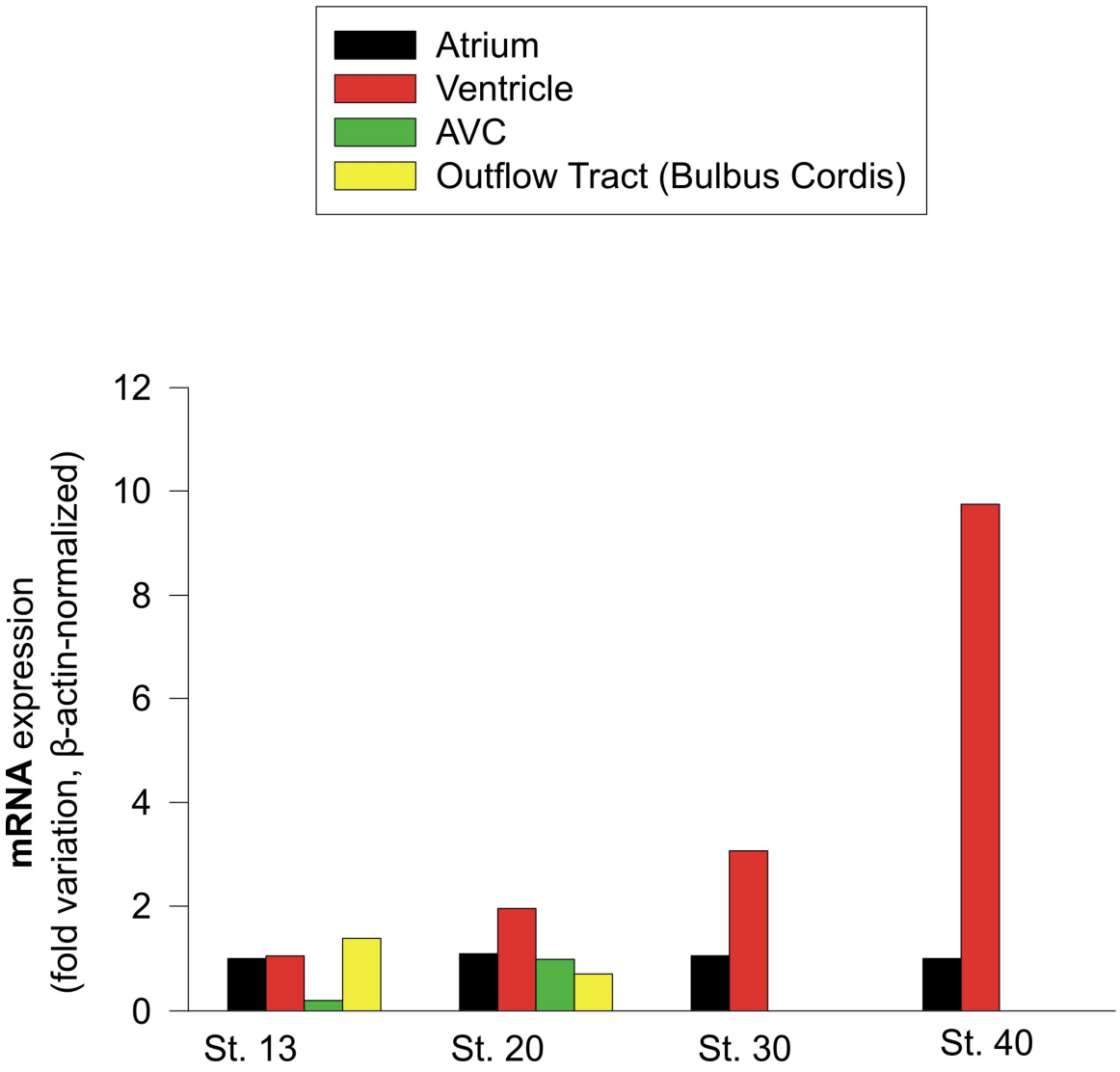

Figure 27. Changes in mRNA levels during development

CHAPTER 4

DISCUSSION

The electrical activity of the heart is critical for embryonic development and it is important to identify the exact mechanism behind it. Different ion channels and membrane transporters are responsible for shaping the electrical activity of the heart and play different roles during development. Also, calcium influx and efflux are very critical under different physiological conditions like ischemia in adult hearts (Lu et al., 2002). Among several known calcium pathways, we identified NCX as one of the most important transporters for maintaining calcium balance in cardiomyocytes. Though it has been identified in adult hearts, its functional role during embryonic development is still unclear. Our results for the first time, demonstrated the functional role of NCX in avian hearts during different stages of development. We also showed that NCX plays an important role in maintaining duration of APs and shaping the electrical activity of heart.

Our results for the first time indicated the importance of NCX in the electrical activity of heart under normal physiological conditions and provide very important supplementary information about NCX and its role in intact hearts. These effects should be discussed in terms of calcium influx through NCX and the role of intracellular calcium on the electrical activity of the heart during development. The role of calcium has already been reviewed in previous chapters and we discuss more details in this chapter. Apart from that, we also found the effect of calcium influx on potassium channels and chlorine channels. This part is particularly important because these channels carry a net positive charge outside to the cell and are important in determining the duration of action

potentials. Also, presence of these channels is still under investigation and not much information is available about its role in development. The importance of our results can also be seen in the light of correlating NCX and these channels for shaping the electrical activity of the heart. Hence, we decided to review this section in discussing the effects of NCX on

1. Calcium influx through NCX
2. Role of NCX in EC coupling
3. Effect of NCX on other channels
4. mRNA expression of NCX in atriums and ventricles
5. Immunostaining of NCX

Before discussing the results for this section, we will review the some of the experimental parameters and selection of particular stages for these experiments. Ideally we should use human heart cells for studying the heart development but it has several ethical and practical problems associated with it. Hence number of different animal models has been used in the past to study heart development. Mouse, chicken and zebra fish are the most popularly studied animal models. Chicken heart has been a well accepted model for studying heart development because of its similarity with human heart development. Listed below down are some of the advantages of using chicken heart as an animal model for studying the role of NCX during development.

- Easy to obtain different stages of development
- Comparatively simpler experimental setup
- Longer biological stability
- Economical

Considering these factors, it was rational to use chicken embryos as our biological model. Also, there are several experimental factors associated with the embryonic development. We need to understand the difference between absolute and relative time for development. Absolute time can be defined as the time when the embryo is developed under steady state conditions and absolute time is the time the embryo actually takes for development. Under ideal conditions these two time frames can be equated with each other and classification can be done as it is done by Hamburger and Hamilton (1992). Apart from this time frame temperature dependence is another concern for all developing animals and constant temperature is important in meeting the absolute and relative time frames (Burggren and Crossley, 2002). In our experiments we maintained a constant temperature of 37.1°C. We observed reduced frequency and changes in action potentials at higher/lower temperatures (data not shown) during our experiments. These conditions were not perfectly met for all experiments and there was some degree of variations. Also it required careful selection of embryos based on visual observation of different physical parameters described in literature. We tried to maintain this accuracy in our experiments but we still feel that the growth may not be uniform in all embryos of the same stage in terms of heart development. Hence, some of the variation observed in amplitude and maximum rate of rise can be attributed to these variations.

As known from the literature review, that the reversal potential for the exchanger can be mathematically expressed as $E_{Na/Ca} = (nE_{Na} - 2E_{Ca})/(n-2)$ (Bers et al., 2003) where n is the coupling ratio of the exchanger. When E_m 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 extrudes calcium through the forward mode for rest of its operation. Most of the studies on exchanger in adult hearts have been focused on its forward mode or the calcium extrusion mode but our results emphasize the importance of reverse mode/calcium entry of the exchanger. This mode has not been given importance in adult hearts because of other well defined calcium influx pathways through voltage gated channels and SR released calcium influx.

In embryonic mouse ventricular myocytes, unlike adult hearts, the sarcoplasmic reticulum is poorly developed and a well defined T-tubule network is absent. Hence, there is lot of dependence on plasma membrane calcium influx to maintain spontaneous activity. Also, part of this influx through L-type calcium channels is not voltage dependent in adult hearts but surprisingly the total calcium currents are strongly voltage dependent in embryonic stages (Schroder et al., 2006). This is possible if L-type calcium channels are voltage dependent during early stages of development and contributes entirely to calcium influx or there exists significant contribution from NCX and other voltage dependent calcium channels during embryonic stages. Researchers have shown that the calcium influx depends on the L-type calcium currents and there is very little dependence on NCX in embryonic ventricular cells derived from rat. Also the T-type channels are not present in early chick ventricles and also blocking of L-type calcium channels completely abolishes the L-type currents (Risso and DeFelice, 1993). Heart beats were not abolished by the blocking of the exchanger at higher concentrations in intact hearts. The drug response graphs show the minimum amount of drug required to produce changes and that was found to be .1 µM and at 10 µM it tends to saturate the response. This is consistent when compared to previous studies (Amran et al., 2004;

Wongcharoen et al., 2006). To further investigate the different effects observed in the atrium and ventricles, we carried out the RT-PCR studies. Surprisingly mRNA levels are equal in both the atrium and ventricles during early stages of development, but it increases rapidly in ventricles and remains almost constant in the atrium in later stages of development. This differential expression level could be one of the possible reasons for different effects in the atrium and ventricles.

Similar to mice, even embryonic chicken ventricles mainly consists of L-type calcium channels and T-type channels do not exist in the 7 day old ventricles (Risso and DeFelice, 1993). The effect of these channels and the calcium influx has been studied through patch clamp technique or by optical measurements. Brotto et al. (1996) have carried out some important experiments to reveal more information about the calcium transients in embryonic chick heart from calcium channels and SR. Their results indicate that calcium transients were abolished completely by the L-type calcium channel blocker but some of the transient were visible with another compound Iso which simulates other calcium influx pathways. Further investigation of cardiac myocytes through patch clamp showed that apart from L-type channel blocking, there was no simulation of T-type current and hence, the calcium current can be due to reverse NCX or through CICR by SR. Our results are consistent with this data and considering the incomplete development of SR during early embryonic stages in cardiac myocytes, NCX can be the only important mechanism for calcium currents along with L-type calcium channels. Our intracellular recordings with reduced extracellular calcium produce similar effects as produced by the blocking of exchanger (data not shown). Hence, we believe that there is a decrease in calcium influx after blocking of the exchanger. Also, all these experiments were

performed on ventricular myocytes. Our results provide information about the changes in the atrium and ventricles during all stages of experiment. Interestingly, the results in the atrium and ventricles also vary during development which is very important and will be discussed in later part of this chapter. We show that NCX can be one of the important Ca^{+2} entry modes during development in intact embryonic hearts and not in isolation only.

In our results, we showed for the first time that NCX is present in as early as stage 21 (Hamburger and Hamilton, 1992) of embryonic chick hearts in both the atrium and ventricles and its effects the electrical activity of the heart during early development. Interestingly, the effects in the atrium and ventricles differ significantly because of blocking of NCX. In the atriums, the increase in duration of APs is minimal during stage 21 and almost doubles at stage 29 with 1µM Kb-R7943. In contrast, ventricles exhibit large changes during stage 21 with not so significant changes during later stages of development. These changes are very significant because of the quantified information they reveal about the overall effect of NCX in the atrium and ventricles during development. We do not know the exact mechanism behind these results and it can be caused by different of mRNA expression levels in the atrium and ventricles. Also, the changes in duration were mainly contributed by changes in decay time of the APs and not so much with the rise time of APs in both the atrium and ventricles. Hence, it is clear that NCX affects the plateau phase of action potentials which is characterized by the calcium entry mode into the cell.

One of the studies with KB-R7943 on adult guinea pig ventricular cells showed a decrease in $I_{Na/Ca}$ at 50mV and -80 mV indicating the non specific effects on forward and

reverse mode of the exchanger. Our experiments carried out under normal physiological conditions are more consistent than these previous findings. Also, the exchanger can be studied under bidirectional conditions instead of unidirectional conditions as in patch clamp (Lu et al., 2002). Our experiments are useful in this regard and also provide more information about the specificity of the exchanger under normal physiological conditions.

The role of calcium influx through SR is also very important for contractile force generated by ventricles and for EC coupling. The contractile force of the pulmonary vein of adult rabbit hearts decreases when treated with the NCX inhibitor (Wongcharoen et al., 2006). SR is poorly developed in embryonic hearts when compared to adult hearts and therefore there is not enough calcium influx through SR for EC coupling in embryonic hearts. NCX may supplement the calcium influx necessary for EC coupling in embryonic heart. Blocking of NCX shows remarkable decrease in spontaneous activity of heart but is not completely abolished even at higher concentrations.

KB-R7943 is capable of blocking voltage gated Na^{+1} channels when administered in high dosage in adult hearts (Watano et al., 1999). We observed that the maximum rate of rise increases during stage 21 and decreases during later stages of development in both the atrium and ventricles. This may be because of sensitivity of voltage gated Na^{+1} channel to KB-R7943 during early stages of development. These effects can be seen with help of our results simultaneously instead of observing in isolation through patch clamp techniques and therefore are physiologically more relevant. The increase in duration can also be attributed to calcium activated potassium channels. Apart from well documented diversity of voltage gated potassium channels there is very little information available about calcium activated potassium channels. It has been

shown that these channels are present in cardiac myocytes and are expressed differentially in the atrium and ventricles and these channels have remarkable effect on membrane repolarization (Xu et al., 2003). This current is important in membrane repolarization. Xu et al. (2003) also shows that these channels depend on intracellular calcium for its activation and produce a long lasting hyperpolrization Xu et al. (2003) used apamin to block calcium activated potassium channels and found an increase in the duration of action potential recorded through patch clamp. Also, the changes were more in the atrium than the ventricles. We also found the increase in duration for all stages of in atrium and ventricles during the hyperpolrization phase. This may be because of two different possibilities. The NCX is responsible for blocking the calcium influx and thereby allowing more time for calcium activated potassium channel to activate or the KB-R7943 used to block NCX may not be a specific blocker and it also blocks these potassium channels. Also, the different effects in atrium and ventricles can also be explained based in the differential distribution of these channels in the atrium and ventricles. These results are very important for investigating the presence of these channels during embryonic development further.

Apart from normal heart development, NCX is also important in case of heart failures, ischaemia induced arrhythmias and arrhythmogenesis during myocardial reperfusion (Pogwizd, 2003; Reppel et al., 2007). In such cases, NCX mediates calcium influx through its reverse mode and triggers SR for uncontrolled calcium overload. Also the late phase of action potential can be disturbed by aberrant excitation and arrhythmias. This phase is mainly contributed by calcium activated potassium channels (Xu et al., 2003). As discussed before, that NCX blocker may be able to modulate these channels

and can act as antiarrythmic agents. Also, since we saw different effects in the atrium and ventricles, it is also possible to directly modify atrium action potentials with no effects on ventricular APs. 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. Our results strengthen the claim of using NCX inhibitors as potential antiarrhythmic agents. In summary, we have shown the functional role of NCX during development of avian heart. These results clearly indicate that NCX is an important calcium influx pathway along with voltage gated calcium channels

Apart from discussing the physiological significance of our results, we would also like to discuss some of the experimental problems encountered during the completion of this project. We performed these experiments initially in normal daylight and found similar effects with a lot of inconsistency. This problem was mainly because of the light sensitive nature of KB-R7943. These lose its effects partially when exposed to normal daylight. Also, we did several sets of experiments with single cardiac cells to investigate the effects of NCX on calcium transients. The cells were loaded with fluo-3 dye as mentioned in the materials and methods chapters. We found that dye abolishes spontaneous activity of cells upon exposure to excitation light. These effects were misinterpreted as effects caused by NCX.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

The embryonic development has always been an important process. There have been numerous studies on understanding different aspects of development. Heart, brain and all other vital organs constitute a large part of these studies and normal development of these organs is very important. Cardiac physiology consists of different factors associated with normal development and synchronizes itself during development. We identify NCX as one of the many factors effecting the normal development of the embryonic heart. Our studies were based on the motivation to understand the role of this exchanger which is relatively less studied in embryonic hearts and contribute to the existing knowledge base about this exchanger.

These results can be concluded in terms of presence and physiological significance of NCX. We have identified the presence of NCX in as early as stage 21 of development in both the atrium and ventricles. The PCR studies conducted show the expression of mRNA levels in the atrium and ventricles during different stages of development. The intracellular recordings are used for the first time to provide experimental evidence regarding the effects of NCX on the electrical activity of heart. We are able to identify that NCX is very important in deciding the total duration of action potential and specifically affects decay time in both the atrium and ventricle. Though effects were seen in the atrium and ventricles, they differ completely during young and older stages of development. This is very interesting and important because it shows that the exchanger not only plays active role during development but also affects the atrium

and ventricles differently. Most of the previous studies with embryonic cardiac cells used ventricular myocytes as mentioned in previous sections and hence, our results help in understanding the role of exchanger in the atrium as well. Considering the fact that NCX affects the plateau phase of action potentials, we found that the reverse mode of the exchanger is very important for calcium entry during early development. In adult cardiomyocytes, NCX is primarily used as a calcium extrusion mode or the forward mode. Our work is important in establishing the importance of the reverse mode of the exchanger during development for calcium influx and setting up EC coupling and generation of contractile forces. Apart from its physiological influence on cardiac development, NCX can also be potentially treated as a therapeutic target and the drugs blocking this exchanger can be used as therapeutic agents. We used KB-R7943 as an NCX blocker which blocks the reverse mode of the exchanger. KB-R7943 is not a very specific blocker for the exchanger and we emphasize the need to develop more potent and specific blockers for advanced studies. Though we have identified its effects on the electrical activity generated by heart, we could not identify the exact mechanism for its activation or inactivation. Also, we could not quantify the amount of calcium carried in and out of the cell in intact hearts. It will also be interesting to investigate the effect of the exchanger on contractile forces generated by developing cardiomyocytes after blocking of the exchanger.

In conclusion, we found that NCX is an important mechanism for calcium transport in developing cardiomyocytes and EC coupling. It is present during the very early stages of development and affects the atrium and ventricles in different proportions.

More studies are needed to identify the exact mechanism for its activation and inactivation in both the atrium and ventricles.

The recordings of action potential using AFM agree consistently with our recordings made through electrophysiological. Hence, it is reasonable to believe that this technique is less probable of producing any external artifacts. The main outcome for this project will be to analyze the effects of NCX on the force generated by single atrial and ventricular cells. We believe that we should be able to quantify the smallest force generated by single cardiac cells for the first time using AFM. Also, we will be able to see the difference in the forces generated by atrium and ventricular cells during different stages of development. This can provide deeper insight into many mechanisms underlying the development. Based on our previous work using intracellular recording with NCX, we believe to see a decrease in force generated by cardiac cells because of less influx through NCX. This will help us understand the effects of NCX in embryonic development in greater details.

In this project we plan to study the effects of NCX on the contractile force generated by the atrium and ventricles by using AFM. AFM is basically used for imaging surfaces at extremely high resolution by scanning the surface. The imaging is based on the interaction between the probe and sample surface. This basic principle can also be used to measure the force generated by single cells. So far, this technique has been primarily used for hard surface imaging. Due to recent advancements in AFM technology and commercially available instrumentation, it can be used to study the force generated by single cells in aqueous medium. Hence, we propose to perform this analysis on single cells before and after blocking of the exchanger with its specific inhibitor KB-R7943. We

also plan to extend this study on different stages of development and using different concentrations of the NCX inhibitor. This will provide deeper insights into the exact role played by NCX in contractile forces generated by cardiac cells.

REFERENCES

Amran MS, Hashimoto K, Homma N. 2004. Effects of sodium-calcium exchange inhibitors, KB-R7943 and SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in vitro, and in computer simulation studies. J Pharmacol Exp Ther 310:83-9.

Arguello C, Alanis J, Pantoja O, Valenzuela B. 1986. Electrophysiological and ultrastructural study of the atrioventricular canal during the development of the chick embryo. J Mol Cell Cardiol 18:499-510.

Arguello C, Alanis J, Valenzuela B. 1988. The early development of the atrioventricular node and bundle of His in the embryonic chick heart. An electrophysiological and morphological study. Development 102:623-37.

Bers DM, Barry WH, Despa S. 2003. Intracellular Na+ regulation in cardiac myocytes. Cardiovasc Res 57:897-912.

Blaustein MP, Lederer WJ. 1999. Sodium/calcium exchange: its physiological implications. Physiol Rev 79:763-854.

Burggren W, Crossley DA, 2nd. 2002. Comparative cardiovascular development: improving the conceptual framework. Comp Biochem Physiol A Mol Integr Physiol 132:661-74.

Davies MP, An RH, Doevendans P, Kubalak S, Chien KR, Kass RS. 1996. Developmental changes in ionic channel activity in the embryonic murine heart. Circ Res 78:15-25.

Hamburger V, Hamilton HL. 1992. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn 195:231-72.

Hilge M, Aelen J, Perrakis A, Vuister GW. 2007. Structural basis for Ca2+ regulation in the Na+/Ca2+ exchanger. Ann N Y Acad Sci 1099:7-15.

Kitchens SA, Burch J, Creazzo TL. 2003. T-type Ca2+ current contribution to Ca2+-induced Ca2+ release in developing myocardium. J Mol Cell Cardiol 35:515-23.

Liu W, Kenji Y, Tobias O, Ryoji I, Jong-Kook L, Kaichiro K. 2002. Developmental changes of Ca(2+) handling in mouse ventricular cells from early embryo to adulthood. Life Sciences 71:1279-92.

Lu J, Liang Y, Wang X. 2002. Amiloride and KB-R7943 in outward Na+ /Ca2+ exchange current in guinea pig ventricular myocytes. J Cardiovasc Pharmacol 40:106-11.

Pogwizd SM. 2003. Clinical potential of sodium-calcium exchanger inhibitors as antiarrhythmic agents. Drugs 63:439-52.

Reppel M, Sasse P, Malan D, Nguemo F, Reuter H, Bloch W, Hescheler J, Fleischmann BK. 2007. Functional expression of the Na+/Ca2+ exchanger in the embryonic mouse heart. J Mol Cell Cardiol 42:121-32.

Reuter H, Pott C, Goldhaber JI, Henderson SA, Philipson KD, Schwinger RH. 2005. Na(+)--Ca2+ exchange in the regulation of cardiac excitation-contraction coupling. Cardiovasc Res 67:198-207.

Risso S, DeFelice LJ. 1993. Ca channel kinetics during the spontaneous heart beat in embryonic chick ventricle cells. Biophys J 65:1006-18.

Schroder EA, Wei Y, Satin J. 2006. The developing cardiac myocyte: maturation of excitability and excitation-contraction coupling. Ann N Y Acad Sci 1080:63-75.

Sperelakis N, Pappano AJ. 1983. Physiology and pharmacology of developing heart cells. Pharmacol Ther 22:1-39.

Takeshima H. 2002. Intracellular Ca2+ store in embryonic cardiac myocytes. Frontiers in Bioscience 7:D1642.

Tohse N, Seki S, Kobayashi T, Tsutsuura M, Nagashima M, Yamada Y. 2004. Development of excitation-contraction coupling in cardiomyocytes. Jpn J Physiol 54:1-6.

Watano T, Harada Y, Harada K, Nishimura N. 1999. Effect of Na+/Ca2+ exchange inhibitor, KB-R7943 on ouabain-induced arrhythmias in guinea-pigs. Br J Pharmacol 127:1846-50.

Wongcharoen W, Chen YC, Chen YJ, Chang CM, Yeh HI, Lin CI, Chen SA. 2006. Effects of a Na+/Ca2+ exchanger inhibitor on pulmonary vein electrical activity and ouabain-induced arrhythmogenicity. Cardiovasc Res 70:497-508.

Xu Y, Tuteja D, Zhang Z, Xu D, Zhang Y, Rodriguez J, Nie L, Tuxson HR, Young JN, Glatter KA, Vazquez AE, Yamoah EN, Chiamvimonvat N. 2003. Molecular identification and functional roles of a Ca(2+)-activated K+ channel in human and mouse hearts. J Biol Chem 278:49085-94.