Changes in regulation of sodium/calcium exchanger of avian ventricular heart cells during embryonic development

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Shepherd N, Graham V, Trevedi B, Creazzo TL. Changes in regulation of sodium/calcium exchanger of avian ventricular heart cells during embryonic development. Am J Physiol Cell Physiol 292: C1942–C1950, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00564.2006.—It has been suggested that the sodium/calcium exchanger NCX1 may have a more important physiological role in embryonic and neonatal hearts than in adult hearts. However, in chick heart sarcolemmal vesicles, sodium-dependent calcium transport is reported to be small and, moreover, to be 3–12 times smaller in hearts at embryonic day (ED) 4–5 than at ED18, the opposite of what would be expected of a transporter that is more important in early development. To better assess the role of NCX1 in calcium regulation in the chick embryonic heart, we measured the activity of NCX1 in chick embryonic hearts as extracellular calcium-activated exchanger current (I_{NCX}) under controlled ionic conditions. With intracellular calcium concentration ([Ca^{2+}]_i) = 47 nM, I_{NCX} density increased from 1.34 ± 0.28 pA/pF at ED2 to 3.22 ± 0.55 pA/pF at ED11 (P = 0.006); however, with [Ca^{2+}]_i = 481 nM, the increase was small and statistically insignificant, from 4.54 ± 0.77 to 5.88 ± 0.73 pA/pF (P = 0.20, membrane potential = 0 mV, extracellular calcium concentration = 2 mM). Plots of I_{NCX} density against [Ca^{2+}]_i were well fitted by the Michaelis-Menton equation and extrapolated to identical maximal currents for ED2 and ED11 cells (extracellular calcium concentration = 1, 2, or 4 mM). Thus the increase in I_{NCX} at low [Ca^{2+}]_i appeared to reflect a developmental change in allosteric regulation of the exchanger by extracellular calcium rather than an increase in the membrane density of NCX1. Supporting this conclusion, RT-PCR demonstrated little change in the amount of mRNA encoding NCX1 expression from ED2 through ED18.

NCX1; chick embryo; allosteric regulation; sodium/calcium exchange current

THE CARDIAC SODIUM/CALCIUM exchanger (NCX1) transports three or possibly four sodium ions (33) across the sarcolemma in exchange for a single calcium ion. The main role of NCX1 in adult mammalian heart muscle is thought to be long-term maintenance of the low diastolic intracellular calcium concentration ([Ca^{2+}]_i; ∼100 nM) (4–6). Hence, the removal of calcium from the cell is called the forward mode, whereas the movement of calcium into the cell is called the reverse mode. Transport by the exchanger is allosterically regulated by [Ca^{2+}]_i, independently of the direction and magnitude of the transport. There have been few studies of the role of NCX1 in embryonic heart, and those have been mainly with mouse heart, in which NCX1 has a much smaller role in excitation-contraction coupling in both adult heart (35) and embryonic heart (29, 32, 39) than in other mammalian hearts. NCX1 knockout mouse embryos die early in gestation, and the heart never beats spontaneously; however, cells isolated from the knockout heart can be electrically stimulated and produce intracellular calcium transients that differ little from those of wild-type cells (29, 40). Targeted deletion of NCX1 in the adult ventricle has little short-term effect on the physiology of the unstressed mouse heart (35). On the other hand, the exchanger is apparently more abundant in neonatal rabbit heart and has a more important role in excitation-contraction coupling than it does in the adult heart (1, 10, 15, 17, 49). These observations are consistent with the suggestions, as yet controversial, that 1) the exchanger may have a more important physiological role in embryonic and neonatal hearts than in adult heart (1) and 2) NCX1 plays an important role in pacemaking (8), particularly in embryonic heart (50).

Electrogenic sodium/calcium exchange was demonstrated in chick embryonic heart cells soon after its description in mammalian cells (26). This form of transport has been linked to the protein encoded by NCX1 in a variety of tissues by numerous lines of evidence (4). In the chick heart, NCX1 has been shown to be important for initial development and to have a marked effect on the heart rate of stage 12 embryos (∼45 h postfertilization) (18, 31). However, in sarcolemmal vesicles from chick heart, sodium-dependent calcium transport is reported to be very small and to increase by up to 12-fold between embryonic day (ED) 4 and ED18 of incubation (45), the opposite of what might be expected of a transporter that becomes less important as development proceeds.

To clarify the role of NCX1 in the embryonic chick heart, we made a quantitative determination of the reverse exchanger current in enzymatically isolated cardiomyocytes at four stages of development of the chick embryo: ED2, ED5, ED11, and ED18 (approximately Hamburger-Hamilton stages 14, 26, 37, and 43, respectively) (18). In stage 14 embryos, the heart tubes, lacking clear regional differences, were excised whole and digested, and only the ventricles of older embryos were used. We find that the maximum current density [i.e., for large [Ca^{2+}]_i, so that allosteric activation (22) is maximized] does not increase significantly with age of the embryo, consistent with there being no change in NCX1 density during development. However, when the current density was submaximal, i.e., when [Ca^{2+}]_i was in the physiological range (100–200 nM), extracellular calcium-activated exchanger current (I_{NCX}) did increase during development but by factors much smaller than found in previous work (36, 45). These results are interpreted to mean that during development there is an increase in the affinity of the allosteric activating site of the exchanger for calcium. The possible structural and physiological correlates of this change are discussed.
METHODS

Cell preparation and culture. Fertilized Arbor Acre chicken eggs (Gold Kist Hatchery, Siler City, NC) were incubated at 37°C and 97% humidity, until they reached the required developmental stage (18). The hearts from 1 ED18 to 10 ED2 embryos were rapidly removed, placed in PBS, and then trimmed of the great vessels and atria (except in ED2 embryos, where the entire heart tube was digested). The ventricles of the ED2 heart tubes were then dissociated with repeated 4- to 8-min exposures to PBS containing trypsin (7.5 U/ml trypsin TL) and/or collagenase (180 U/ml collagenase type II; Worthington), DNase I (11 μg/ml; Worthington), and BSA (fatty-acid free, 1 mg/ml) at 37°C. Cells were cultured overnight, except where trypsin-inhibiting solution containing 1.8 mM calcium (method modified collected from each digestion and diluted into a bicarbonate-buffered, Worthington), and BSA (fatty-acid free, 1 mg/ml) at 37°C. Cells were

Electrophysiological recording and analysis. A drop of cells suspended in medium was placed in the experimental chamber, allowed to settle, and then superfused with chick Ringer solution containing (in mM) 142 NaCl, 4 KCl, 5 HEPES, 1.8 MgCl2, and 1.8 CaCl2, pH 7.4. A spherical cell of uniform appearance, 13 to 20 μm in diameter, was selected and then lifted gently with a large-tipped pipette (from Refs. 12 and 13). The cells were cultured overnight, except where noted, in DeHaan 21212 medium (1.8 mM CaCl2) in a humidified 5% CO2-95% air atmosphere at 37°C.

The extracellular solution was imme-

diately switched to one that was nominally calcium free and contained (in mM) 150 NaCl, 2 BaCl2, 2 CsCl, 0.01 verapamil, 0.05 ouabain, 4 MgCl2, 0 CaCl2, and 5.5 dextrose. This standard extracellular solution prevented interference by potassium and calcium currents (33) and the sodium pump. In some experiments, the solution contained 200 μM niflumic acid or 200 μM DIDS to block chloride conductance (Sigma; diluted from 200 nM stocks in DMSO). In other experiments, we added either 10 mM caffeine or 200 μM tetracaine. All additions to the standard solutions were included in both the control solution and the calcium-containing solutions that were used to activate ICSCX.

Patch pipettes had a resistance between 1.4 and 3.9 MΩ when filled with a solution containing (in mM) 20 NaCl, 20 TEACl, 50 EGTA, 0–43.1 Ca2+, 40 HEPES, 10 MgATP, 3 MgCl2, and 5 Tris2creatinic phosphate and sufficient CsOH to bring pH to 7.4 (~120 for pcH 7.3). Matsuoka and Hilgemann (33) reported severe contractures in whole cell preparations when BAPTA was used to buffer intracellular calcium but not when EGTA was used; hence, we chose to buffer calcium with EGTA. Calculated free calcium concentration in the pipette solution ([Ca2+]pip) was between 0 and 480 nM (WinMAXC, C. Patton, Stanford University). Under steady-state conditions, the myoplasmic calcium concentration, [Ca2+]im, should be identical to [Ca2+]pip. When reverse exchanger current is activated, [Ca2+]im may vary slightly from [Ca2+]pip, and it is possible, although we present evidence to the contrary, that the calcium concentration just inside the cell membrane (subsarcolemmal calcium concentration; [Ca2+]ss) and near the exchanger could be significantly larger than [Ca2+]im. We distinguish among these calcium pools where it seems appropriate, but in general we use [Ca2+]pip to indicate the highly buffered [Ca2+]im in our experiments and [Ca2+]im when discussing more physiological conditions or measured intracellular values.

Reverse exchange current was activated by rapidly switching the perfusate solution from the calcium-free solution (4 mM Mg2+) to one in which 1–4 mM Mg2+ was replaced by Ca2+ (Fig. 1A) (28). Extracellular calcium activation of exchange current is preferable to other

![Fig. 1. Recording of extracellular calcium-activated exchange current (ICSCX) in an isolated embryonic day (ED) 11 ventricular cell. A: for each current-voltage (I-V) relationship, current (top trace, smoothed by 25-point running average) was measured as the membrane potential (Em) was changed in a ramp fashion from the holding potential of -40 to +60 mV at 0.4 V/s and then from +60 to -80 mV at the same rate for 350 ms (middle trace). This voltage pattern was applied 3 times at 6-s intervals, with extracellular calcium concentration ([Ca2+]im) being increased from nominally 0 to 4 mM ~200 ms before the second voltage change (bottom trace, optically recorded motion of solution jets). This entire pattern was repeated at ~30-s intervals with a different [Ca2+]im until each concentration had been tested twice. Calcium concentration in the pipette solution ([Ca2+]pip) = 47 nM, sodium concentration in the pipette solution ([Na+]pip) = 20 mM, membrane capacitance (Cm) = 7.2 ± 0.7 pF, and resistance (Ri) = 3.4 MΩ. B: superimposition of the 3 current records from A, on an expanded time scale and without smoothing. C: I-V relationships from the records in B smoothed by 25-point running average. a: I-V relationship of the control ([Ca2+]im = 0 mM) during both the positive-going (top portion) and the negative-going (bottom portion) voltage ramps. The 2 limbs of the control are parallel, separated by the capacitive current (CmV/dV). b: I-V relationship of the test ([Ca2+]im = 4 mM) during both the positive-going (top portion) and the negative-going (bottom portion) voltage ramps. The 2 limbs of the control are again parallel, but the current is a much steeper function of voltage. b-(a+c)/2: Subtracted I-V relationship, i.e., test current minus the average of the controls, showing that the relationship of the calcium-activated current during the positive-going ramp (a, extracted at 4-mV intervals) is identical to that during the negative-going ramp (line).]
methods of measurement to avoid interference by other ionic currents (23), and rapid exchange of the extracellular solution (half-time of ~40 ms) is essential to permit a very brief activation and thus avoid altering the intracellular ion concentrations (42). A test voltage in the form of a ramp from -40 to +60 mV (positive going) followed immediately by a ramp from +60 to -80 mV (negative going; Fig. 1A) was applied to the cell, and the digitized current was recorded with pCLAMP8 software, a 200B clamp amplifier, and Digidata 1300 board (Axon Instruments, Burlingame, CA). Test voltages were applied at 6-s intervals in groups of three, and calcium was applied just before and during the second test voltage (Fig. 1A). With no extracellular calcium (Fig. 1, A and B, a and c), the currents in the two limbs of the voltage change were parallel, separated by the current due to the cell capacitance (Fig. 1C, line a). A current-voltage (I-V) relation for \( I_{SCX} \) was determined by taking the difference between the calcium-activated current waveform and the average of the currents in the bracketing controls, using only the negative-going portion of the waveform, from +60 to -80 mV, herein referred to as the ramp (Fig. 1C). The difference method for isolating \( I_{SCX} \) depends on there being no other current changes due to the application of extracellular calcium, which is assured in part by the nature of the solutions used, as described above, and in part by having good control of the intracellular ion concentrations, as we document in RESULTS. It should be noted that the difference current actually represents a change in \( I_{SCX} \), since there is an inward (negative) \( I_{SCX} \) under the control conditions due to the inclusion of calcium in the pipette solution and sodium in the extracellular solution (transport in the forward mode). This current is small at positive potentials, and, in any case, \( [Ca^{2+}]_{\text{pip}} \) is never large enough to significantly reduce calcium-activated reverse mode transport by competing with intracellular sodium (34). For simplicity, we call the difference current \( I_{SCX} \) rather than \( \Delta I_{SCX} \).

Current magnitudes were normalized to the cell capacitance for comparison among the different cell groups. Cell capacitance was found by integrating the current during a 3-mV hyperpolarizing pulse, calculating a capacitance, and then averaging four such calculated values.

Measurement of \([Ca^{2+}]_i\) during activation of the exchanger. In some experiments, the intracellular solutions contained 50 \( \mu \)M fura 2 to permit monitoring of \([Ca^{2+}]_i\). Excitation wavelengths were 340 and 380 nm, and the emitted light was passed through a 510-nm filter and amplified by a photomultiplier tube (DeltaRam; PTI, Monmouth Junction, NJ). \([Ca^{2+}]_i\) was calculated from 340 nm-to-380 nm emission ratio (R) by standard methods \([Ca^{2+}]_i = \frac{\beta K_{1/2}}{R - R_{\text{min}}} (R_{\text{max}} - R)^{-1} \) (16), using an in vitro calibration \((R_{\text{min}} = 0.2, R_{\text{max}} = 7, \beta = 9, K_{1/2} = 200 \text{ nM})\) for \([Ca^{2+}]_i\). Changing the pipette solution during a single experiment. In one series of experiments, the \([Ca^{2+}]_\text{pip}\) was varied during the experiment by means of a small tube inside the pipette, the tip of the tube (~50 \( \mu \)m diameter) being ~0.4 mm from the pipette tip and the distal end of the tube connected to a reservoir via a valve (43). In describing the results of such experiments, we use \([Ca^{2+}]_i\) to indicate the measured intracellular calcium concentration.

RT-PCR and cloning of chick \( NCX \). Total RNA was isolated from embryonic chicken heart tissue using the Qiagen RNeasy mini-kit. One-step RT-PCRs were performed with the use of the Invitrogen SuperScript one-step RT-PCR kit with Platinum Taq. For each reaction, 10 ng of total RNA and 0.2 \( \mu \)M of each primer were used. cDNA was synthesized at 50°C for 30 min and then denatured for 2 min at 94°C. Amplification was carried out for 35 cycles with the following protocol: denaturation for 30 s at 94°C, annealing for 30 s at 50–55°C, and extension for 1 min at 72°C. The primers used to clone chick \( NCX \) were designed with predicted chick \( NCX \) sequences from GenBank (accession no. XM_415002) and the Ensembl Genome Browser (Ensembl Transcript ID: ENSGALT0000013920). Six sets of primers were used to amplify six overlapping PCR products and subsequently generate a sequence for the entire coding region of chick \( NCX \) (GenBank accession no. DQ987923). The primers used are shown in Table 1. PCR products were subsequently subcloned into the pGEM-T easy vector (Promega) and transformed into Top 10 \( E. \) coli competent cells (Invitrogen). Clones were then selected using IPTG/X-Gal and grown in LB medium in the presence of 100 \( \mu \)g/ml ampicillin. Plasmid DNA was purified with use of the Promega Wizard plus Miniprep DNA purification system. The presence of specific inserts was checked by using EcoRI endonuclease digestion and further confirmed by DNA sequencing and comparison with previously published predicted sequences.

For semi-quantitative analysis of \( NCX \) expression during embryological development, RT-PCR was performed using primer set 4. RT-PCRs were run as described above. RT-PCRs for \( GAPDH \) were run as a loading control using the following primers: sense primer 5'-GGGAACCTTACCTGGAATGG-3' and anti-sense primer 5'-ACCAGGAAAAACCTTGGACG-3'. These primers give rise to a product of 264 bp corresponding to nucleotides 727-991 of chicken \( GAPDH \) (GenBank accession no. AFO47874).

Presentation of results. The original current records and difference currents in Figs. 1A, 1B, and 2C were smoothed for presentation by 25-point adjacent averaging (Origin). All numerical data are given as means ± SE except where indicated. Statistical differences between groups were calculated by Student’s t-test.

### RESULTS

Isolation and measurement of \( I_{NCX} \) in embryonic chick heart cells. We first show that we can elicit in the chick cells a current that has characteristics described in the literature as corresponding to \( I_{NCX} \) under these experimental conditions. These characteristics include the following: 1) application of extracellular calcium elicits an outward current at all membrane potentials; 2) the current is not time dependent; 3) the I-V relation of the current is approximately exponential; 4) the outward current (carried by sodium ions) requires the presence of intracellular calcium ions (allosteric activation). In our experiments, outward \( I_{NCX} \) current (positive current, reverse mode of the exchanger) was activated by the application of extracellular calcium concentration ([Ca\(^{2+}\)]\( _{lo}\), 1, 2, or 4 mM), with a sodium concentration in the pipette solution of 20 mM and [Ca\(^{2+}\)]\( _{\text{pip}} \) between 0 and 480 nM (47 nM in Fig. 1, ED11 cell). The I-V of the activated current was determined by

<table>
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Table 1. Primers used to clone chick \( NCX \) (GenBank accession no. DQ98723)
applying a voltage ramp 200–250 ms after the start of the solution change (Fig. 1Ab). The same voltage ramp was applied without changing the extracellular solution before (pre-test control, Fig. 1Aa) and after (posttest control, Fig. 1Ac) the application of the calcium-containing solution. The application of 4 mM \([\text{Ca}^{2+}]_o\) (Fig. 1, A and B, first arrow) resulted in the membrane current at the holding potential (holding current) becoming rapidly more positive, achieving a new steady state within 200 ms. The more positive holding current was maintained throughout the application of \([\text{Ca}^{2+}]_o\), returning to the control level only after its removal (Fig. 1, A and B, second arrow). The current measured during the voltage ramps was strongly outward at positive potentials (Fig. 1Cb).

The entire I-V of the calcium-activated current during the positive-going voltage ramp (Fig. 1C) was identical to the I-V during the negative-going ramp (Fig. 1C); i.e., there was no detectable time dependence of the extracellular calcium-activated current at any potential. The absence of time dependence is important because \(I_{\text{NCX}}\) should be a steady-state current at any voltage under constant ionic conditions and, in fact, implies that the ionic conditions have not changed during the test voltage ramps, as discussed below.

The magnitude of the activated current depended strongly on \([\text{Ca}^{2+}]_o\) at all stages of development (Figs. 2 and 4), and the voltage dependence of the current was always approximately exponential. Furthermore, as shown in the lower records in Fig. 2, A and B, the extracellular calcium-activated current in chick embryonic heart cells is allosterically regulated by calcium, consistent with the description of the intact exchanger in other heart cells (2, 3, 22, 27). With \([\text{Ca}^{2+}]_{\text{pip}} = 0 \text{ mM}\), the application of extracellular calcium activated no current at all (Fig. 2, A and B, lower records), showing the strict dependence of reverse-mode exchanger activity on intracellular calcium at both ED2 and ED11. Thus properties of the extracellular calcium-activated current measured in the chick myocyte correspond well with the known properties of \(I_{\text{NCX}}\), and we conclude that we have, in fact, activated \(I_{\text{NCX}}\) in these experiments, although not necessarily only \(I_{\text{NCX}}\).

Although the method of eliciting \(I_{\text{NCX}}\) as extracellular calcium-activated current was adapted from one of the earliest demonstrations of the existence of \(I_{\text{NCX}}\) (28), and it has been argued that this method is the best way to study \(I_{\text{NCX}}\) (23), activation of the exchanger by raising \([\text{Ca}^{2+}]_i\) will result in a large flux of calcium into the cell. Given the relatively large surface-to-volume ratio of the cells used in our studies, a large influx of \(\text{Ca}^{2+}\) could conceivably raise either the \([\text{Ca}^{2+}]_o\) or \([\text{Ca}^{2+}]_i\), or both, despite the large buffering capacity of the intracellular solutions (50 mM EGTA).

A rise in \([\text{Ca}^{2+}]_o\) could, at least conceptually, affect \(I_{\text{NCX}}\) in several ways. It could, for example, activate other currents, the most likely being chloride channels (44, 51). Alternatively, if incoming calcium were to accumulate under the membrane or to release calcium from intracellular stores, the measured current would become more inward at the holding potential because of both increased transport and increased allosteric activation. Outward current would increase because of increased allosteric activation (48), although this might be balanced by the shift in transport. It was to preclude all such possibilities that we carefully examined the time and voltage dependence of current in each experiment to look for signs of intracellular concentration changes, e.g., the I-V relation crossing the voltage axis at negative potential. We also elicited current with three concentrations of extracellular calcium to look for signs of systematic changes in the shape of the I-V and \([\text{Ca}^{2+}]_o\) dependence of current that correlated with the current magnitude.

The finding that there was no time dependence of the extracellular calcium-activated current is in itself a strong argument against a change in \([\text{Ca}^{2+}]_o\) during the measurement period. Any such change would lead to a time-dependent alteration of \(I_{\text{NCX}}\), particularly the current at the holding potential, contrary to our observations (Fig. 1). It is highly unlikely that there could be canceling effects on multiple conductances under all experimental conditions. We conclude that activation of the exchanger current by extracellular calcium, under our experimental conditions, does not increase \([\text{Ca}^{2+}]_i\) to a degree that would alter the measured exchanger current.

To support this conclusion, we compared our measured currents to the meticulously compiled properties of the exchanger in guinea pig ventricular cells (34). Matsuoka and Hilgemann studied the properties of the deregulated exchanger under a wide variety of ionic conditions, using giant patches of membrane from guinea pig ventricular cells and chloride-free

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**Fig. 2.** I-V relations of \([\text{Ca}^{2+}]_o\)-activated current in ED2 and ED11 cells. **A.** Broken lines at top: mean of subtracted I-V relationships for the indicated \([\text{Ca}^{2+}]_i\), from 4 experiments such as those depicted in Fig. 1 (ED2, \([\text{Ca}^{2+}]_o\) = 47 nM). Smooth lines represent theoretical relationships calculated from the model of Matsuoka and Hilgemann (34). The calculated \(I_{\text{NCX}}\) for all \([\text{Ca}^{2+}]_i\), were scaled by the factor that was needed to match the model curve to the data for 0 mV and \([\text{Ca}^{2+}]_o = 1 \text{ mM}\) (scale factor = 0.14). **B.** Bottom: mean of subtracted I-V relationships for various \([\text{Ca}^{2+}]_o\) (n = 2; \([\text{Ca}^{2+}]_o = 0 \text{ mM}, 50 \text{ mM EGTA}, \text{no added calcium})\). **B.** Top: as in A, but for cells from ED11 embryos (n = 11; \([\text{Ca}^{2+}]_o = 47 \text{ nM}\)). Scale factor needed to match the model curve to the data for 0 mV and \([\text{Ca}^{2+}]_o = 1 \text{ mM}\) is 0.32. **B.** Bottom: mean of subtracted I-V relationships for various \([\text{Ca}^{2+}]_o\) (n = 3; \([\text{Ca}^{2+}]_o = 0 \text{ mM}, 50 \text{ mM EGTA}, \text{no added calcium})\).
solutions, summarizing their data in mathematical form (34). Using the mathematical model (APPENDIX), we calculated I-V curves for intracellular sodium concentration ([Na\(^+\)]\(_i\)) = 20 mM, [Ca\(^{2+}\)]\(_i\) = 47 nM, extracellular sodium concentration = 150 mM, and [Ca\(^{2+}\)]\(_o\) = 0, 1, 2, or 4 mM and then took the difference currents as we did experimentally (Fig. 2, A and B, smooth lines). The model I-V relations were scaled for each day’s data set (ED2 or ED11) by matching the I-V for 1 mM [Ca\(^{2+}\)]\(_i\), and using that scaling factor for the other two [Ca\(^{2+}\)]\(_i\). The scaling factor is necessary both because the model equations were not scaled for membrane area and because the patch preparation was deregulated with respect to [Ca\(^{2+}\)]\(_i\), thereby maximizing the current for any given set of conditions (20). The voltage dependence of the measured current is well described by the patch model (Fig. 2). The [Ca\(^{2+}\)]\(_i\) dependence of the current at 0 mV was also well described by the patch model (Fig. 2), and we show below that this is true throughout development from ED2 to ED18 (see Fig. 4). If our data were affected by an increase in [Ca\(^{2+}\)]\(_o\) this is true throughout development from ED2 to ED18 (see Fig. 2). If our data were affected by an increase in [Ca\(^{2+}\)]\(_o\), it was possible that myoplasmic calcium, [Ca\(^{2+}\)]\(_i\), might rise because of calcium influx during the [Ca\(^{2+}\)]\(_o\)-activated currents. The change in [Ca\(^{2+}\)]\(_i\) could conceivably alter subsequent currents (48), and this would affect the measured calcium dependence of the [Ca\(^{2+}\)]\(_o\)-activated current. Although the strong intracellular calcium buffering in our experiments makes this particular kind of self-regulation of the exchanger unlikely, such an effect has been observed in experiments with unbuffered intracellular solutions in Ferret heart cells (48).

To test this idea, we measured [Ca\(^{2+}\)]\(_i\) as it was continuously varied over a range from 0 to 480 nM while activating $I_{\text{SCX}}$ with 4 mM [Ca\(^{2+}\)]\(_o\), at ~30-s intervals (Fig. 3). This was done by rupturing the cell patch with a pipette solution containing no calcium, then changing the pipette solution to one containing 480 nM free calcium, and finally returning to a pipette solution containing no calcium, with all pipette solutions containing 50 μM fura 2 (see METHODS). In this experiment and two similar experiments, [Ca\(^{2+}\)]\(_o\) dropped rapidly from its resting value [100–200 nM (11)] to the pipette value (0 nM; 6 s) on rupture of the membrane, reflecting the ease of dialyzing these small cells (Fig. 3A). Once started, ~200 s were needed for the 480 nM Ca\(^{2+}\) to reach the cell. The delay was unavoidable because both the delay and the rate of change of [Ca\(^{2+}\)] were dependent on the distance of the calcium source (i.e., the tip of the small tube within the pipette) to the cell, and the rate of change of [Ca\(^{2+}\)] had to be slow enough that [Ca\(^{2+}\)] was nearly constant during the three ramps used to define each activation of exchange current (protocol as in Fig. 1A). Activation of the exchanger by 4 mM [Ca\(^{2+}\)]\(_o\) raised [Ca\(^{2+}\)]\(_i\) by ~30 nM when the control [Ca\(^{2+}\)]\(_o\) was 450 nM and ~10 nM when control [Ca\(^{2+}\)] was
200 nM (Fig. 3B, time points a and b), consistent with the smaller \( I_{\text{NCX}} \) observed at the lower \([\text{Ca}^{2+}]_i\) (Fig. 3C). For a control \([\text{Ca}^{2+}]_o\), smaller than 200 nM, the activation of \( I_{\text{NCX}} \) did not produce a measurable change in \([\text{Ca}^{2+}]_i\). Thus the rise in \([\text{Ca}^{2+}]_i\) was small relative to the initial \([\text{Ca}^{2+}]_i\); and had no effect on the magnitude of the current at any potential, indicating that calcium sensed by the exchanger was not significantly affected by the calcium influx due to activation of the exchanger.

Thus we conclude that the \([\text{Ca}^{2+}]_o\)-activated current is entirely and accurately \( I_{\text{NCX}} \) under our experimental conditions. This allows us to make a quantitative study of developmental changes in the exchange current.

**Age dependence of \( I_{\text{NCX}} \).** Although we did find, in the initial experiments described above (Figs. 1 and 2), an increase in \( I_{\text{NCX}} \) with embryonic age, we did not find the steep increase in exchanger activity with age that has been reported in other preparations (Fig. 4). The increase in \( I_{\text{NCX}} \) between ED2 and ED11 was only 2.4-fold, with little further increase by ED18. This is much smaller than changes reported for sodium-dependent calcium transport in vesicles from chick embryonic heart (6- and 2-fold, respectively, estimated from Fig. 3 in Ref. 45) but similar to the developmental changes in radiocalcium transport by whole heart (36).

Because all of our initial experiments were made with \([\text{Ca}^{2+}]_\text{pip}\) = 47 nM, it seemed possible that the intracellular calcium-dependent allosteric regulation of the exchanger (22) changed with embryonic development such that more \([\text{Ca}^{2+}]_i\), was needed to activate the exchanger in the older embryos. That is, it was possible that the amount of exchanger actually increased substantially during development as previously reported, but the increase was partly masked by a decreased allosteric activation by intracellular calcium. Therefore, we studied the regulation of \( I_{\text{NCX}} \) by intracellular calcium at two stages of development: ED2 and ED11.

**Regulation of \( I_{\text{NCX}} \) by \([\text{Ca}^{2+}]_o\) in ED2 and ED11 cells.** To study regulation of the exchanger by \([\text{Ca}^{2+}]_\text{pip}\), we mixed pipette solutions containing 47 nM with ones containing 480 nM calcium in amounts appropriate to give 84 and 200 nM. These, plus a calcium-free pipette solution, were adequate to extrapolate maximal current values of the exchange current in \( I_{\text{NCX}} \), which is the major current in this voltage range (Fig. 5). For the ED2 cells, \( K_{1/2} \) for \([\text{Ca}^{2+}]_i\) was 188 nM (mean of the values for \([\text{Ca}^{2+}]_i\) = 1, 2, and 4 mM), whereas for ED11 the mean value was 46 nM (Fig. 5). The difference in \( K_{1/2} \) was very similar at all \([\text{Ca}^{2+}]_o\); i.e., it was not dependent on the magnitude of the current and so could not be ascribed, for example, to changes in the \([\text{Ca}^{2+}]_o\) due to larger currents in the ED11 cells. The extrapolated maximal current values for ED11 at all \([\text{Ca}^{2+}]_o\) were identical, or nearly so, to those for ED2. The magnitudes of the extrapolated maximal current values of the exchange current in chick cells (Fig. 5) are very similar to values calculated from the model given in the Appendix (4.0, 6.4, and 9.0 pA/pF for \([\text{Ca}^{2+}]_o\) = 1, 2, and 4 mM, respectively). However, more importantly, this same model gives a maximal outward current, i.e., with \([\text{Na}^{+}]_i\) = 300 mM and \([\text{Ca}^{2+}]_o\) = 10 mM, of \( \approx 27 \) pA/pF, in the middle of the range of values for the giant patches from guinea pig ventricular cells, 20–30 pA/pF (24).

Our primary conclusion is that the density of NCX1 in the sarcolemma of embryonic chick heart cells does not change appreciably between ED2 and ED18, contrary to previous conclusions drawn on the basis of transport studies (36, 45). Second, the increase in exchanger current that we observed between ED2 and ED11 in the physiological range of \([\text{Ca}^{2+}]_\text{pip}\) (95% increase at 84 nM and 43% increase at 200 nM) was due to a developmental change in the affinity for intracellular calcium of the allosteric regulatory site of the exchanger that does not involve a change in the primary structure of NCX1. These conclusions are based on electrophysiological measure-
ments of $I_{\text{NCX}}$, and our RT-PCR data are consistent with those conclusions.

**Constant ionic conditions.** Even if the calcium-activated current were purely $I_{\text{NCX}}$, a change in $[\text{Ca}^{2+}]$ or $[\text{Na}^{+}]$ during the acquisition of an $I-V$ relation could still affect the magnitude of the exchanger current. However, the calculated amount of sodium moved during a single activation would have a negligible effect on $[\text{Na}^{+}]_i$, and we have shown that when the exchanger is activated $[\text{Ca}^{2+}]_i$ does not rise to an extent that could affect the relationship between current and $[\text{Ca}^{2+}]_{\text{pip}}$ (Fig. 3). Furthermore, because the $I-V$ relation of the $[\text{Ca}^{2+}]_{\text{ss}}$-activated current was time independent (Fig. 1C), any change in ionic conditions near the membrane that affected the exchanger or activated a second current must be an instantaneous function of voltage. Such an effect of $[\text{Ca}^{2+}]_{\text{ss}}$ on the exchanger would be indistinguishable from an intrinsic property of the exchanger in our experiments. The steep slope of the measured $I$-$V$ curves at positive potentials could conceivably be due to such an effect. However, instantaneous self-activation by $[\text{Ca}^{2+}]_{\text{ss}}$ cannot be a large effect or it would not be possible to see the effects of $[\text{Ca}^{2+}]_{\text{pip}}$ on allosteric activation; i.e., a large change in $[\text{Ca}^{2+}]_{\text{ss}}$ would swamp $[\text{Ca}^{2+}]_{\text{pip}}$. Indeed, Weber et al. (48) found an increase in exchanger allosteric activation due to calcium influx via the exchanger itself, but the increase could be accounted for by the observed cumulative increase in $[\text{Ca}^{2+}]_i$ rather than an instantaneous increase in $[\text{Ca}^{2+}]_{\text{ss}}$. Hence, in our experiments, in which the intracellular solutions are strongly calcium buffered, rapid self-activation is implausible.

On the other hand, we cannot positively exclude the activation of a conductance other than $I_{\text{NCX}}$ if that conductance were an instantaneous function of the $[\text{Ca}^{2+}]_{\text{ss}}$, which would in turn be an instantaneous function of $I_{\text{NCX}}$ and hence of voltage. However, the failure to block any such current with chloride channel blockers, and the good match between the calcium dependence of $I_{\text{NCX}}$ in the chick cells and the chloride-free giant patch preparation, are consistent with our conclusion that we have measured $I_{\text{NCX}}$ in the absence of interfering effects of other currents or changes in intracellular ionic concentrations.

**Comparison with previous results.** The currents that we measured were much larger than expected from an extrapolation of the results of the vesicle study (45). The sodium-dependent calcium flux at ED4 in the sarcolemmal vesicles of chick heart was $\sim 1 \mu g \cdot kg^{-1} \cdot s^{-1}$ (45), corresponding to $2.5 \mu mol \cdot l^{-1} \cdot cytosol^{-1} \cdot s^{-1}$ (14). For an embryonic chick cell with a volume of $\sim 1.8 \mu l$ (11)] and a surface area of 7.1 pF (707 $\mu m^2$, corresponding to a spherical cell of 15 $\mu m$ diameter), this is equivalent to $2.5 \mu mol \cdot l^{-1} \cdot s^{-1} \times 10^9$ Coul/mol $\times 10^{-12}$ liters cytosol = 0.25 pA, i.e., a current density of $\sim 0.035$ pA/pF . This is extremely small compared with the currents that we measured in intact cells and also much smaller than current densities calculated from the model equations for the ionic conditions of the flux study, i.e., $\sim 2$ pA/pF. Currents equivalent to the measured fluxes (calculated as above) are in Fig. 4, inset (bottom line). It is not obvious why the sodium-dependent calcium transport in the vesicles was so small nor why transport increased so steeply with development. Because neither the sidedness nor the initial $[\text{Na}^+]$ of the vesicles is known, it would not be useful to speculate on this point.

On the other hand, a study of sodium-dependent calcium flux in isolated embryonic ventricles found an increase in flux of two- to threefold between ED5 and ED18 (36), similar to what we found for $I_{\text{NCX}}$ in isolated cells for physiological levels of $[\text{Ca}^{2+}]$. Likewise, our data are consistent with protein expression studies in mouse heart that showed a decline of membrane NCX1 of 40% between days post coitum 9.5 and 18 days post coitum (32), i.e., a relatively stable level throughout development.

**Allosteric regulation.** The apparent change in the $K_{1/2}$ of the regulatory site for intracellular calcium during development was unexpected and could be due to a number of factors. The $K_{1/2}$ of activation by $[\text{Ca}^{2+}]_{\text{pip}}$ did not depend on the magnitude of the measured current (Fig. 5); therefore, the change in $K_{1/2}$ with development cannot be attributed to $I_{\text{NCX}}$-induced changes in the $[\text{Ca}^{2+}]_i$. Our RT-PCR indicates that the change in allosteric regulation does not involve different isoforms of NCX1 being expressed as the embryo develops (Fig. 6 and text). On the other hand, it is known that the ryanodine
receptors of the sarcoplasmic reticulum (SR) in chick heart cells are elaborated over the time period ED2–ED12 (37), and it is possible that the two events are related. This relation, if it exists, does not involve the calcium contents of the SR in any way, because neither 10 mM caffeine, which empties the SR of calcium, nor 200 μM tetracaine, which prevents the release of calcium from the SR, had any effect on 10 mM caffeine, which empties the SR of calcium from the SR, had any effect on pacemaker current in the embryonic chick heart. Furthermore, the lipid environment of the exchange, known to strongly affect NCX1 activity (21), may change with development.

Physiological implications. Sodium/calcium exchange is generally thought to be the major path for calcium efflux in heart cells from many species (6, 7), with the mouse heart being a possible exception (19, 29, 35, 38). We find that the exchanger is expressed at a nearly constant level throughout development of the chick heart, consistent with its essential role in calcium regulation.

It is thought by some investigators that intracellular calcium release and the consequent exchanger current play significant roles in generating the diastolic depolarization of pacemaker cells in the adult heart (8, 47) and even more so in embryonic cells (46, 50). We find that the exchanger density is probably sufficient to support such a scheme, since membrane depolarization due to SR calcium release and consequent InCXC have been shown under a variety of conditions (4), even in adult mammalian ventricle, where background potassium currents are much larger than in pacemaker cells. In preliminary experiments, we found much more caffeine-releasable calcium in ED2 cells than in ED11 cells (Shepherd and Creazzo, unpublished observation), presumably due to the lack of SR calcium-release channels in the junctional regions of ED2 cells (37). Spontaneous release from overloaded SR could generate an inward, depolarizing current as the calcium is pumped from the cell by the exchanger. This would contribute to the diastolic depolarization, whereas Ca2+ entry during the subsequent action potential would refill the SR, continuing the cycle of uptake and spontaneous release. This idea is speculative but is consistent with the observation that the fraction of cultured chick heart cells that beat spontaneously decreases markedly over the first 2 wk of development (13).

In conclusion, we find that, in the chick, the membrane density of the cardiac sodium/calcium exchanger does not change during development but that allosteric regulation of the exchanger changes such that activation of the exchanger requires less intracellular calcium in older embryos. The exchanger density is similar to that found in guinea pig ventricle; therefore, the exchanger probably plays an important role in calcium transport throughout development, as it does in the adult mammalian myocardium, and could contribute to the pacemaker current in the embryonic chick heart.

APPENDIX

Matsuoka and Hilgemann (34) measured I_{NCX} under a variety of zero-trans conditions in giant membrane patches from guinea pig ventricular cells. The resulting I-V relations were fitted simultaneously with systems of equations that were derived from consecutive models of transport by the exchanger. In these models, transport was effected by occlusion and deocclusion of either three sodium ions or one calcium ion, and the models, designated E2, E4, and E8, had 2, 4, or 8 different occluded states, respectively. We have used the E2 model throughout this paper, although the E4 model gave similar results. The current (in pA) calculated with the original equations was scaled by a factor of 11 to match the magnitude (in pA/pF) of the extrapolated maximal currents measured in chick embryonic ventricular cells in the present study (Fig. 5). The model equations, as implemented in JSim software (National Simulation Resource, University of Washington, Seattle, WA), are as follows.

// E2 model of Na/Ca exchange (Matsuoka and Hilgemann, 1992)
math ncx { real Domain V; V.min = -100; V.max = 60; V.delta = 10; // voltage range // ion concentrations, mM real ca0 = 1; // extracellular calcium nao = 145; // extracellular sodium cai = 0.00005; // intracellular calcium na1 = 20; // intracellular sodium // dissociation constants, mM real kci = 0.0162; // intracellular calcium kcn = 11.31; // extracellular sodium calcium//intracellular sodium k1 = 125.9; // intracellular sodium k2 = 11.31; // intracellular sodium k3 = 11.31; // intracellular sodium k1n = 243.5*125.9; // extracellular sodium k2n = 11.31; k3n = 11.31; // extracellular sodium // transport rates, 1/seconds real kna = 1000; // sodium kca = 1744; // calcium // fractional charge movement with ion occlusion reaction real qci = 0.254; // extracellular calcium qco = 0; // intracellular calcium qni = 381; // intracellular sodium qno = 0.365; // extracellular sodium // calculation of fractional binding site occupation real di(V), doo(V), k12(V), k21(V), fco(V), fci(V), f3no(V), f3ni(V), e1(V), e2(V), inaca(V);

// calculation of distribution of enzyme states k1 = f3ni*kna+ f1* kca;

k2 = f3no*kna+ fco*kca;

e1 = k21/(k12+k21); e2 = k12/(k12+k21);

// calculation of net transport

inaca = (-e2* f3no*kna- e1* f3ni*kna)/11;

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