Conformational changes of a Ca\textsuperscript{2+}-binding domain of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger monitored by FRET in transgenic zebrafish heart

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The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) is a sarcolemmal protein that catalyzes the electrogenic countertransport of Na\textsuperscript{+} and Ca\textsuperscript{2+} (transport ratio is about 3 Na\textsuperscript{+}:1 Ca\textsuperscript{2+}) (19). Depending on the gradients of Na\textsuperscript{+} and Ca\textsuperscript{2+} and membrane potential, the exchanger can function in either the Ca\textsuperscript{2+}-efflux (forward) or Ca\textsuperscript{2+}-influx (reverse) mode. NCX is the primary Ca\textsuperscript{2+} efflux mechanism of cardiac myocytes and removes Ca\textsuperscript{2+} brought into cells by the L-type Ca\textsuperscript{2+} channel on a beat-to-beat basis. By extruding Ca\textsuperscript{2+} from the cell, NCX plays an important role in the regulation of cardiac contractility. Abnormal Ca\textsuperscript{2+} cycling caused by altered NCX expression and activity has been associated with cardiac pathophysiology, including ischemia-reperfusion injury, heart failure, and arrhythmias (7, 17, 18).

The transport activity of the exchanger is allosterically regulated by cytosolic Ca\textsuperscript{2+} (25). Ca\textsuperscript{2+} binds to two Ca\textsuperscript{2+}-binding domains (CBD1 and CBD2) located in the large intracellular loop and activates the exchanger (1, 4, 9, 10, 13). The function of CBD1 has been analyzed in most detail. Mutations of acidic residues (e.g., D447V or D498I) within CBD1 intracellular loop and activates the exchanger (1, 4, 9, 10, 13).

CBD1 further decreases Ca\textsuperscript{2+} affinity (15). Different apparent Ca\textsuperscript{2+} affinities have been reported for Ca\textsuperscript{2+} regulation. For example, half-maximal concentration values obtained with giant excised patches range from 100 to 400 nM (5), whereas much lower half-maximal concentration values (20–80 nM) have been suggested from experiments using intact cells (12). Previous studies from our laboratory applied the noninvasive fluorescence resonance energy transfer (FRET) technique to monitor Ca\textsuperscript{2+}-induced conformational changes of CBD1. We demonstrated that this Ca\textsuperscript{2+} regulatory site of the exchanger can sense changes in intracellular Ca\textsuperscript{2+} in cultured neonatal cardiac myocytes during excitation-contraction (EC) coupling (15).

Here, we present the use of the zebrafish Danio rerio as an expression system for studying the Ca\textsuperscript{2+} regulation of the cardiac NCX in vivo. We generated transgenic zebrafish with cardiac-specific expression of the CBD1 linked to yellow and cyan fluorescent protein. Ca\textsuperscript{2+} binding to CBD1 induces conformational changes, as detected by fluorescence resonance energy transfer. With this transgenic fish model, we were able to monitor conformational changes of the Ca\textsuperscript{2+} regulatory domain of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in intact hearts. Treatment with the positive inotropic agents ouabain and isoproterenol increased both Ca\textsuperscript{2+} transients and Ca\textsuperscript{2+}-induced changes in fluorescence resonance energy transfer. The results indicate that Ca\textsuperscript{2+} regulation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger domain CBD1 changes with inotropic state. The transgenic fish models will be useful to further characterize the regulatory properties of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in vivo.

Ca\textsuperscript{2+}-binding domain; sodium/calcium exchange; zebrafish; fluorescence resonance energy transfer

METHODS

Zebrafish maintenance and generation of transgenic lines. Zebrafish were maintained under standard conditions (26). The AB strain was used as the wild-type line. To generate transgenic zebrafish expressing cardiac-specific expression of the CBD1 linked to yellow (YFP) and cyan fluorescent protein (CFP), using FRET, we monitored conformational changes of the Ca\textsuperscript{2+} regulatory domain of NCX during EC coupling in the myocardium of intact zebrafish. With this transgenic fish model, we demonstrate that the increase in Ca\textsuperscript{2+} transients of intact hearts induced by the positive inotropic agents ouabain and isoproterenol leads to an increase in the state of activation of CBD1 and, therefore, most likely of the intact NCX.

THE NA\textsuperscript{+}/CA\textsuperscript{2+} EXCHANGER (NCX) is a sarcolemmal protein that catalyzes the electrogenic countertransport of Na\textsuperscript{+} and Ca\textsuperscript{2+} (transport ratio is about 3 Na\textsuperscript{+}:1 Ca\textsuperscript{2+}) (19). Depending on the gradients of Na\textsuperscript{+} and Ca\textsuperscript{2+} and membrane potential, the exchanger can function in either the Ca\textsuperscript{2+}-efflux (forward) or Ca\textsuperscript{2+}-influx (reverse) mode. NCX is the primary Ca\textsuperscript{2+} efflux mechanism of cardiac myocytes and removes Ca\textsuperscript{2+} brought into cells by the L-type Ca\textsuperscript{2+} channel on a beat-to-beat basis. By extruding Ca\textsuperscript{2+} from the cell, NCX plays an important role in the regulation of cardiac contractility. Abnormal Ca\textsuperscript{2+} cycling caused by altered NCX expression and activity has been associated with cardiac pathophysiology, including ischemia-reperfusion injury, heart failure, and arrhythmias (7, 17, 18).

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Animal experiments were carried out in accordance with protocols and guidelines established by the National Institutes of Health and were approved by the University of California Los Angeles Animal Research Committee.

**Microinjection of morpholino oligonucleotide.** An antisense morpholino oligonucleotide (20), 5'-CATGTTTTGCTCTGA-CACGCCA-3', targeting the cardiac troponin T translation start codon and flanking 5' sequence, was synthesized by Gene Tools (Philomath, OR). Approximately 4 ng oligonucleotide were injected into one- to two-cell stage YFP-CBD1-CFP transgenic embryos. Cardiac phenotypes of the injected embryos were examined at 30 and 48 h postfertilization.

**FRET measurements.** Hearts of 2-day-old transgenic zebrafish embryos were dissected out in Tyrode solution supplemented with 1.8 mM CaCl₂. Cytochalasin D (20 μM) was included in the solution to suppress contraction-related artificial signals. Fluorescent imaging was performed with a Nikon Eclipse TE300 microscope equipped with a ×40 oil objective (numerical aperture 1.2) and excitation and dichroic filters appropriate for CFP excitation, as described previously (15). The samples were excited at wavelengths for CFP absorption (royal blue LED, Lumileds; San Jose, CA). YFP and CFP emission were monitored simultaneously using the Dual View (Optical Insights, Tucson, AZ) image splitter, equipped with a 505-nm long-pass dichroic filter to separate the CFP and YFP signals, a CFP emission filter (480/30), and a YFP emission filter (535/40) (15,16). YFP and CFP images were captured with a Cascade 512B digital camera (Photometrics, Tucson, AZ). YFP and CFP emissions were measured online in real time, and the ratio between YFP and CFP emission was calculated as an indicator of FRET. The FRET ratio was corrected by subtracting the background signal (measured from areas without fluorescent heart sample). Exposure times were optimized for each experiment, but varied between 80 and 200 ms and were recorded at a rate between 2 and 5 Hz. LED illumination, camera exposure, and data acquisition were controlled by MetaFluor Imaging software (Molecular Devices, Sunnyvale, CA). All experiments were performed at room temperature.

**Ca²⁺ imaging.** Wild-type embryos were injected with 1 nl of 10 mg/ml calcium green-1 dextran (70,000 molecular weight, Molecular Probes, Eugene, OR) at the one-cell stage. Hearts of 2-day-old embryos were dissected out in Tyrode solution, supplemented with 1.8 mM CaCl₂ and 20 μM cytochalasin D. Calcium green fluorescence was detected with the same microscope as described above with YFP cube (excitation 500/20 nm, emission 535/30 nm; dichroic long wave pass 515). Images were acquired every 200 ms with a Cascade 512B digital camera and analyzed with MetaFluor Imaging 6.1 software.

**RESULTS**

**Ca²⁺ handling in zebrafish heart.** Intracellular Ca²⁺ regulation during cardiac EC coupling has been studied in great detail in a variety of species. However, very little is known about Ca²⁺ handling during EC coupling in embryonic zebrafish myocardium. Before developing the use of transgenic zebrafish to monitor Ca²⁺ regulation of NCX, we performed initial experiments to begin to assess the contributions of various pathways to cytoplasmic Ca²⁺ regulation in wild-type zebrafish heart.

To measure Ca²⁺ transients, we injected calcium green dextran into wild-type zebrafish embryos at the one-cell stage and imaged calcium green fluorescence from isolated zebrafish hearts at 2 dpf. By visual inspection, we observed repetitive Ca²⁺ fluorescent waves traveling from the atrium to the ventricle (not shown). Ca²⁺ transients accompanied spontaneous contractions (Fig. 1A). To assess sarcoplasmic reticulum (SR) function, we recorded Ca²⁺ transients before and after the addition of caffeine (5 mM). Caffeine increased both diastolic and peak systolic intracellular Ca²⁺ concentration, as well as the amplitude of Ca²⁺ transients (Fig. 1B). The data indicate the presence of a functional SR, although the effects of caffeine were relatively modest. Compared with mammalian adult myocardium, the SR in fish myocardium is underdeveloped, has lesser ability to store and release Ca²⁺, and has lesser importance in EC coupling (3). We also tested the contribution of Ca²⁺ influx through L-type Ca²⁺ channels to EC coupling. As shown in Fig. 1C, the dihydropyridine, nifedipine (10 μM), resulted in cessation of Ca²⁺ transients and beating. These results indicate that the L-type Ca²⁺ channel has an essential role in EC coupling in the 2-day-old embryonic zebrafish heart.

**FRET monitoring of conformational changes of CBD1.** As previously reported (15), we have expressed CBD1 (amino acids 371–508) of the NCX with the fluorescent probes YFP and CFP on the NH₂- and COOH-termini, respectively. This construct, YFP-CBD1-CFP, undergoes conformational changes upon binding Ca²⁺ (15). The conformational motions of the CBD can be monitored by changes of FRET measured as the YFP-to-CFP fluorescence ratio. To study the Ca²⁺-dependent regulation of the exchanger in intact hearts and in vivo, we made transgenic zebrafish with cardiac-specific expression of YFP-CBD1-CFP. We also created transgenic zebrafish expressing...
YFP-CBD1-CFP with two mutations, D447V and D498I. These mutations have been shown to induce a decreased Ca\(^{2+}\)-binding affinity in the intact NCX and abolish Ca\(^{2+}\)-induced FRET in YFP-CBD1-CFP (11, 15).

We isolated hearts from zebrafish embryos at 2 dpf. Spontaneous contractions of the hearts were monitored, and changes of FRET were measured as changes in the YFP-to-CFP ratio. As shown in Fig. 2A, YFP and CFP emissions oscillated in opposite directions as embryonic hearts expressing YFP-CBD1-CFP contracted. The YFP-to-CFP fluorescence ratio was maximal during diastole when cytoplasmic Ca\(^{2+}\) was decreased and minimal during systole when cytoplasmic Ca\(^{2+}\) was elevated. The data indicate that the Ca\(^{2+}\) regulatory site of NCX undergoes conformational changes on a beat-to-beat basis during EC coupling. Ca\(^{2+}\)-induced FRET changes were not observed in hearts expressing mutant YFP-CBD1-CFP (D447V/D498I) (Fig. 2B). This confirms that the FRET signals from beating hearts expressing YFP-CBD1-CFP were not artificial. The magnitude of cardiac contraction was reduced by the presence of cytochalasin D (20 μM) to minimize possible motion artifacts.

To monitor conformational changes of YFP-CBD1-CFP in the myocardium of intact zebrafish, we injected cardiac tropinin T (tnnt2) morpholino at the one-cell stage to uncouple EC coupling and silence the heart (20). This allowed Ca\(^{2+}\) transients to occur in the absence of contraction. In vivo images were obtained at 2 dpf. The tnnt2 morpholino-injected fish exhibit some pericardial edema (arrow in Fig. 3A, right) and an absence of cardiac contraction and blood flow. We detected rapid changes in the FRET signal at an average rate of about 10 frames/s with a ×20 oil immersion lens.

**Fig. 2.** Fluorescence resonance energy transfer (FRET) studies in transgenic zebrafish hearts expressing yellow fluorescent protein (YFP)-Ca\(^{2+}\)-binding domain 1 (CBD1)-cyan fluorescent protein (CFP) (A) or YFP-CBD1-CFP mutant (D447V/D498I) (B). Shown are representative FRET (YFP/CFP) images of transgenic zebrafish hearts (ventricle, shown in pseudocolor) and the corresponding changes in the YFP-to-CFP ratio (black, top traces), YFP (black), and CFP (gray) emissions (bottom traces) during spontaneous contractions. The decrease in the ratio of YFP/CFP emissions correlates with cardiac contraction (not shown). Pseudocolor scale bars are shown to the left of the images. Dissected zebrafish hearts were kept in Tyrode solution at room temperature. Data were collected at 2 frames/s with a ×40 oil immersion lens. Data are representative of several experiments.

**Fig. 3.** FRET measurements from intact transgenic zebrafish embryos. A: epifluorescent images of control (a) and tnnt2 morpholino-injected (b) transgenic zebrafish embryos expressing YFP-CBD1-CFP at 2 days postfertilization. Arrow indicates the presence of pericardial edema in morpholino-injected fish. B: representative recording of changes in YFP (black) and CFP (gray) emission and the YFP-to-CFP ratio in an intact tnnt2 morpholino-injected YFP-CBD1-CFP transgenic zebrafish. Data were collected at 6 frames/s with a ×20 oil immersion lens.
120/min (Fig. 3B), consistent with the average heart rate of wild-type zebrafish at 2 dpf.

The conformation of CBD1 is affected by inotropic state. Using FRET, we assessed whether Ca$^{2+}$ regulation of NCX is altered by the inotropic state of the heart. Positive inotropic agents (ouabain or isoproterenol) were added to isolated transgenic zebrafish hearts expressing YFP-CBD1-CFP to monitor effects on FRET. Sample traces and summary data are shown in Fig. 4. Both ouabain and isoproterenol increased the changes in FRET between contraction and relaxation in a concentration-dependent manner. Ouabain (0.1 and 1 μM) significantly increased the changes (Δ) in FRET to 230 ± 50% (n = 5, P < 0.05) and 280 ± 40% (n = 4, P < 0.05) of control values, respectively. Treatment of the heart with 0.5 μM isoproterenol had a modest effect on ΔFRET (130 ± 10% of control values, n = 4, P = 0.15). A higher concentration of isoproterenol (1 μM) led to a significant stimulation (170 ± 20% of control values, n = 4, P < 0.05).

We also examined the effects of these concentrations of ouabain and isoproterenol on Ca$^{2+}$ transients in wild-type hearts (Fig. 5). As shown in Fig. 5A, ouabain and isoproterenol increased the magnitudes of the Ca$^{2+}$ transients. Ca$^{2+}$ transient amplitudes were significantly increased by 0.1 μM (130 ± 10% of control, n = 4, P < 0.01) and 1 μM (140 ± 10% of control, n = 4, P < 0.01) ouabain, as well as by 0.5 μM (120 ± 10% of control, n = 4, P < 0.05) and 1 μM isoproterenol (130 ± 10% of control, n = 4, P < 0.05) (Fig. 5B). The effects of the inotropic agents on Ca$^{2+}$ transients correlated with the changes of FRET. These data suggest that the positive inotropic agents ouabain and isoproterenol raised intracellular Ca$^{2+}$ and activated NCX. There are no previous studies indicating that the activation state of the NCX is
controlled by regulatory Ca\(^{2+}\) during changes in inotropic state.

**DISCUSSION**

We describe the use of zebrafish myocardium for FRET-based detection of conformation changes of the CBD of the NCX expressed in the cytoplasm. The zebrafish offers several distinct advantages as a model system, including external fertilization, rapid development, and the ability to easily up- and downregulate gene expression. The optical clarity of the zebrafish embryo provides a unique opportunity to monitor physiological processes in excised hearts and in vivo by fluorescence. Here we generated transgenic zebrafish expressing CBD1 of NCX with the fluorophores YFP and CFP linked to its NH\(_2\)- and COOH-termini. Using noninvasive FRET, we monitored Ca\(^{2+}\)-induced conformation changes of CBD1 in isolated, spontaneously contracting zebrafish heart and in intact zebrafish in vivo.

We did initial assessment of the contributions of Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release to intracellular Ca\(^{2+}\) regulation during EC coupling in zebrafish embryonic myocardium. Unlike mammals in which SR Ca\(^{2+}\) release is prominent in EC coupling, in lower vertebrates, such as the fish, the SR is generally a poorly developed organelle of lesser importance in EC coupling (2, 3, 6). Depletion of SR Ca\(^{2+}\) in zebrafish myocardium with caffeine increased diastolic Ca\(^{2+}\), as well as Ca\(^{2+}\) transients. However, caffeine did not stop the repetitive Ca\(^{2+}\) transients, implying that Ca\(^{2+}\) entry across the sarcolemmal membrane is sufficient for a relatively synchronous and uniform rise in whole cell intracellular Ca\(^{2+}\) concentration. The relative importance of SR Ca\(^{2+}\) release to EC coupling is subject to temperature and adrenergic regulation, and prominent species-specific differences exist among fish (21–24). Ca\(^{2+}\) transients were completely abolished by the L-type Ca\(^{2+}\) channel blocker, nifedipine, confirming that transsarcolemmal Ca\(^{2+}\) influx through the dihydropyridine receptor is essential to EC coupling in cardiomyocytes from embryonic zebrafish.

To monitor the conformational changes of the CBD of NCX in intact myocardium, we developed the use of zebrafish as an expression system for CBD1 tagged with CFP and YFP. This fusion protein has previously been shown to change conformation upon binding Ca\(^{2+}\), as indicated by FRET changes in human embryonic kidney cells and in rat neonatal cardiac myocytes (15). Our zebrafish model allows us to monitor changes in conformation of CBD1 during EC coupling in living zebrafish myocardium and in excised hearts. The FRET changes were monitored at a much higher frequency than occurred in the more slowly contracting neonatal myocytes (15). The FRET signal correlated well with cardiac contraction: maximal and minimal FRET occurred during relaxation and contraction, respectively. The changes in FRET were not motion artifacts, as we uncoupled EC coupling with cytochalasin D in isolated heart and with cardiac troponin T morpholino in living zebrafish. Furthermore, FRET changes were not observed in control experiments using YFP-CBD1-CFP mutant (D447V/D498I) transgenic fish, which has decreased Ca\(^{2+}\) affinity (11).

Combining FRET with intracellular Ca\(^{2+}\) imaging, we examined whether the inotropic agents ouabain and isoproterenol modulate Ca\(^{2+}\) regulation of NCX in zebrafish heart. We found that the increased Ca\(^{2+}\) transients induced by ouabain and isoproterenol increased the \(\Delta F\)RET signals from YFP-CBD1-CFP. The strong implication is that increased Ca\(^{2+}\) transients directly lead to enhanced activation of the NCX through the increased binding of regulatory Ca\(^{2+}\). Increased exchange activity could augment both the upstroke and relaxation of Ca\(^{2+}\) transients through reverse and forward mode exchange, respectively. There has not previously been experimental evidence directly linking Ca\(^{2+}\) regulation of the exchanger to inotropic state. Our experiments suggest that direct Ca\(^{2+}\)-dependent regulation of NCX may be involved in an inotropic stimulation of NCX. The data are also inconsistent with reports suggesting that the apparent affinity of NCX is 20–80 nM (12, 14). If this were the case, Ca\(^{2+}\) binding would likely be saturated during diastole, and FRET changes would not be observed in these experiments.

In summary, we have developed the use of zebrafish as a novel expression system to monitor conformations of a CBD of the NCX in living myocardium. We hope to expand these studies to include the use of full-length NCX constructs. We expect this model will help us better understand the physiological function of the exchanger and the roles of cytoplasmic factors in the beat-to-beat regulation of NCX activity.

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