Targeting and Stability of Na/Ca Exchanger 1 in Cardiomyocytes Requires Direct Interaction with the Membrane Adaptor Ankyrin-B

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Na/Ca exchanger activity is important for calcium extrusion from the cardiomyocyte cytosol during repolarization. Animal models exhibiting altered Na/Ca exchanger expression display abnormal cardiac phenotypes. In humans, elevated Na/Ca exchanger expression/activity is linked with pathophysiological conditions including arrhythmia and heart failure. Whereas the molecular mechanisms underlying Na/Ca exchanger biophysical properties are widely studied and generally well characterized, the cellular pathways and molecular partners underlying the specialized membrane localization of Na/Ca exchanger in cardiac tissue are essentially unknown. In this report, we present the first direct evidence for a protein pathway required for Na/Ca exchanger localization and stability in primary cardiomyocytes. We define the minimal structural requirements on ankyrin-B for direct Na/Ca exchanger interactions. Moreover, using ankyrin-B mutants that lack Na/Ca exchanger binding activity, and primary cardiomyocytes with reduced ankyrin-B expression, we demonstrate that direct interaction with the membrane adaptor ankyrin-B is required for the localization and post-translational stability of Na/Ca exchanger 1 in neonatal mouse cardiomyocytes. These results raise exciting new questions regarding potentially dynamic roles for ankyrin proteins in the biogenesis and maintenance of specialized membrane domains in excitable cells.

Normal cardiac function requires the coordinate activity of a collection of ion channels and transporters to regulate myocyte depolarization and repolarization. One key component of cardiomyocyte excitation-contraction coupling is the precise cellular management of cytosolic calcium concentrations during systole and diastole. During cardiac depolarization, extracellular Ca2+ enters the cytosol via the L-type Ca2+ channel. This inward calcium current triggers calcium-induced calcium release through ryanodine receptors (RyR2) on the junctional sarcoplasmic reticulum (SR). This massive release of calcium from internal stores is essential for cardiomyocyte contraction. For proper myocyte relaxation, the influx of calcium into the cytosol must be precisely balanced by extrusion of calcium from the cytosol. Dysfunction in cardiomyocyte calcium-handling mechanisms may lead to a number of cardiac phenotypes including extrasystoles and arrhythmia, apoptosis, and/or heart failure (1, 2). Whereas calcium is actively transported into the SR via the SR Ca2+ ATPase (SERCA2), a secondary mechanism for cytosolic calcium extrusion is the plasma membrane Na/Ca exchanger, which transports calcium down its concentration gradient in exchange for sodium (3). In addition to regulating calcium efflux, the Na/Ca exchanger may also function in reverse mode to cause inward Ca2+ flux during the initial depolarization of the myocyte (4).

The cardiac Na/Ca exchanger is a nine transmembrane protein encoded by human NCX1. Normal function of the Na/Ca exchanger requires proper biophysical properties and precise localization at excitable cardiac membranes including the sarcolemma and cardiomyocyte transverse-tubule surfaces (5–9). Whereas the biophysical features of the cardiac Na/Ca exchanger are widely studied and well defined (10–13), the molecular mechanisms required for the targeting and retention of the Na/Ca exchanger at specialized membrane domains in cardiomyocytes are unknown.

Ankyrins are a family of adaptor proteins which associate with a group of structurally diverse ion channels and transporters including the Na/Ca exchanger (14–16), the Na/K ATPase, voltage-gated Na+ channels, and the anion exchanger (reviewed in Ref. 17). Multiple lines of evidence predict a role for ankyrin polypeptides in the proper localization and stability of the Na/Ca exchanger at the cardiomyocyte plasma membrane. Ankyrin polypeptides directly bind to the cardiac Na/Ca exchanger with high affinity (14, 16). Moreover, reduction of ankyrin-B expression (product of ANK2) in mice homozygous (ankyrin-B−/−) or heterozygous (ankyrin-B+/-) for a null mutation in ankyrin-B display decreased expression and abnormal localization of the Na/Ca exchanger 1 in both neonatal and adult cardiomyocytes (15, 16, 18). Whereas these data predict a role for ankyrin-B in Na/Ca exchanger membrane localization,
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there is no clear evidence to support a direct role for ankyrin-B in Na/Ca exchanger membrane expression and stability.

Here we define the molecular requirements of 220-kD ankyrin-B for cardiac Na/Ca exchanger binding. Moreover, we report that direct interaction between ankyrin-B and Na/Ca exchanger is essential for the expression, localization, and post-translational stability of Na/Ca exchanger in primary cardiomyocytes. Specifically, we show that direct ankyrin-B-Na/Ca exchanger binding requires multiple charged tips on the β-hairpin loops, which connect adjacent α-helices of specific ANK repeats on the ankyrin-B membrane-binding domain. These data demonstrate that the Na/Ca exchanger binding site is distinct from the binding site for the inositol 1,4,5-trisphosphate receptor (InsP3R) (19). Using ankyrin-B mutants and ankyrin-B−/− cardiomyocytes, we demonstrate that direct ankyrin-B-Na/Ca exchanger interactions are necessary for proper localization of the Na/Ca exchanger. Moreover, metabolic labeling experiments reveal that direct ankyrin-B-Na/Ca exchanger interactions are not only required for membrane localization, but are also required for post-translational stability of Na/Ca exchanger in cardiomyocytes. Finally, we present data that ankyrin-B interactions with Na/Ca exchanger 1 are likely important for normal cardiomyocyte regulation.

EXPERIMENTAL PROCEDURES

Animals—Mice used in these studies were neonatal (postnatal day 1 or 2) WT C57BL/6 mice and ankyrin-B−/− littermates (C57BL/6). Animals were handled according to approved protocols and animal welfare regulations of the Animal Care and Use Committee.

Immunofluorescence—Neonatal cardiomyocytes were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed in warm 2% paraformaldehyde (37 °C). Cells were blocked/permeabilized in PBS containing 0.075% Triton X-100 and 2 mg/ml bovine serum albumin, and incubated in primary antisera overnight at 4 °C. Following washes (PBS plus 0.1% Triton X-100), cells were incubated in secondary antisera (Alexa 488, 568; Molecular Probes) for 4 h at 4 °C and mounted using Vectashield (Vector) and No. 1 coverslip. Images were collected on a Zeiss 510 Meta confocal microscope (63 power oil, 1.4 NA, pinhole equals 1.0 Airy Disc or 40 power water, 1.2 NA, pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software. Both channels were collected on PMT3 using sequential scans to eliminate channel bleed-through. Identical imaging protocols (laser strength, pinhole size, gain, etc.) were used when comparing Na/Ca exchanger intensity in myocytes transfected with ankyrin-B mutants. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment.

Ankyrin-B and Na/Ca Exchanger 1 Constructs—GFP-220-kDa ankyrin-B mutant constructs were generated using standard molecular techniques and QuikChange Mutagenesis (Stratagene). Loop mutations were designed to replace ANK repeat β-hairpin loop tip residues with two alanine residues (19). The mutated region was subcloned into a native GFP-220-kD ankyrin-B plasmid, and the plasmid was completely sequenced to verify that no additional mutations were introduced. Mutants were sequenced and expressed in HEK293 cells to confirm full-length protein expression. Full-length human NCX1 cDNA was cloned from a human heart library into pBacPak9 (Clontech) using standard molecular techniques and sequenced to ensure that no mutations were introduced by the cloning procedure.

Binding Studies—GFP-220-kDa ankyrin-B and mutants were expressed in HEK293 cells and purified using affinity-purified GFP Ig coupled to protein A-agarose beads. Briefly, cells were lysed in homogenization buffer plus 1.0% Triton X-100 and 0.5% deoxycholate (16). The extract was centrifuged at 100,000 × g, and the supernatant was incubated with GFP affinity-purified Ig coupled to protein A-Sepharose. The beads were washed with homogenization buffer plus 1.0% Triton X-100. Purified proteins were incubated with 10 μg of affinity-purified GFP Ig or control Ig coupled to protein A-Sepharose beads for 4 h at 4 °C. The beads were washed four times with homogenization buffer plus 1.0% Triton X-100. Protein bound to each mutant GFP-220-kD ankyrin-B was eluted, analyzed by immunoblot, normalized for relative GFP ankyrin-B expression, and then compared with WT GFP-220-kD ankyrin-B binding.

Statistics—When appropriate, data were analyzed using a two-tailed Student’s t test, and values less than p < 0.05 were considered significant. Values are expressed as the mean ± S.D.

Na/Ca Exchanger Protein Purification—For purification of Na/Ca exchanger, a His tag was engineered to the C terminus. Human Na/Ca exchanger 1 was expressed in SF21 insect cells using a generated recombinant baculovirus as described (16). The Na/Ca exchanger was purified using Ni-nitrilotriacetic acid-Sepharose as described (16).

Metabolic Labeling/Pulse-Chase Experiments—Neonatal cardiomyocytes were incubated in Dulbecco’s modified Eagle’s medium (DMEM) without methionine for 2 h followed by a 1–3-h pulse period in DMEM containing [35S]methionine (100 μCi/ml) at 37 °C. Cells were washed and chased in normal medium plus 2 mM methionine for defined times (up to 72 h). Detergent-soluble lysates were prepared and NCX1 was immunoprecipitated using affinity-purified NCX1-specific monoclonal Ig (R3F1). Equal counts were immunoprecipitated to correct for small variations in protein concentrations. Radiolabeled Na/Ca exchanger was quantitated for each time point by phosphorimaging.

Neonatal Cardiomyocytes—To generate the primary cardiomyocyte cultures, hearts were dissected from P1 or P2 mice and placed in 1 ml of Ham’s F-10. Atrial tissue was removed, and the ventricular chambers were rinsed to remove any remaining blood. Hearts were transferred into 1.5 ml of 0.05% trypsin, 200 μM EDTA in Ham’s F-10 medium (Mediatech). Hearts were minced into ∼20 small pieces using forceps and small scissors and incubated in the trypsin/EDTA medium at 37 °C. Following 15 min, the heart pieces were mixed by a Pasteur pipette and incubated for an additional 15 min. A mixture of 200 μl of soybean trypsin inhibitor (2 mg/ml; Worthington) and 200 μl of collagenase (0.2 mg/ml; 1980 units/mg Sigma) was incubated with the cells for 35–50 min at 37 °C. The cell suspension was pelleted, resuspended in “Complete Medium” (40% DMEM, 40% Ham’s F-10, 20% fetal calf serum), and plated on plastic dishes. Following 5 h, the non-anchored cells (cardiomyocytes) were aspirated from the plate, pelleted, resuspended in Complete Medium, and plated on coverslips or Mat-
Direct Interaction between Ankyrin-B and Na/Ca Exchanger 1 Requires β-Hairpin Loop Tips of ANK Repeats 16–18—We previously demonstrated that Na/Ca exchanger 1 co-immunoprecipitates with 220-kD ankyrin-B from detergent-soluble lysates of rat heart using affinity-purified ankyrin-B Ig (15, 16). We also demonstrated the reverse that 220-kD ankyrin-B co-immunoprecipitates with Na/Ca exchanger 1 from detergent-soluble fractions of rat heart using affinity-purified Na/Ca exchanger Ig (16). Ankyrin-B is co-localized with Na/Ca exchanger 1 over cardiac transverse tubules (16). Furthermore, mice with reduced ankyrin-B expression display reduced Na/Ca exchanger 1 expression (15), as well as aberrant localization of the transporter over the transverse tubule network (15, 16). To establish whether direct interactions between ankyrin-B and Na/Ca exchanger are required for the membrane localization of cardiac Na/Ca exchanger 1, we first determined the structural requirements on 220-kD ankyrin-B for Na/Ca exchanger binding activity.

220-kD ankyrin-B contains four major structural domains including a membrane-binding domain (24 ANK repeats), spectrin-binding domain, death domain, and C-terminal domain (Fig. 1A). Our previous experiments implicate the membrane-binding domain as necessary for high affinity direct interactions between ankyrin-B and Na/Ca exchanger (16) (kDa equals 5 nm); therefore, we focused on this domain in our binding experiments.

To assess the structural requirements on ankyrin-B for direct Na/Ca exchanger 1 binding, we used a series of ankyrin-B membrane-binding domain mutants designed based on the crystal structure of the closely related ankyrin-R membrane-binding domain (21). This structure consisted of ANK repeats 13–24 and revealed that the ANK repeats fold as pairs of antiparallel α-helices connected with a β-hairpin loop (21). The ANK repeats come together to form a large super helix, which surrounds a central cavity (21). Based on this structure, as well as other structures of proteins that contain consecutive ANK repeats, the β-hairpin loops between the α-helices are highly accessible to the solvent. Moreover, the tips of these β-hairpin loops are highly variable and have been implicated in mediating a number of protein interactions (22), including the interaction of ankyrin-B with the InsP₃ receptor (19).

The first series of ankyrin-B mutants harbored two alanine substitutions in the variable β-hairpin loop tips connecting each pair of ANK repeats within the membrane-binding domain (ANK repeats 1–24, Fig. 1B). Mutagenesis was performed in the context of GFP-tagged ankyrin-B (220 kD). Mutant plasmids were completely sequenced to verify that no additional mutations were introduced during PCR amplification. Wild-type ankyrin-B, as well as each GFP ankyrin-B mutant was expressed in HEK293 cells. Expressed proteins were immunopurified from the detergent-soluble fraction of the cells using immobilized affinity-purified GFP Ig and incubated with purified Na/Ca exchanger 1 derived from baculoviral expression (see "Experimental Procedures"). The quantity of pure Na/Ca exchanger 1 bound to each mutant was determined by immunoblot using an affinity-purified Na/Ca exchanger 1 Ig. Using similar methods, we determined the relative level of GFP-ankyrin-B mutant expression for each data point. The
amount of Na/Ca exchanger 1 bound to each ankyrin-B mutant was corrected for relative to GFP-ankyrin-B expression. The mutant ankyrin-B constructs bound to Na/Ca exchanger 1 at levels similar to that of wild-type ankyrin-B ($n = 5$, $p > 0.05$). These results demonstrate that binding activity between ankyrin-B for Na/Ca exchanger 1 is not mediated by an individual ANK repeat $\beta$-hairpin loop (Fig. 2).

Because the direct interaction between ankyrin-B and the InsP$_3$ receptor requires multiple $\beta$-hairpin loop tips (19), we next tested the binding of purified Na/Ca exchanger to a series of ankyrin-B membrane-binding domain mutants, which harbor alanine substitutions in various combinations of consecutive ANK repeat $\beta$-hairpin loop tips (e.g. ANK repeats 5–6, 4–6, 3–6, 2–6, 1–6; see Fig. 1C). Binding analysis was performed as described above using GFP-ankyrin-B mutants expressed and immunopurified from HEK293 cells. Mutations to the $\beta$-hairpin loop tips of multiple ANK repeats including 1–6, 7–12, and 19–24 had no effect on ankyrin-B binding to purified Na/Ca exchanger 1 (Fig. 3). In contrast, mutations to the $\beta$-hairpin loop tips of consecutive ANK repeats 13–18 significantly reduced Na/Ca exchanger 1 binding to ankyrin-B. Further analysis revealed that the binding domain between Na/Ca exchanger 1 and ankyrin-B resides across the $\beta$-hairpin loop tips of ANK repeats 16–18 (Fig. 3). These results establish that multiple $\beta$-hairpin loop tips are required for the direct binding of ankyrin-B to the Na/Ca exchanger. Moreover, these results demonstrate that the Na/Ca exchanger associates with the membrane-binding domain of ankyrin-B on a site distinct from the InsP$_3$ receptor binding site (Figs. 3 and 4).

Ankyrin-B ANK Repeats 16–18 Are Required for Normal Expression and Localization of Na/Ca Exchanger in the Sarcolemma of Primary Cardiomyocytes—In contrast to neonatal cardiomyocytes derived from wild-type mice, myocytes derived from ankyrin-B-deficient mice (ankyrin-B$^{-/-}$ and ankyrin-B$^{+/+}$ mice) display reduced spontaneous contraction rates and aberrant intracellular calcium release (15, 16, 18). Moreover, neonatal cardiomyocytes with reduced ankyrin-B expression display decreased expression of Na/Ca exchanger levels by immunoblot (NCX levels reduced 50–60%; (18), see also supplemental Fig. S1). Finally, ankyrin-B$^{+/+}$ cardiomyocytes display decreased Na/Ca exchanger membrane expression by immunofluorescence (Fig. 5, A and B, (15, 18)). Specifically, in neonatal cardiomyocytes derived from ankyrin-B$^{-/-}$ mice, Na/Ca exchanger is expressed only at cellular sites where ankyrin-B expression is not reduced (see arrows in Fig. 5B). Abnormal cellular phenotypes are rescued by exogenous expression of wild-type ankyrin-B CDNA (Fig. 5C), but not with ankyrin-B variants harboring loss-of-function mutations associated with human arrhythmia (15, 18). Accordingly, we used this cardiomyocyte ankyrin-B rescue assay to determine whether direct interaction between Na/Ca exchanger and ankyrin-B is required for the proper localization of Na/Ca exchanger in cardiomyocytes. Specifically, we analyzed Na/Ca exchanger 1 expression by immunofluorescence in neonatal ankyrin-B$^{+/+}$ cardiomyocytes expressing various GFP-ankyrin-B membrane-binding domain mutants (Fig. 6).

Ankyrin-B$^{+/+}$ cardiomyocytes were transfected with GFP-220-kD ankyrin-B and GFP-220-kD ankyrin-B ANK repeat mutants R1–6, R7–12, R13–18, and R19–24 (Fig. 1C). Similar to wild-type GFP-220-kD ankyrin-B, GFP-220-kD ankyrin-B
mutants R1–6, R7–12, and R19–24 rescued the expression and localization of Na/Ca exchanger (all mutants were localized similar to endogenous ankyrin-B; Fig. 6, A, B, and D). In contrast, expression of GFP-220-kD ankyrin-B mutant R13–18 did not rescue the normal expression/localization of Na/Ca exchanger even though the mutant GFP fusion protein was expressed and localized similar to endogenous ankyrin-B and other GFP ankyrin-B mutants (Fig. 6C, NCX1 localization similar to untransfected ankyrin-B++/+ cardiomyocytes, see Fig. 5B).

In fact, expression of additional GFP-220-kD ankyrin-B mutants revealed that as few as three consecutive ANK repeats (R16–18) rendered GFP-220-kD ankyrin-B unable to rescue the normal localization of the Na/Ca exchanger (Fig. 6E). In contrast, expression of the ankyrin-B R13–18 mutant (also R16–18, not shown) lacking Na/Ca exchanger binding activity rescued the normal expression of InsP3 receptor in ankyrin-B++/+ cardiomyocytes (Supplemental Fig. S2). Expression of single mutants 220-kD ankyrin-B R16 (Fig. 6H), R17 (Fig. 6I), or R18 (Fig. 6J) as well as double mutants R16–17 (Fig. 6F) and R17–18 (Fig. 6G) restored the normal expression/localization pattern of Na/Ca exchanger. Therefore, these data demonstrate that the ankyrin-B structural requirements for Na/Ca exchanger binding (R16–18) are also essential for the proper expression and localization of the Na/Ca exchanger in primary cardiomyocytes (Fig. 6). Moreover, mutation of these loop tips (R16–18) does not affect ankyrin-B activity for binding (19) or targeting (Supplemental Fig. S2) of the InsP3 receptor.

**Direct Interaction with Ankyrin-B Is Required for Na/Ca Exchanger Stability in Primary Cardiomyocytes**—Na/Ca exchanger expression is reduced in cardiac tissue and in single cardiomyocytes with reduced ankyrin-B expression (Fig. 5B, Supplemental Fig. S1) (15, 18). Moreover, previous findings demonstrate no difference in Ncx1 mRNA levels between wild-type and ankyrin-B++/+ hearts by Northern blot analysis (15). Therefore, the mechanism underlying the decrease in Na/Ca exchanger 1 expression in cells with reduced ankyrin-B is likely post-translational. To determine whether ankyrin-B expression is required for stability of Na/Ca exchanger protein, we measured the half-life of Na/Ca exchanger in neonatal cardiomyocytes isolated from wild-type and ankyrin-B++/+ mice. Using standard biosynthetic labeling techniques (see “Experimental Procedures”), primary cardiomyocytes were labeled with [35S]methionine for 3 h and then washed. Primary cells were then chased in [35S]methionine-free medium for defined times up to 72 h as determined by previous determination of Na/Ca exchanger half-life (23). Following each chase time, primary cells were collected. Proteins from each collection time were solubilized (using a protocol previous determined to solubilize >90% of Na/Ca exchanger protein from neonatal car-
compared with wild-type cardiomyocytes (Fig. S3), cardiomyocytes were labeled with [35S]methionine, expression in myocytes. F–J, expression in myocytes transfected with mutants R16–17, R16, R17, and R18 is rescued to normal levels. Note that GFP-220-kDa ankyrin-B mutants are localized to endogenous ankyrin-B when expressed in cardiomyocytes. R17–18, R16, R17, and R18 is rescued to normal levels. Note that GFP-220-kDa ankyrin-B mutants are localized similar to endogenous ankyrin-B when expressed in cardiomyocytes. Scale bars equal 10 microns.

FIGURE 6. Direct interactions between ankyrin-B and Na/Ca exchanger are required for normal expression and localization of Na/Ca exchanger in neonatal cardiomyocytes. Localization of GFP-220-kDa ankyrin-B mutants and Na/Ca exchanger 1 in transfected ankyrin-B+/− cardiomyocytes. A, B, D, GFP-220-kDa ankyrin-B mutants R1–6, R7–12, and R19–24 rescue Na/Ca exchanger 1 localization. C, in contrast, localization of Na/Ca exchanger 1 in ankyrin-B−/− cardiomyocytes is not rescued by transfection of GFP-220-kDa ankyrin-B mutant R13–18, and is similar to the Na/Ca exchanger expression pattern observed in untransfected ankyrin-B+/− cardiomyocytes (see Fig. 5B). E, GFP-220-kDa ankyrin-B mutant R16–18 cannot rescue Na/Ca exchanger expression in myocytes. F–J, Na/Ca exchanger expression in myocytes transfected with mutants R16–17, R17–18, R16, R17, and R18 is rescued to normal levels. Note that GFP-220-kDa ankyrin-B mutants are localized similar to endogenous ankyrin-B when expressed in cardiomyocytes. Scale bars equal 10 microns.

diomyocytes (16)), and Na/Ca exchanger was immunopurified using affinity-purified Ig against Na/Ca exchanger 1. Radiolabeled immunopurified Na/Ca exchanger was analyzed by SDS-PAGE and phosphorimaging. Consistent with previous observations (23), our experiments demonstrate that the half-life of Na/Ca exchanger in wild-type cardiomyocytes is ~27.2 h (Fig. 7A). Decreased expression of ankyrin-B in ankyrin-B+/− cardiomyocytes leads to reduced stability of Na/Ca exchanger 1 protein. Specifically, Na/Ca exchanger half-life in ankyrin-B+/− cells was ~17.8 h (Fig. 7A), a 44.6% decrease in half-life compared with wild-type cardiomyocytes (p < 0.05). Therefore, our results suggest that ankyrin-B is required for the posttranslational stability of Na/Ca exchanger in primary cardiomyocytes.

To assess whether the difference in Na/Ca exchanger stability in wild-type and ankyrin-B+/− cardiomyocytes is caused by direct interactions between ankyrin-B and the exchanger, we measured the Na/Ca exchanger half-life in ankyrin-B+/− cardiomyocytes exogenously expressing wild-type GFP-ankyrin-B or GFP-ankyrin-B lacking binding activity for the Na/Ca exchanger (R16–18; see Fig. 3). Following a 24-h transfection (t½ of ankyrin-B was determined to be ~12.4 h; Supplemental Fig. S3), cardiomyocytes were labeled with [35S]methionine, chased for defined times, and the stability of Na/Ca exchanger spontaneous contractions in neonatal cells (15, 18–20, 24, 25). These abnormal cellular phenotypes are rescued to normal by expression of wild-type ankyrin-B (15, 18, 20, 24). To determine whether these abnormal phenotypes were the result of loss of ankyrin-B binding to the Na/Ca exchanger and/or InsP3 receptor, we analyzed spontaneous contractions in ankyrin-B+/− cardiomyocytes expressing various ankyrin-B membrane-binding domain mutants as outlined in Fig. 1.

Abnormal spontaneous contractions (Fig. 8A) as well as calcium dynamics (not shown) were rescued by transfection of wild-type ankyrin-B or ankyrin-B constructs with mutations to an individual ANK repeat β-hairpin loop tip. Moreover, the majority of ankyrin-B constructs with multiple mutations in the β-hairpin loops were sufficient to rescue normal cardiomyocyte contraction and calcium dynamics. However, ankyrin-B variants R13–18, R14–18, R15–18, and R16–18 were unable to rescue normal spontaneous contraction rates (Fig. 8B) in ankyrin-B+/− cardiomyocytes, despite having similar targeting and expression patterns as wild-type ankyrin-B. ANK repeats R16–18 are required for direct ankyrin-B interaction with Na/Ca exchanger. ANK repeats R22–24 are required for ankyrin-B direct interaction with InsP3 receptor (19). These data suggest that loss of ankyrin-B-InsP3 receptor interaction is likely not responsible for abnormal spontaneous contractions as described above. As demonstrated in Fig. 7B, expression of GFP-220-kDa ankyrin-B restored Na/Ca exchanger half-life in ankyrin-B+/− cardiomyocytes to near normal levels (n = 3). In contrast, expression of GFP-220-kDa ankyrin-B R16–18 at equivalent levels did not rescue Na/Ca exchanger half-life in ankyrin-B+/− cardiomyocytes (Fig. 7B). Because these experiments were performed in the context of a whole cell, we cannot completely rule out the possibility that an intermediate protein may be mediating the interaction between ankyrin-B and Na/Ca exchanger. However, the combination of our direct binding data and targeting data strongly suggests that ankyrin-B facilitates normal posttranslational Na/Ca exchanger stability in vivo by association with the exchanger.

Ankyrin-B ANK Repeats 16–18 Are Required for Neonatal Cardiomyocyte Spontaneous Contractions—Adult and neonatal cardiomyocytes with reduced ankyrin-B expression display abnormal intracellular calcium homeostasis resulting in early and delayed afterdepolarizations in adult ankyrin-B+/− cells and aberrant
in ankyrin-deficient cardiomyocytes. Although we cannot formally exclude the possibility that other proteins may interact with ankyrin-B at the R16–18 site, our data strongly support an important role for direct ankyrin-B-Na/Ca exchanger interactions in normal neonatal cardiomyocyte function.

**DISCUSSION**

We present the first direct evidence for a protein pathway required for Na/Ca exchanger localization and stability in primary cardiomyocytes. Specifically, we identify the binding site for Na/Ca exchanger in the membrane-binding domain of ankyrin-B. Moreover, we demonstrate that proper membrane localization and post-translational stability of Na/Ca exchanger 1 in neonatal mouse cardiomyocytes is dependent upon the direct interaction between Na/Ca exchanger and ankyrin-B.

The importance of Na/Ca exchanger function for normal cardiac development and function is well established, and clearly demonstrated by Na/Ca exchanger animal models. Global knock-out of Ncx1 in mice leads to embryonic lethality due to cardiac dysfunction (26–29). Mice with a cardiac-specific knock-out of Ncx1 display a 20–30% decrease in contractility by 7.5 weeks (30). Surprisingly, these mice live to adulthood (likely due to adaptations in inward calcium current), although their cardiac function decreases with age (30). Cardiomyocytes isolated from mice overexpressing Na/Ca exchanger 1 exhibit decreased calcium transients and reduced excitation-contraction coupling despite preserved SR calcium load and peak L-type calcium current (31, 32). In both overexpression and
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knock-out Ncx1 models, mice are highly susceptible to heart failure in response to stress (30, 31).

The requirement for Na/Ca exchanger activity for normal cardiac function is conserved in metazoans. For example, Zebrafish trembler (tre) mutants express a cardiac-specific NCX1 truncation resulting in abnormal calcium transients and unsynchronized cardiac contractions (33, 34). While no direct link between human NCX1 gene variants and cardiac dysfunction has been identified to date, elevated Na/Ca exchanger expression/activity has been associated with pathophysiological conditions in humans and animal models such as arrhythmia and heart failure (35–41).

Since the cloning of the cardiac Na/Ca exchanger in 1992, the molecular cardiology field has primarily focused on elucidating the molecular properties underlying Na/Ca exchanger biophysical properties in myocytes. Notably, these studies have identified a number of intracellular factors that bind to and regulate Na/Ca exchanger transport activity. These factors include both calcium and sodium ions, as well as protons, phosphatidylino-sitol 4,5-bisphosphate (PIP2), inhibitor peptide (XIP), ATP, and proteases (42–54).

More recently, multiple groups have identified potential in vivo cytoplasmic-binding proteins for Na/Ca exchanger that may serve to regulate its localization and/or function. Schulze et al. (55) demonstrated an association between Na/Ca exchanger and a macromolecular complex including protein kinase A, protein kinase C, protein phosphatase PP2A, and muscle protein kinase A锚oring protein (mAKAP). Additionally, calcineurin, 14-3-3 and F-actin have been shown to associate with the Na/Ca exchanger and modulate its activity (56–58). Finally, both caveolin 3 and annexin A5 may also associate with Na/Ca exchanger in vivo (59, 60). Although it is likely that one or more of these proteins may influence the cellular pathways responsible for the localization and stability of Na/Ca exchanger in cardiomyocytes, in vivo data to support the physiological relevance of these interactions is not yet available.

We and other groups (14, 16) have demonstrated that ankyrin associates with Na/Ca exchanger with high affinity. Moreover, we have shown that cardiomyocytes from mice with reduced ankyrin-B expression display reduced expression and abnormal localization of Na/Ca exchanger (15, 16). These results, while indirect, strongly suggest a role for direct ankyrin-B-Na/Ca exchanger interactions in normal Na/Ca exchanger localization. Our new data demonstrate that the direct interaction between ankyrin-B and Na/Ca exchanger is required for normal localization and stability of Na/Ca exchanger in neonatal cardiomyocytes. While neonatal cardiomyocytes do not display the same degree of membrane complexity of an adult ventricular cardiomyocyte, our data strongly predict that in adult cardiomyocytes, the placement of the Na/Ca exchanger at specialized T-tubule membrane sites distinct from the L-type calcium channel/RyR, dyad (9, 16) requires ankyrin-B. Consistent with these predictions, ankyrin-B+/− adult cardiomyocytes display reduced Na/Ca exchanger at these sites, abnormal calcium transients and delayed afterpolarizations (15). We hypothesize that loss of ankyrin-B-dependent targeting/stabilization of Na/Ca exchanger in heart could be a contributing factor to the cardiac phenotypes observed in ankyrin-B/type 4 long QT syndrome, an autosomal-dominant arrhythmia disorder characterized by prolonged QT, interval, cardiac arrhythmia, and risk of sudden death (15, 18, 61). Interestingly, one specific missense mutation associated with type 4 long QT syndrome (E1425G) in the spectrin-binding domain of ankyrin-B disrupts ankyrin-B-Na/Ca exchanger binding (16). Whereas exogenous expression of wild-type ankyrin-B cDNA in ankyrin-B+/− and ankyrin-B−/− cells can rescue the localization of Na/Ca exchanger (see Fig. 5 and Ref. 15), the human ankyrin-B variant E1425G lacking Na/Ca exchanger binding activity cannot rescue this phenotype (15, 18). The fact that this missense mutation does not reside in the Na/Ca exchanger binding site (R16–18) suggests that additional regulatory mechanisms, possibly intramolecular interactions, may mediate the appropriate binding and localization of ankyrin-associated integral membrane proteins.

The inability of the ankyrin-B R16–18 mutant (lacks NCX1 binding activity) to rescue the abnormal ankyrin-B+/− cardiomyocyte phenotypes suggests a role for direct ankyrin-B-Na/Ca exchanger interactions in normal cardiomyocyte contractility, calcium dynamics, and exchanger localization and stability. However, these data do not formally exclude the possibility that a second protein or protein complex may associate with this same site on ankyrin-B to regulate contraction rates/calcium dynamics. Moreover, our ability to rescue select ankyrin-B+/− cardiomyocyte phenotypes with ankyrin-B cDNA does not exclude the possibility that secondary features (ankyrin-B-independent) of ankyrin-B+/− cardiomyocytes may also influence cardiomyocyte cellular properties including Na/Ca exchanger stability and cardiomyocyte spontaneous contractions. Future experiments to determine cellular phenotypes in a cardiomyocyte expressing Na/Ca exchanger that lacks ankyrin-B-binding activity (in a Na/Ca exchanger-null background) will be necessary to conclusively prove the role for direct ankyrin-B-Na/Ca exchanger interactions for normal neonatal cardiomyocyte spontaneous contractions, Na/Ca exchanger expression and localization, and Na/Ca exchanger post-translational stability.

A definitive role for ankyrin-B function in Na/Ca exchanger localization and activity is not yet known. Based on the role of ankyrin-R as a static membrane scaffold in the red blood cell (62, 63), ankyrin-B is predicted to function similarly in the cardiomyocyte to stabilize membrane ion channels and transporters once they reach specific membrane domains. However, ankyrin-B may play multiple roles in the cellular delivery, as well as the scaffolding of a specific ion channel or transporter. In support of a cellular chaperone role for ankyrin-B, we recently determined that ankyrin-B-dependent interactions with the cardiomyocyte cytoskeleton (β2-spectrin) are not required for ankyrin-B-based channel/transporter localization in cardiomyocytes (25). In addition to roles in ion channel and transporter delivery and scaffolding, ankyrin-B may also regulate ion channel and transporter biophysical properties. In support of this hypothesis, our group demonstrated that a cardiac voltage-gated Na+ channel 1.5 mutant, which lacks ankyrin-G binding activity displays abnormal activation and inactivation (64). These results were recently supported by an independent
group studying ankyrin-G interactions with neuronal voltage-gated Na, channel Na$_1$6 (65). Together, these two studies suggest that ankyrin polypeptides may play an unexpected role in channel/transporter biophysical properties. Clearly, additional studies to further characterize ankyrin-based pathways will be critical for defining the diversity of roles for cardiac ankryns.

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