ANKYRIN-B REGULATES KIR6.2 MEMBRANE EXPRESSION AND FUNCTION IN HEART

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Ankyrin polypeptides are critical for normal membrane protein expression in diverse cell types, including neurons, myocytes, epithelia, and erythrocytes. Ankyrin dysfunction results in defects in membrane expression of ankyrin-binding partners (including ion channels, transporters, and cell adhesion molecules), resulting in aberrant cellular function and disease. Here we identify a new role for ankyrin-B for cardiac cell biology. We demonstrate that cardiac sarcolemmal K_ATP channels directly associate with ankyrin-B in heart via the K_ATP channel alpha-subunit, Kir6.2. We demonstrate that primary myocytes lacking ankyrin-B display defects in Kir6.2 protein expression, membrane expression, and function. Moreover, we demonstrate a secondary role of ankyrin-B in regulating K_ATP channel gating. Finally, we demonstrate that ankyrin-B forms a membrane complex with K_ATP channels and the cardiac Na/K ATPase, a second key membrane transporter involved in the cardiac ischemia response. Collectively, our new findings define a new role for cardiac ankyrin polypeptides for regulation of ion channel membrane expression in heart.

Ankyrins are multivalent adapter proteins required for the proper membrane expression of ion channels, transporters, cell adhesion molecules, and structural and signaling molecules in excitable and non-excitable cells (1). Three genes (ANK1, ANK2, ANK3) encode a host of structurally-similar but functionally-distinct ankyrin polypeptides (ankyrin-R, ankyrin-B, ankyrin-G, respectively) with specific roles in erythrocyte membrane structure, cardiac excitability, polarized epithelia ion regulation, and neuronal development. In heart, ankyrins regulate membrane excitability by coordinating the expression of voltage-gated Na⁺ and Ca²⁺ channels, cytoskeletal elements, key membrane transporters and pumps, and signaling proteins (2,3). The importance of cardiac ankyrins for normal physiology is demonstrated by human disease associated with dysfunction in ankyrin-based pathways. For example, dysfunction in the ankyrin-G-based cellular pathway for voltage-gated Na⁺ channel membrane expression is associated with the potentially-fatal Brugada syndrome cardiac arrhythmia due to reduced membrane sodium current (4,5).

In heart, ankyrin-B is critical for regulating membrane protein expression, with ankyrin-B dysfunction linked to cardiovascular disease in humans and mice (6-10). Humans harboring ANK2 loss-of-function gene variants display a complex cardiac phenotype that may include sinus node disease, conduction defects, ventricular arrhythmia, and sudden death (6-8,10). Mice lacking one functional allele of Ank2 (ankyrin-B⁻/⁻ mice) display similar phenotypes and have been utilized to identify and validate potential ankyrin-B protein partners that contribute to the human disease phenotype (6). More recently, ANK2 variants have been linked with arrhythmia susceptibility in the general human population (11). Furthermore, ankyrin-B dysfunction has been identified following myocardial infarction (12,13), suggesting an
important role for ankyrin-B in regulating the heart’s response to common, acquired forms of ischemic heart disease. Unfortunately, despite the link between ankyrin-B and cardiac disease, we still lack fundamental information regarding the identity of the cast of ankyrin-B protein partners in vivo, and the potential role of ankyrin-B in the regulation of membrane-binding partners.

Here, we define a new role of ankyrin-B in cardiovascular cell biology by demonstrating a role for ankyrin-B in KATP channel regulation. Ankyrin-B associates with the cardiac KATP channel via Kir6.2, a key component of the cellular machinery required for intrinsic cardioprotection from ischemia. Hearts and isolated cardiomyocytes lacking ankyrin-B display loss of Kir6.2 membrane expression and decreased membrane $I_{\text{KATP}}$. Moreover, we demonstrate that ankyrin-B regulates cardiac KATP channel gating. Finally, we demonstrate that ankyrin-B coordinates a complex of the KATP channel and Na/K ATPase. Together, these data define new roles for ankyrin-B in cardiac membrane protein expression, identify a new in vivo membrane partner for ankyrin-B in heart, and define potential new roles of ankyrin-B for regulating cardiac function in health and disease. Moreover, these new data, combined with recent findings linking ankyrin-B with Kir6.2 membrane expression in pancreatic beta cells(14), suggest that the ankyrin-B cellular pathway has evolved to modulate membrane protein expression across functionally-diverse excitable cell types.

**Experimental Procedures**

**Electrophysiology.** Mice were sacrificed after deep anesthesia with 2.5% Avertin at a dose of 0.2 ml/10 g [10 g of tribromoethanol alcohol + 10 ml of tert-amyl alcohol with the addition of 1 mg/ml of heparin (187 USP units/mg)]. Ventricular myocytes were isolated from Langendorff-perfused hearts not subjected to ischemia, as previously described (15). $I_{\text{KATP}}$ from ventricular myocytes was recorded with inside-out patch-clamp configuration of the patch-clamp technique using an amplifier (Axopatch-200B, Axon Instruments, Foster City, CA), monitored and stored pClamp-10 data acquisition system (Molecular Devices Co, Sunnyvale, CA) (15). Tip resistance was 1.5–3 MΩ when pipettes were filled with intracellular solution, and series resistance compensation was routinely set at >85% in all experiments. All recordings were obtained at room temperature (23–25°C). Standard bath (intracellular) and pipette (extracellular) solution used in patch-clamp experiments was KINT (in mM: KCl 140, K⁺-HEPES 10, K’EGTA 10, pH 7.3 with KOH). $I_{\text{KATP}}$ was evoked by a membrane potential of -50 mV (pipette potential +50 mV) at six different concentrations of ATP: 0, 10, 25, 50, 100 and 1000 μM. Data were filtered at 5 kHz. Off-line analysis was performed using ClampFit and Microsoft Excel programs. Steady-state dependence of membrane current on [ATP] was obtained by calculating the relative current indexed to zero ATP ($I_{\text{rel}}$). The data were fitted using the Hill equation: $I_{\text{rel}} = 100/(1+([\text{ATP}]/K_{1/2})^H$ where $I_{\text{rel}}$ is the relative current, $K_{1/2}$ is the concentration causing half-maximum blockade, and H is the Hill coefficient. Single channel $I_{\text{KATP}}$ recordings were obtained from inside-out patches using fire-polished pipettes, (resistance ~9–12-MΩ), when filled with pipette solution containing (mM): 140 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES-KOH, pH 7.3. Cardiac cells were superfused with intracellular solution containing (mM): 140 KCl, 1 MgCl₂, 5 EGTA, 5 HEPES-KOH, pH 7.3, in the absence or presence of ATP, and recordings made at room temperature (23–25°C) as described (15). Single-channel recordings in the inside-out configuration were measured at a holding potential of +60 mV. Signal was low-pass filtered at 1 kHz and sampled every 50-ms. The threshold for judging the open state of KATP channels was set at half of the single channel amplitude. The $nPo$, where $n$ represents the number of channels in the patch and $Po$ the probability of each channel to open, was assessed using Clampfit-10 software.

**Animals.** Mice were age-matched wild type and ankyrin-B⁻/⁻ male littermates. Ankyrin-B⁻/⁻ mice were generated by backcrossing at least 20 generations (99.8% pure) into the C57Bl/6 background (Jackson Labs). Animals were handled according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee (IACUC). Both wild type and ankyrin-B⁻/⁻ mice were housed in
the same facility, consumed the same diet, provided water *ad libitum*, and kept on identical 12 hour light/dark cycles.

*Tissue preparation and homogenization.* For immunoblotting and co-immunoprecipitation analysis, heart tissues were flash-frozen in liquid nitrogen and ground into a fine powder. The powder was resuspended in three volumes of ice-cold homogenization buffer (50 mM Tris HCl (pH 7.35), 10 mM NaCl, 0.32 M sucrose, 5 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, 1 mM AEBSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and homogenized. The homogenate was centrifuged at 1,000 x *g* at 4 ºC to remove nuclei. Triton X-100 and deoxycholate were added to the post-nuclear supernatant for final concentrations of 1.5% Triton X-100 and 0.75% deoxycholate. The lysate was pelleted at 100,000 x *g* for one hour at 4°C. The resulting supernatant was quantitated by bicinchoninic acid assay (BCA) prior to analysis.

*Antibodies.* The following primary antibodies were used for immunoblotting protocols and/or immunofluorescent staining: anti-ankyrin-B (polyclonal and monoclonal), anti-ankyrin-G (polyclonal), anti-Kir6.2 (Alomone), anti-Kir6.1 (Alomone), anti-SUR1 (Santa Cruz), anti-SUR2A (Santa Cruz), anti-NHERF1 (polyclonal), anti-NCX1 (RDI), anti-NKA (Upstate), anti-SERCA2 (polyclonal), anti-Ca,1.2 (polyclonal), and anti-Na,1.5 (polyclonal).

*Co-immunoprecipitation.* Protein-A-conjugated agarose beads (Rockland) were incubated with either control IgG or affinity-purified anti-ankyrin-B, anti-Kir6.2, anti-NCX, or anti-NKA in Co-IP binding buffer (phosphate buffered saline (PBS) with 0.1% Triton X-100 and protease inhibitor cocktail (Sigma)) for 12 hours at 4°C. Beads were centrifuged and washed three times in ice-cold PBS. 100 μg wild type heart lysate or 200 μg ankyrin-B−/− heart lysate were added to the washed beads, along with protease inhibitor cocktail and Co-IP binding buffer, and incubated for 12 hours at 4°C. The reactions were washed three times in ice-cold Co-IP buffer. The samples were eluted and the proteins separated by SDS-PAGE prior to immunoblotting.

*Pulldown analysis using Kir6.2 ABM peptide.* A biotinylated Kir6.2 oligopeptide (GQRFVIPAEEDGR; Biosynthesis, Inc.) was constructed with an SGSG linker between the biotin and the Kir6.2 peptide sequence. Additionally, a biotinylated Kir6.1 oligopeptide (analogous to Kir6.2 oligopeptide sequence; GHRFVSIVTEEGR; Biosynthesis, Inc.) was constructed with an SGSG linker between the biotin and the Kir6.1 peptide sequence. Twenty micrograms of oligopeptide were conjugated to 40 μl bead volume of streptavidin beads (Thermo Scientific) for four hours at 4°C in Binding buffer (PBS + 1% Triton X-100 + protease inhibitor cocktail). The beads were centrifuged and washed three times in binding buffer and 100 μg freshly-prepared wild type heart lysate were added to the washed beads, along with 500 μl of Binding buffer and protease inhibitors. The reactions were incubated overnight at 4°C. The reactions were centrifuged and washed three times in binding buffer prior to elution, SDS-PAGE, and immunoblotting with anti-ankyrin-B Ig.

*Cell culture.* HEK293 cells were maintained in Dulbecco’s Modified Essential Media (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone) and 0.1% penicillin/streptomycin. Cells were cultured at 37ºC in 5% CO₂.

*K_{ATP} constructs.* Human Kir6.2 (*KCNJ11*; NP_000516) and human SUR1 (*ABCC8*; NP_000343.2) were cloned from the GAL4 human cardiac cDNA library (Clontech) and subcloned into pcDNA3.1+ (Invitrogen). Human SUR2A (*ABCC9*; NP_005682.2) in pcDNA3.1+ was a generous gift from Dr. Leonid Zingman (University of Iowa). All constructs were thoroughly sequenced prior to experimentation.

*Transfection.* Cultured cells were split 24 hours prior to transfection (30% confluence at time of transfection). Qiagen Effectene reagent and corresponding manufacturer’s instructions were used to transfect cells with 0.2 μg Kir6.2 pcDNA3.1+, with or without 0.2 μg SUR1 pcDNA3.1+ and/or 0.2 μg SUR2A pcDNA3.1+. Transfection was carried out for nine hours at 37 ºC and the cells allowed to recover overnight(16-18). After 48 hours, the cells were lysed, centrifuged at high speed to remove debris, and
the supernatant used in subsequent co-immunoprecipitation and immunoblotting experiments.

Imaging of adult cardiomyocytes. Adult ventricular cardiomyocytes were stained according to the protocol in Morrissey, et al. (19). Briefly, single, dissociated myocytes were plated onto fibronectin-coated Mattek plates and incubated at 37°C for 15 minutes to allow attachment before fixation. Myocytes were fixed in 4% paraformaldehyde for 15 minutes at room temperature, followed by permeabilization with ice-cold 100% methanol at -20°C for five minutes. Myocytes were then washed with Ca²⁺ and Mg²⁺-free PBS. Washed myocytes were incubated with 0.1% Triton X-100 (in PBS) for 15 minutes at room temperature, followed by washing (2 x five minutes) with room temperature PBS, and blocking (5% fetal bovine serum in PBS; 2 x 10 minutes). The cells were incubated with primary antibody (1 hour at room temperature), washed (3 x 10 minutes in PBS-serum), and incubated with secondary antibodies (45 minutes at room temperature). Following washing (4 x 10 minutes) with PBS, the cells were mounted with VectaShield and covered with #1 glass coverslips.

Pulldown experiments. 10 μg AnkB MBD-GST, AnkB SBD-GST, AnkB CTD-GST, AnkG MBD-GST, and GST alone were incubated with glutathione agarose (Amersham Biosciences) overnight at 4 °C. The beads were centrifuged and washed three times in binding buffer. One hundred micrograms of wild type heart lysate were added to the beads, along with 500 μl binding buffer and protease inhibitor cocktail and the reactions incubated for 12 hours at 4 °C. The beads were centrifuged and washed three times in binding buffer. The proteins were eluted, separated by SDS-PAGE and immunoblotted using anti-Kir6.2 or –Kir6.1 Ig.

Statistics. Data are presented as a mean ± SE, and statistical significance was estimated using a Student’s t-test or ANOVA, as appropriate (Sigma Stat). Post-hoc comparisons after ANOVA were performed using the Holm-Sidak test. The null hypothesis was rejected for p ≤ 0.05.

RESULTS

K_{ATP} channel subunit expression is decreased in the ankyrin-B⁻/⁻ heart

The ATP-sensitive potassium channel (K_{ATP}) is one of several ion channels and pumps linked with the heart’s response to ischemia (20,21). Notably, this channel complex has been previously associated with ankyrin in pancreatic beta cells (14). We investigated K_{ATP} channel subunit expression in ankyrin-B⁻/⁻ mouse heart lysates by immunoblot. We first evaluated cardiac K_{ATP} alpha (Kir6.1 and Kir6.2) and beta (SUR1, SUR2A) K_{ATP} channel subunit expression. We observed decreased Kir6.2 expression in ankyrin-B⁻/⁻ ventricular tissue, compared to wild-type littermate hearts (Fig. 1B; decreased ~46%, n=4, p<0.01). Notably, we also observed a parallel decrease in the expression of both K_{ATP} channel beta subunits, SUR1 and SUR2A (Fig. 1C-D; decreased 51% and 47%, respectively; n=4, p<0.01) in ankyrin-B⁻/⁻ ventricle. Kir6.1, while present in mouse ventricle, was not altered by ankyrin-B deficiency (Fig. 1F; N.S.; n=4). As expected, ankyrin-B expression was significantly reduced in ankyrin-B⁻/⁻ whole heart lysates (Fig. 1A; 57%; n = 4, p<0.001), while the expression levels of the related ankyrin gene product, ankyrin-G and NHERF1 (loading control) were unchanged (Fig. 1E,G; N.S.; n=4). Consistent with previous findings (6) , Na/Ca exchanger (NCX) and Na/K ATPase expression levels were reduced in ankyrin-B⁻/⁻ ventricle (Fig. 1H-I, n=4, p<0.05).

Kir6.2 associates with ankyrin-B in heart

Considering that Kir6.2 protein expression was significantly reduced in ankyrin-B⁻/⁻ heart with no difference in mRNA levels of KCNJ11 (data not shown), we evaluated the ability of ankyrin-B to associate with Kir6.2 in heart. Co-immunoprecipitation analysis using detergent-soluble lysates from adult mouse left ventricle revealed that affinity-purified anti-ankyrin-B Ig co-immunoprecipitated Kir6.2 from heart lysate (Fig. 2A). Conversely, anti-Kir6.2 Ig co-immunoprecipitated ankyrin-B from detergent-soluble lysates (Fig. 2B). We observed no interaction between ankyrin-B or Kir6.2 with control Ig (Fig. 2A-B). Moreover, we observed no interaction of Kir6.1 with ankyrin-B using an
identical assay (Fig. 2C-D). These data support an in vivo interaction between ankyrin-B and Kir6.2, but not Kir6.1 in heart.

We further evaluated the cardiac ankyrin-B/Kir6.2 interaction using pull-down experiments from mouse detergent-soluble heart lysates. Ankyrin-B is comprised of three structural domains termed the membrane-binding domain (MBD), spectrin-binding domain (SBD), and C-terminal domain (CTD) (22). Notably, purified GST-ankyrin-B MBD, but not GST-ankyrin-B SBD or ankyrin-B CTD interacted with Kir6.2 from cardiac lysates (Fig. 2E). This interaction was specific for ankyrin-B versus ankyrin-G, as purified GST-ankyrin-G MBD (74% identical to ankyrin-B MBD at amino acid level) lacked Kir6.2 binding activity (Fig. 2E). Conversely, identical assays demonstrated that ankyrin-B MBD-GST was unable to associate with Kir6.1 from detergent-soluble heart lysates (Fig 2F). Thus, cardiac ankyrin-B associates with Kir6.2 via its MBD and this interaction is specific for ankyrin-B versus ankyrin-G. Finally, we tested the requirements on Kir6.2 for cardiac ankyrin-B binding. Our group previously identified an eight amino acid motif in the C-terminal domain of Kir6.2 necessary for ankyrin-B association in pancreas (14). A biotinylated version of this peptide was used to evaluate the ability to pull down ankyrin-B from heart lysate. As demonstrated in Figure 2G, this peptide interacted with ankyrin-B from heart lysate, with no appreciable pull-down in the control reaction (Fig. 2G; streptavidin beads alone). Likewise, the corresponding peptide from Kir6.1 was unable to pull-down ankyrin-B from detergent-soluble heart lysates (Fig. 2H). Collectively, these findings support an interaction between cardiac ankyrin-B and the alpha-subunit Kir6.2 of the cardiac K<sub>ATP</sub> channel complex.

Ankyrin-B forms a ternary complex with Kir6.2 and SUR1/SUR2A

Interaction between Kir6.2 and K<sub>ATP</sub> channel SUR subunits regulate K<sub>ATP</sub> channel function (23). In fact, mice harboring mutant K<sub>ATP</sub> alpha and beta subunits where this association is affected display significant dysfunction (24-26). To determine whether association with ankyrin-B affects association of Kir6.2 with SUR beta subunits, HEK293 cells (express endogenous ankyrin-B, but lack Kir6.2, SUR1, or SUR2A) were transfected with Kir6.2 cDNA in the presence of absence of SUR1 or SUR2A. As expected, Kir6.2 Ig co-immunoprecipitated both SUR1 and SUR2A from co-transfected cells (Fig. 3A). Likewise, ankyrin-B Ig co-immunoprecipitated Kir6.2 in all cells with Kir6.2 cDNA (Fig. 3B). Notably, Kir6.2 also co-immunoprecipitated ankyrin-B with SUR1 and SUR2A demonstrating the presence of a ternary complex between these proteins in heterologous cells (Fig. 3A). Additionally, these data suggest that the interaction of Kir6.2 with ankyrin-B does not block association of either SUR beta subunit with Kir6.2. Importantly, despite the reduced levels of SUR1 and SUR2A in ankyrin-B<sup>+/−</sup> cardiac lysates, we did not observe association of SUR1 or SUR2A with ankyrin-B in transfected cells lacking Kir6.2 expression (Fig. 3A-B). Collectively, these data demonstrate the presence of a ternary complex of ankyrin-B with Kir6.2 and SUR1/SUR2A. Furthermore, these new data demonstrate that this ternary complex is mediated by both ankyrin-B and SUR beta subunit interactions with Kir6.2.

Ankyrin-B<sup>+/−</sup> cardiomyocytes display decreased Kir6.2 membrane expression and reduced I<sub>KATP</sub>

Based on reduced Kir6.2 expression in ankyrin-B<sup>+/−</sup> heart, we evaluated the role of ankyrin-B deficiency on Kir6.2 expression in single isolated cardiomyocytes. Consistent with previous findings (19), Kir6.2 was localized primarily to the transverse-tubule network of isolated adult mouse cardiomyocytes (Fig. 4A). Loss of ankyrin-B resulted in decreased levels of Kir6.2 immunostaining throughout the cardiomyocyte, and particularly across the transverse-tubule network (Fig. 4B). Thus, loss of Kir6.2 by immunoblot (Fig. 1B) was paralleled by decreased Kir6.2 immunostaining in primary ankyrin-B<sup>+/−</sup> isolated cardiomyocytes (Fig. 4B). While ankyrin-B has previously been shown to affect the membrane localization of Na/Ca exchanger and Na/K ATPase, ankyrin-B loss did not affect the localization of other critical cardiac ion channels and transporters including Cav1.2, Nav1.5, or SERCA (6,9).
We performed functional electrophysiological analysis of $K_{\text{ATP}}$ channel function to quantitatively evaluate the effects of ankyrin-B on Kir6.2 membrane expression in heart. $I_{\text{KATP}}$ was analyzed in excised membrane patches from wild-type and ankyrin-B$^{+/\text{c}}$ cardiomyocytes at 0 micromolar ATP to promote opening of all membrane-bound $K_{\text{ATP}}$ channels (current inhibited by ATP). In agreement with immunoblot and immunostaining results, we observed a nearly 50% decrease in membrane $I_{\text{KATP}}$ density in ankyrin-B$^{+/\text{c}}$ cardiomyocytes compared with wild-type myocytes (Fig. 4C). In summary, ankyrin-B directly associates with Kir6.2 and is necessary for normal Kir6.2 membrane expression in cardiomyocytes.

Ankyrin-B regulates $K_{\text{ATP}}$ channel ATP sensitivity and Po

We next analyzed the role of ankyrin-B for cardiac $I_{\text{KATP}}$ membrane regulation. Notably, we observed differences in $I_{\text{KATP}}$ ATP sensitivity in ankyrin-B$^{+/\text{c}}$ cardiomyocytes. Specifically, $I_{\text{KATP}}$ (analyzed in excised inside-out membrane patches) in ankyrin-B$^{+/\text{c}}$ myocytes was less sensitive to inhibitory ATP when compared with wild-type myocytes (Fig. 5A-D; $K_{1/2}$ ([ATP] causing half-maximal inhibition= 24.38 µM for WT and 41.47 µM for ankyrin-B$^{+/\text{c}}$ and $p<0.01$). The slope index was equivalent between cardiomyocyte genotypes (Fig. 5E; $p>0.05$).

Finally, we measured the single channel open probability of $K_{\text{ATP}}$ channels from WT and ankyrin-B$^{+/\text{c}}$ mouse cardiomyocytes. In the absence of ATP, we observed a significant difference between Po in ankyrin-B$^{+/\text{c}}$ cardiomyocytes compared with WT cardiomyocytes (Fig. 6A-C 0.62±0.03 and 0.48±0.05, respectively; $n=10$; $p<0.05$). This increased Po in ankyrin-B$^{+/\text{c}}$ cardiomyocytes may represent a compensatory response for the decreased $I_{\text{KATP}}$. Thus, our collective data demonstrate that loss of ankyrin-B significantly reduces the membrane localization of $K_{\text{ATP}}$ channels and alters the regulation of residual membrane $K_{\text{ATP}}$ channels.

Ankyrin-B organizes complexes of functionally-related membrane proteins

In heart and other tissues, $K_{\text{ATP}}$ channel function has been tightly linked with the activity of the Na/K ATPase (27,28). Notably, work from our group and others has demonstrated direct high affinity interaction of ankyrin with Na/K ATPase (ankyrin-B in heart), and loss of membrane Na/K ATPase in ankyrin-deficient cells (9,29-31). Given our new data demonstrating that ankyrin-B associates with Kir6.2 in heart, as well as previous evidence that both ankyrin-B and ankyrin-R can form hetero-complexes between two ankyrin-binding proteins (9,32), we tested whether ankyrin-B could form a multi-protein complex of Na/K ATPase and Kir6.2 in heart. To test this complex, we conducted a series of co-immunoprecipitation experiments using detergent-soluble lysates of adult mouse heart. Ankyrin-B Ig co-immunoprecipitated Na/K ATPase and Kir6.2, as well as the cardiac Na/Ca exchanger (Fig. 7A; also ankyrin-B partner (33)). Na/K ATPase Ig co-immunoprecipitated ankyrin-B, Na/Ca exchanger, and Kir6.2 (Fig. 7C). Moreover, Kir6.2 Ig co-immunoprecipitated ankyrin-B, Na/K ATPase, and Na/Ca exchanger from heart lysates (Fig. 7B). Finally, Na/Ca exchanger Ig co-immunoprecipitated 220 kDa ankyrin-B, Na/K ATPase, and Na/Ca exchanger (Fig. 7D). Notably, consistent with previous studies, we did not observe association of ankyrin-B-targeted proteins with other cardiac membrane or structural proteins including Cav1.2, Nav1.5, or SERCA2 (Fig. 7E-F). These mutual co-immunoprecipitations provide evidence for a macro-molecular complex in heart containing ankyrin-B associated with Na/K ATPase, Kir6.2, and the Na/Ca exchanger.

We further examined whether ankyrin-B was required for mutual co-immunoprecipitation of Na/Ca exchanger, Na/K ATPase, and Kir6.2 by comparing wild type and ankyrin-B$^{+/\text{c}}$ hearts (deficient in ankyrin-B, see Fig. 2A). Ankyrin-B$^{+/\text{c}}$ hearts express reduced levels of 220 kDa ankyrin-B, Na/K ATPase, and Kir6.2 (Fig. 1). Strikingly, ankyrin-B$^{+/\text{c}}$ heart lysates exhibited over 70% loss in the ability of ankyrin-B Ig to co-immunoprecipitate Kir6.2, Na/K ATPase, and Na/Ca exchanger, even when the quantity of lysate was increased (doubled in these experiments) to equalize the starting amount of wild type ankyrin-B levels (Fig. 7A). Moreover, a
similar reduction in Na/Ca exchanger co-immunoprecipitation of the Na/K ATPase and Kir6.2 occurred using doubled ankyrin-B+/- lysates (Fig. 7D). Na/K ATPase also failed to associate with a significant fraction of Na/Ca exchanger or Kir6.2 from ankyrin-B+/- double lysates (Fig. 7C). Finally, Kir6.2 Ig immunoprecipitated minimal levels of Na/K ATPase and Na/Ca exchanger from ankyrin-B+/- double lysates (Fig. 7B). These data suggest that a specialized population of ankyrin-B, that is decreased in the ankyrin-B+/- mouse heart, is essential for ankyrin-B interactions with Na/K ATPase, Na/Ca exchanger, and Kir6.2.

DISCUSSION

Our new findings demonstrate a role of ankyrin-B for Kir6.2 membrane expression and regulation in heart. KATP channels have now been recognized to serve a cardioprotective role in ischemia via a KATP-channel-mediated shortening of the cardiac action potential (34). Notably, acute global ischemia has a greater negative impact on heart function in Kir6.2 knockout animals compared to wild-type (reviewed in (35,36)). Additionally, KATP channel activity has been implicated in the mechanism of ischemic preconditioning (21,37,38).

Ankyrin-B has previously been identified as a critical component required for cardiac Na/K ATPase membrane expression (6,7,9). Numerous studies have demonstrated a role for the Na/K ATPase in the heart’s response to ischemia reperfusion (39). Notably, a functional link between Na/K ATPase and KATP has also been described (27). Haruna and colleagues demonstrated that a coordinate interaction between the KATP channel and Na/K ATPase modulates ischemic preconditioning (27). Here, we identify a potential molecular link between the KATP channel and Na/K ATPase. Specifically, we demonstrate that ankyrin-B regulates Kir6.2 and Na/K ATPase membrane expression, and may directly couple Kir6.2 and Na/K ATPase in heart. Thus, our new data suggest that ankyrin-B may provide a functional/physical linkage between the sarcolemmal KATP channel and the Na/K ATPase.

Our group has recently described a role for ankyrin-B in the membrane expression and metabolic regulation of the KATP channel in pancreatic beta cells (14). While pancreatic beta cells express only Kir6.2 and SUR1 subunits, the heart expresses Kir6.1, Kir6.2, SUR1, and SUR2A subunits. There has been little published data regarding the mechanisms required for regulating the membrane expression of specific KATP channel populations in heart. Our data suggest that ankyrin-B associates specifically with Kir6.2-containing channels, as ankyrin-B and Kir6.1 do not interact in the heart. These data argue that Kir6.1-containing cardiac KATP channels are regulated by an ankyrin-B-independent mechanism. Furthermore, our data suggest that inhibitory gating regulation by ATP of Kir6.2-containing KATP channels is dependent on ankyrin-B. An exciting future direction in the field will be to define additional components of the ankyrin-B/Kir6.2 macromolecular complex in heart.

For many years, the ion channel field has struggled to identify the molecular link between the KATP channel and the cytoskeleton (40-44). The presence of an adapter complex was first proposed in the mid 1990’s, based on a series of elegant experiments that demonstrated a striking stimulatory effect of actin filament disrupting agents on KATP channel opening (42). Specifically, disruption of actin cytoskeleton has been shown to activate KATP channel activity in excitable cells by reducing the sensitivity of KATP channels to ATP-dependent channel closure (40-44). Thus, impairment in ATP sensitivity indicates a transduction pathway of inhibitory gating signals determined by the integrity of the submembrane cytoskeletal network. This suggests that the association of KATP channels with the cytoskeleton is critical to the modulation of ligand-dependent regulation. Considering that ankyrins link ion channels, such as voltage-gated sodium channels, the Na/K ATPase, anion exchanger, and the Na/Ca exchanger, to the actin-based cytoskeleton (22), our new data may finally provide new insight on the identity of the link between the cardiac cytoskeleton and KATP channel function.

Finally, while our data demonstrate a requirement of ankyrin-B for Kir6.2 membrane
expression, the specific cellular role(s) for cardiac ankyrin-B remain unclear. Similar to the role of ankyrin-R in the erythrocyte plasma membrane (45-47), cardiac ankyrin-B may act as a membrane scaffolding protein to link Kir6.2 with the underlying actin- and spectrin-based cytoskeleton. Alternatively, ankyrin-B may play critical roles in the trafficking and/or membrane retention of Kir6.2 channels to/at the cardiomyocyte plasma membrane. A third option is that ankyrin-B has multiple roles in the active trafficking of Kir6.2 to the plasma membrane, as well as key roles in the retention and stabilization of Kir6.2 in relation to the plasma membrane and cytoskeleton. An important future goal in the field will be to identify the specific cellular roles of ankyrin polypeptides in heart.
REFERENCES


**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1. Decreased K\textsubscript{ATP} channel subunit expression in ankyrin-B\textsuperscript{+/-} hearts. Detergent-soluble ankyrin-B\textsuperscript{+/-} heart lysates express significantly less A) ankyrin-B, B) Kir6.2, C) SUR1, D) SUR2A, H) NKA, and I) NCX1. There were no significant differences in the expression of E) ankyrin-G, F) Kir6.1, or G) NHERF1 (loading control). J) Quantitative data demonstrating a significant decrease in the expression of ankyrin-B, NCX1, NKA, and K\textsubscript{ATP} channel proteins in ankyrin-B\textsuperscript{+/-} detergent-soluble mouse heart lysates compared to wild type. Expression of proteins was normalized to wild-type expression.

Fig. 2. Ankyrin-B associates with Kir6.2 in heart. Ankyrin-B Ig co-immunoprecipitates Kir6.2 from detergent-soluble heart lysate (A) and Kir6.2 Ig co-immunoprecipitates ankyrin-B from detergent-soluble heart lysate (B; note no interaction with control Ig). The association with ankyrin-B is specific to Kir6.2 given that ankyrin-B Ig does not co-immunoprecipitate Kir6.1 (D) nor does Kir6.1 Ig co-immunoprecipitate ankyrin-B (C) from detergent-soluble heart lysates (note no interaction with control Ig). E-F) The ankyrin-B membrane-binding domain (MBD), but not spectrin-binding domain (SBD) or C-terminal domain (CTD) associates with Kir6.2 (E), but not Kir6.1 (F) from detergent-soluble heart lysate (note no interaction with GST control beads). Moreover, Kir6.2 specifically associates with the MBD of ankyrin-B and not the structurally-similar MBD of ankyrin-G (E). G) A biotinylated peptide based on a previously-determined ankyrin-binding motif of Kir6.2 is sufficient to associated with ankyrin-B from detergent-soluble heart lysates, while the analogous peptide from Kir6.1 was unable to bind ankyrin-B (note no interaction with streptavidin control beads).

Fig. 3. Ankyrin-B, Kir6.2, and SUR1/SUR2A associate in a ternary complex. A-B) Co-immunoprecipitation experiments demonstrate association of ankyrin-B, Kir6.2, and SUR1/SUR2A. HEK293 cells (express ankyrin-B, but not Kir6.2, SUR1, or SUR2A; C, first lane) were transfected with Kir6.2, and/or SUR1/SUR2A as noted in lower panel. Cell lysates were immunoprecipitated with either A) Kir6.2 Ig or B) ankyrin-B Ig, and bound protein was analyzed using SDS-PAGE and immunoblot using ankyrin-B, Kir6.2, SUR1, and SUR2A antibodies. Note that Kir6.2 expression is required for ankyrin-B/SUR1 and ankyrin-B/SUR2A association. C) Expression control demonstrating that HEK293 cells express transfected proteins.

Fig. 4. Ankyrin-B\textsuperscript{+/-} myocytes display decreased Kir6.2 membrane expression and reduced I\textsubscript{KATP}. Kir6.2 expression in (A) wild-type and (B) ankyrin-B\textsuperscript{+/-} adult mouse cardiomyocytes. Note reduced Kir6.2 expression in ankyrin-B\textsuperscript{+/-} cardiomyocytes, particularly overlying the transverse-tubule network. Scale bar equals 10 microns; nuclei are stained (purple) with topo-3AM 633. (C) Functional measurements of I\textsubscript{KATP} in wild-type and ankyrin-B\textsuperscript{+/-} adult cardiomyocytes. Measurements were made at 0 mM ATP to open all available membrane K\textsubscript{ATP} channels. Note that I\textsubscript{KATP}/patch was decreased nearly 50% in ankyrin-B\textsuperscript{+/-} cardiomyocytes (n=51 myocytes/genotype; p<0.005).

Fig. 5. Ankyrin-B regulates myocyte I\textsubscript{KATP} ATP sensitivity. (A-D) In excised inside-out membrane patches, K\textsubscript{ATP} channels in ankyrin-B\textsuperscript{+/-} myocytes were less sensitive to inhibitory ATP than in wild-type myocytes. C) Mean K\textsubscript{1/2} for ankyrin-B\textsuperscript{+/-} =41.47 μM; for wild-type=24.38 μM /L respectively (p<0.01). Data in A-B depict I\textsubscript{KATP} recorded from cell membrane patches excised from wild-type and ankyrin-B\textsuperscript{+/-} ventricular myocytes under various ATP concentrations applied to the cytoplasmic membrane face. D) Steady-state dependence of membrane currents on [ATP] normalized to the 0 μM ATP condition (I\textsubscript{KATP, REL}). Lines correspond to least squares fits of the Hill equation (see methods). At all corresponding ATP concentrations, I\textsubscript{KATP} is significantly greater in ankyrin-B\textsuperscript{+/-} than in wild-type ventricular myocytes, indicating that K\textsubscript{ATP} channels of ankyrin-B\textsuperscript{+/-} ventricular myocytes were less sensitive to inhibitory ATP than those of wild-type. E) The slope index is the same in both genotypes (p>0.05).
**Fig. 6.** $K_{\text{ATP}}$ channels from wild-type and ankyrin-B$^{+/−}$ mice exhibit different open probability in the absence of ATP. (A-B) Examples of single channel recordings from wild-type and ankyrin-B$^{+/−}$ cardiomyocytes. The calibration bars indicate 200 ms (abscissa) and 5 pA (ordinate). (B) Bar graphs represent calculated single channel $I_{K_{\text{ATP}}}$ open probability in wild-type and ankyrin-B$^{+/−}$ mice. The $P_o$ was significant greater in ankyrin-B$^{+/−}$ than in wild-type myocytes (0.62±0.03 and 0.48±0.05, respectively; $p<0.05$; $n=10$).

**Fig. 7.** Ankyrin-B forms a macromolecular complex with $K_{\text{ATP}}$, Na/K ATPase (NKA), and Na/Ca exchanger (NCX) that is reduced in ankyrin-B$^{+/−}$ heart. A-D) Detergent-soluble lysates from wild-type and ankyrin-B$^{+/−}$ mouse hearts were used for co-immunoprecipitations with indicated antibodies (IB, immunoblot; IP, immunoprecipitation). Co-immunoprecipitations of ankyrin-B$^{+/−}$ lysates used doubled amounts of input lysate to compensate for the reduction of ankyrin-B. E-F) Control co-immunoprecipitations using detergent-soluble lysates from wild-type and ankyrin-B$^{+/−}$ mouse hearts demonstrating no interaction between NKA and NCX with SERCA2, Ca$_{1.2}$, and Na$_{1.5}$.
Figure 1

A. WT AnkB +/-
IB: AnkB

B. WT AnkB +/-
IB: Kir6.2

C. WT AnkB +/-
IB: SUR1

D. WT AnkB +/-
IB: SUR2A

E. WT AnkB +/-
IB: Ankg

F. WT AnkB +/-
IB: Kir6.1

G. WT AnkB +/-
IB: NHERF1

H. WT AnkB +/-
IB: Na/K ATPase

I. WT AnkB +/-
IB: NCX

J. Relative Expression
(normalized for loading control)

- Wildtype
- Ankyrin-B +/-

* Indicates significance.
Figure 4

A wild-type

B ankyrin-B^{+/−}

C

IK_{ATP} (pA/patch)

<table>
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<th>ankyrin-B^{+/−}</th>
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*
Figure 5

A

ATP μM

0

25

100

1000

0

25

100

1000

20 s

B

ATP μM

0

25

100

1000

0

25

100

1000

20 s

C

K_{12}(μM)

0

10

20

30

40

50

wild-type

ankyrin-B+/−

(51)

(51)

D

[ATP] μM

1

10

100

1000

IK_{ATP} Relative Current

0.0

0.2

0.4

0.6

0.8

1.0

wild-type (n=51)

ankyrin-B+/− (n=51)

*** p<0.0001

** p<0.005

* p<0.05

E

Slope Index

0.0

1.0

1.5

2.0

wild-type

ankyrin-B+/−

(51)

(51)
Figure 6

A  wild-type

B  ankyrin-B^{+/-}

$5 \text{ pA}$  $200 \text{ ms}$

C  Open Probability (Po)

$0.0$  $0.2$  $0.4$  $0.6$  $0.8$

wild-type  ankyrin-B^{+/-}

(10)  (10)

*
Figure 7

A

Ctrl Ig | WT lysate | 2x AnkB+/− lysate

IB: Kir6.2
IB: NCX
IB: NKA

IP: AnkR Ig

B

Ctrl Ig | WT lysate | 2x AnkB+/− lysate

IB: AnkB
IB: NCX
IB: NKA

IP: Kir6 Ig

C

Ctrl Ig | WT lysate | 2x AnkB+/− lysate

IB: AnkB
IB: Kir6.2
IB: NCX

IP: NKA Ig

D

Ctrl Ig | WT lysate | 2x AnkB+/− lysate

IB: AnkB
IB: Kir6.2
IB: NCX
IB: NKA

IP: NCX Ig

E

Input | Ctrl Ig | NKA Ig

IB: SERCA2
IB: Cav1.2
IB: Nav1.5

F

Input | Ctrl Ig | NCX Ig

IB: SERCA2
IB: Cav1.2
IB: Nav1.5