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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 in cardiac cell biology. We demonstrate that cardiac sarcolemmal K_{ATP} channels directly associate with ankyrin-B in heart via the K_{ATP} channel α-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 for 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 in 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*, and *ANK3*) encode a host of structurally similar but functionally distinct ankyrin polypeptides (ankyrin-R, ankyrin-B, and ankyrin-G, respectively) with specific roles in erythrocyte membrane structure, cardiac excitability, polarized epithelial ion regulation, and neuronal development. In heart, ankyrins regulate membrane excitability by coordinating the expression of voltage-gated Na^+ and Ca^{2+} 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 K_{ATP} channel regulation. Ankyrin-B associates with the cardiac K_{ATP} 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_{KATP}. Moreover, we demonstrate that ankyrin-B regulates cardiac K_{ATP} channel gating. Finally, we demonstrate that ankyrin-B coordinates a complex of the K_{ATP} 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 for ankyrin-B in 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 killed 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 heparin (187 USP units/mg). Ventricular myocytes were

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isolated from Langendorff-perfused hearts not subjected to ischemia, as described previously (15). I_{KATP} from ventricular myocytes was recorded with inside-out patch-clamp configuration of the patch-clamp technique using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA), monitored, and stored using a pCLAMP 10 data acquisition system (Molecular Devices, Inc., Sunnyvale, CA) (15). Tip resistance was 1.5–3 megohms 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). The standard bath (intracellular) and pipette (extracellular) solution used in patch-clamp experiments was 140 mM KCl, 10 mM K^+-HEPES, and 10 mM K^+-EGTA (pH 7.3) with KOH. I_{KATP} was evoked by a membrane potential of −50 mV (pipette potential of +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. The steady-state dependence of membrane current on [ATP] was obtained by calculating the relative current indexed to zero ATP (I_{rel}). The data were fitted using the Hill equation: $I_{rel} = 100/(1 + ([ATP]/K_{50})H$, where I_{rel} is the relative current, K_{50} is the concentration causing half-maximal blockade, and H is the Hill coefficient. Single-channel I_{KATP} recordings were obtained from inside-out patches using fire-polished pipettes (resistance of ~9–12 megohms) when filled with pipette solution containing 140 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES-KOH (pH 7.3). Cardiac cells were superfused with intracellular solution containing 140 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 5 mM HEPES-KOH (pH 7.3) in the absence or presence of ATP, and recordings were 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. The signal was low pass-filtered at 1 kHz and sampled every 50 ms. The threshold for judging the open state of K_{ATP} channels was set at half of the single-channel amplitude. nP_o, where n represents the number of channels in the patch and P_o 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 ImmunoResearch Laboratories). Both wild-type and ankyrin-$B^{+/-}$ mice were housed in the same facility, consumed the same diet, were provided water *ad libitum*, and were kept on identical 12-h 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 3 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 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and homogenized. The homogenate was centrifuged at 1000 × *g* at 4 °C 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 × *g* for 1 h at 4 °C. The resulting supernatant was quantitated by BCA assay 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 and anti-Kir6.1 (Alomone), anti-SUR1 and anti-SUR2A (Santa Cruz Biotechnology), anti-NHERF1 (polyclonal), anti-NCX1 (RDI), anti-Na/K-ATPase (Upstate), anti-SERCA2 (polyclonal), anti-$Ca_v1.2$ (polyclonal), and anti-$Na_v1.5$ (polyclonal).

Co-immunoprecipitation—Protein A-conjugated agarose beads (Rockland Immunochemicals, Inc.) were incubated with either control IgG or affinity-purified anti-ankyrin-B, anti-Kir6.2, anti-NCX1, or anti-Na/K-ATPase antibody in co-immunoprecipitation binding buffer (PBS with 0.1% Triton X-100 and protease inhibitor mixture (Sigma)) for 12 h at 4 °C. Beads were centrifuged and washed three times with ice-cold PBS. 100 μg of wild-type heart lysate or 200 μg of ankyrin-$B^{+/-}$ heart lysate was added to the washed beads, along with protease inhibitor mixture and co-immunoprecipitation binding buffer, and incubated for 12 h at 4 °C. The reactions were washed three times with ice-cold co-immunoprecipitation buffer. The samples were eluted, and the proteins were separated by SDS-PAGE prior to immunoblotting.

Pulldown Analysis Using Kir6.2 Ankyrin-B-binding Motif Peptide—A biotinylated Kir6.2 oligopeptide (GQRFVPIV-AEEDGR; Biosynthesis, Inc.) was constructed with an SGSG linker between the biotin and Kir6.2 peptide sequence. Additionally, a biotinylated Kir6.1 oligopeptide (analogous to the Kir6.2 oligopeptide sequence; GHRFVSIVTEEEGV; Biosynthesis, Inc.) was constructed with an SGSG linker between the biotin and Kir6.1 peptide sequence. 20 μg of oligopeptide was conjugated to a 40-μl bead volume of streptavidin beads (Thermo Scientific) for 4 h at 4 °C in binding buffer (PBS, 1% Triton X-100, and protease inhibitor mixture). The beads were centrifuged and washed three times with binding buffer, and 100 μg of freshly prepared wild-type heart lysate was added to the washed beads, along with 500 μl of binding buffer and protease inhibitors. The reactions were incubated overnight at 4 °C and then centrifuged and washed three times with binding buffer prior to elution, SDS-PAGE, and immunoblotting with anti-ankyrin-B Ig.

Cell Culture—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS (HyClone) and 0.1% penicillin/streptomycin. Cells were cultured at 37 °C in 5% CO_2.

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 h prior to transfection (30% confluence at time of transfection). Effectene reagent (Qiagen) was used to transfect cells with 0.2 μg of Kir6.2 pcDNA3.1$^+$ with or without 0.2 μg of SUR1 pcDNA3.1$^+$ and/or 0.2 μg of SUR2A pcDNA3.1$^+$ following the manufac-

turer's instructions. Transfection was carried out for 9 h at 37 °C, and the cells were allowed to recover overnight (16–18). After 48 h, the cells were lysed and centrifuged at high speed to remove debris, and the supernatant was used in subsequent co-immunoprecipitation and immunoblotting experiments.

Imaging of Adult Cardiomyocytes—Adult ventricular cardiomyocytes were stained according to the protocol described by Morrissey *et al.* (19). Briefly, single dissociated myocytes were plated onto fibronectin-coated MatTek plates and incubated at 37 °C for 15 min to allow attachment before fixation. Myocytes were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with ice-cold 100% methanol at −20 °C for 5 min. Myocytes were then washed with Ca^{2+}- and Mg^{2+}-free PBS. Washed myocytes were incubated with 0.1% Triton X-100 (in PBS) for 15 min at room temperature, followed by washing (2× 5 min) with room temperature PBS and blocking (2× 10 min) with 5% fetal bovine serum in PBS. The cells were incubated with primary antibody (1 h at room temperature), washed (3× 10 min in PBS serum), and incubated with secondary antibodies (45 min at room temperature). Following washing (4× 10 min) with PBS, the cells were mounted with VECTASHIELD and covered with No. 1 glass coverslips.

Pulldown Experiments—10 μg of AnkB[3] MBD-GST, AnkB SBD-GST, AnkB CTD-GST, AnkG MBD-GST, or GST alone was incubated with glutathione-agarose (Amersham Biosciences) overnight at 4 °C. The beads were centrifuged and washed three times with binding buffer. 100 μg of wild-type heart lysate was added to the beads, along with 500 μl of binding buffer and protease inhibitor mixture, and the reactions were incubated for 12 h at 4 °C. The beads were centrifuged and washed three times with binding buffer. The proteins were eluted, separated by SDS-PAGE, and immunoblotted using anti-Kir6.2 or anti-Kir6.1 Ig.

Statistics—Data are presented as the mean ± S.E., and statistical significance was estimated using Student's *t* test or analysis of variance, as appropriate (SigmaStat). Post hoc comparisons after analysis of variance were performed using the Holm-Sidak test. The null hypothesis was rejected for $p \leq 0.05$.

RESULTS

K_{ATP} Channel Subunit Expression Is Decreased in 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 previously been associated with ankyrin in pancreatic beta cells (14). We investigated K_{ATP} channel subunit expression in ankyrin-$B^{+/-}$ mouse heart lysates by immunoblotting. We first evaluated cardiac K_{ATP} channel α-subunit (Kir6.1 and Kir6.2) and β-subunit (SUR1 and SUR2A) expression. We observed decreased Kir6.2 expression in ankyrin-$B^{+/-}$ ventricular tissue compared with wild-type littermate hearts (~46% decrease; $n = 4$; $p < 0.01$) (Fig. 1*B*). Notably, we also observed a parallel decrease in the expression of both K_{ATP} channel β-subunits, SUR1 and SUR2A (51 and 47% decreases, respectively; $n = 4$; $p < 0.01$) in ankyrin-$B^{+/-}$ ventricle (Fig. 1, *C* and *D*). Although present in mouse ventricle, Kir6.1 was not altered by ankyrin-B deficiency (not significant; $n = 4$) (Fig. 1*F*). As expected, ankyrin-B expression was significantly reduced in ankyrin-$B^{+/-}$ whole heart lysates (57% decrease; $n = 4$; $p < 0.001$) (Fig. 1*A*), whereas the expression levels of the related ankyrin gene product, ankyrin-G, and NHERF1 (loading control) were unchanged (not significant; $n = 4$) (Fig. 1, *E* and *G*). Consistent with previous findings (6), NCX ($\underline{Na}^{2+}/\underline{Ca}^{2+}$ exchanger) and Na/K-ATPase expression levels were reduced in ankyrin-$B^{+/-}$ ventricle ($n = 4$; $p < 0.05$) (Fig. 1, *H* and *I*).

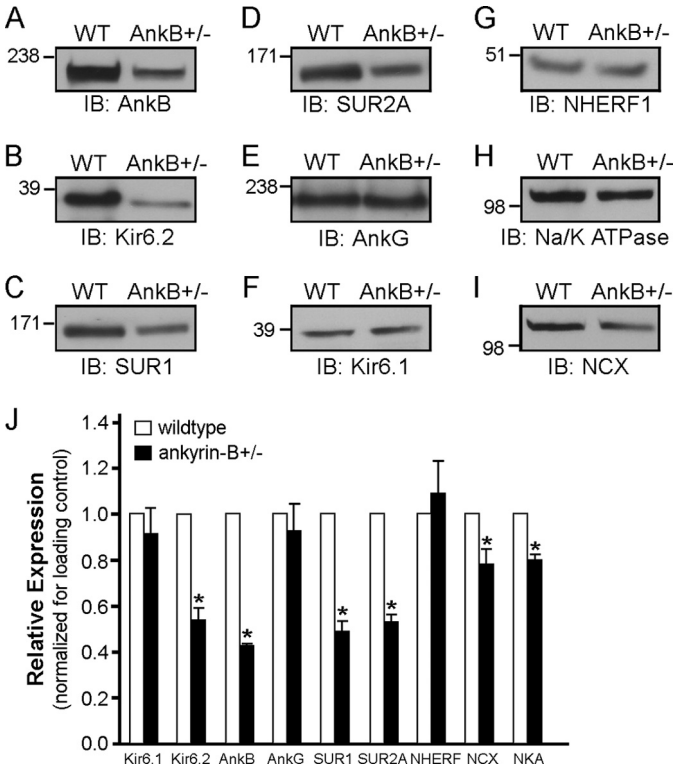

FIGURE 1. **Decreased K_{ATP} channel subunit expression in ankyrin-$B^{+/-}$ hearts.** Detergent-soluble ankyrin-$B^{+/-}$ heart lysates expressed significantly less ankyrin-B (*A*), Kir6.2 (*B*), SUR1 (*C*), SUR2A (*D*), Na/K-ATPase (*H*), and NCX1 (*I*). There were no significant differences in the expression of ankyrin-G (*E*), Kir6.1 (*F*), or NHERF1 (loading control; *G*). *J*, quantitative data demonstrating a significant decrease in the expression of ankyrin-B, NCX1, Na/K-ATPase, and K_{ATP} channel proteins in ankyrin-$B^{+/-}$ compared with wild-type detergent-soluble mouse heart lysates. Expression of proteins was normalized to wild-type expression. *IB*, immunoblot. *, $p < 0.05$.

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. 2*A*). Conversely, anti-Kir6.2 Ig co-immunoprecipitated ankyrin-B from detergent-soluble lysates (Fig. 2*B*). We observed no interaction between ankyrin-B or Kir6.2 and control Ig (Fig. 2, *A* and *B*). Moreover, we observed no interaction of Kir6.1 with ankyrin-B using an identical assay (Fig. 2, *C* and *D*). These data support an *in vivo* interaction between ankyrin-B and Kir6.2, but not Kir6.1, in heart.

[3] The abbreviations used are: AnkB, ankyrin-B; MBD, membrane-binding domain; SBD, spectrin-binding domain; CTD, C-terminal domain.

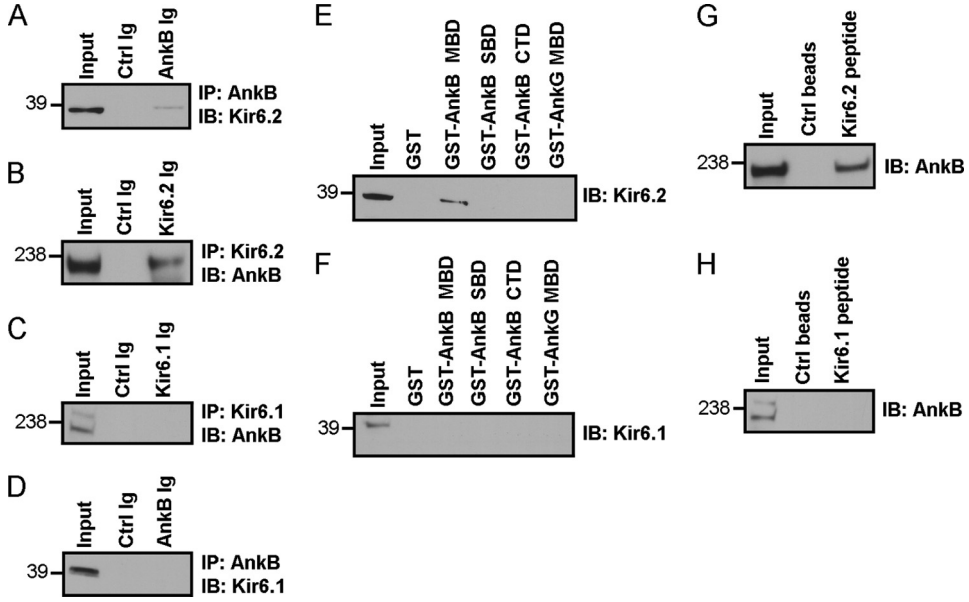

FIGURE 2. **Ankyrin-B associates with Kir6.2 in heart.** Anti-ankyrin-B Ig co-immunoprecipitated Kir6.2 (*A*) and anti-Kir6.2 Ig co-immunoprecipitated ankyrin-B (*B*) from detergent-soluble heart lysates (note no interaction with control Ig). The association with ankyrin-B was specific to Kir6.2 given that anti-ankyrin-B Ig did not co-immunoprecipitate Kir6.1 (*D*) and anti-Kir6.1 Ig did not co-immunoprecipitate ankyrin-B (*C*) from detergent-soluble heart lysates (note no interaction with control Ig). The ankyrin-B MBD, but not SBD or CTD, associated with Kir6.2 (*E*), but not Kir6.1 (*F*), from detergent-soluble heart lysates (note no interaction with GST control beads). Moreover, Kir6.2 specifically associated with the MBD of ankyrin-B and not the structurally similar MBD of ankyrin-G (*E*). A biotinylated peptide based on a previously determined ankyrin-binding motif of Kir6.2 was sufficient to associate with ankyrin-B from detergent-soluble heart lysates (*G*), whereas the analogous peptide from Kir6.1 was unable to bind ankyrin-B (*H*) (note no interaction with streptavidin control beads). *IB*, immunoblot; *IP*, immunoprecipitation.

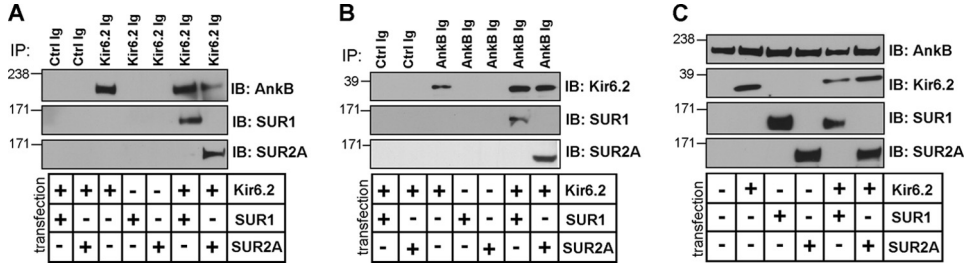

FIGURE 3. **Ankyrin-B, Kir6.2, and SUR1/SUR2A associate in a ternary complex.** *A* and *B*, co-immunoprecipitation experiments demonstrated association of ankyrin-B, Kir6.2, and SUR1/SUR2A. HEK293 cells (which express ankyrin-B, but not Kir6.2, SUR1, or SUR2A; *C*, *first lane*) were transfected with Kir6.2 and/or SUR1/SUR2A as indicated. Cell lysates were immunoprecipitated (*IP*) with either anti-Kir6.2 Ig (*A*) or anti-ankyrin-B Ig (*B*), and bound protein was analyzed by SDS-PAGE and immunoblotting (*IB*) using anti-ankyrin-B, anti-Kir6.2, anti-SUR1, and anti-SUR2A antibodies. Note that Kir6.2 expression was required for ankyrin-B/SUR1 and ankyrin-B/SUR2A association. *C*, expression control demonstrating that HEK293 cells express transfected proteins.

We further evaluated the cardiac ankyrin-B/Kir6.2 interaction using pulldown experiments with mouse detergent-soluble heart lysates. Ankyrin-B is composed of three structural domains: MBD, SBD, and CTD (22). Notably, purified GST-AnkB MBD, but not GST-AnkB SBD or GST-AnkB CTD, interacted with Kir6.2 from cardiac lysates (Fig. 2*E*). This interaction was specific for ankyrin-B *versus* ankyrin-G, as purified GST-AnkG MBD (74% identical to AnkB MBD at the amino acid level) lacked Kir6.2 binding activity (Fig. 2*E*). Conversely, identical assays demonstrated that AnkB MBD-GST was unable to associate with Kir6.1 from detergent-soluble heart lysates (Fig. 2*F*). 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 of Kir6.2 for cardiac ankyrin-B binding. Our group previously identified an 8-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 Fig. 2*G*, this peptide interacted with ankyrin-B from heart lysate, with no appreciable pulldown in the control reaction (streptavidin beads alone) (Fig. 2*G*). Likewise, the corresponding peptide from Kir6.1 was unable to pull down ankyrin-B from detergent-soluble heart lysates (Fig. 2*H*). Collectively, these findings support an interaction between cardiac ankyrin-B and the α-subunit Kir6.2 of the cardiac K_{ATP} channel complex.

Ankyrin-B Forms a Ternary Complex with Kir6.2 and SUR1/SUR2A—Interactions between Kir6.2 and K_{ATP} channel SUR subunits regulate K_{ATP} channel function (23). In fact, mice harboring mutant K_{ATP} α- and β-subunits in which this association is affected display significant dysfunction (24–26). To determine whether association with ankyrin-B affects association of Kir6.2 with SUR β-subunits, HEK293 cells (which express endogenous ankyrin-B but lack Kir6.2, SUR1, and SUR2A) were transfected with Kir6.2 cDNA in the presence or absence of SUR1 or SUR2A. As expected, anti-Kir6.2 Ig co-immunoprecipitated both SUR1 and SUR2A from cotransfected cells (Fig. 3*A*). Likewise, anti-ankyrin-B Ig co-immunoprecipitated Kir6.2 in all cells with Kir6.2 cDNA (Fig. 3*B*). Notably, Kir6.2 also co-immunoprecipitated ankyrin-B with SUR1 and SUR2A (Fig. 3*A*), demonstrating the presence of a ternary complex between these proteins in heterologous cells. Additionally, these data suggest that the interaction of Kir6.2 with ankyrin-B does not block the association of either SUR β-subunit with Kir6.2. Importantly, despite the reduced levels of SUR1 and SUR2A in ankyrin-$B^{+/-}$ cardiac lysates, we did not observe an association of SUR1 or SUR2A with ankyrin-B in transfected cells lacking Kir6.2 expression (Fig. 3, *A* and *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

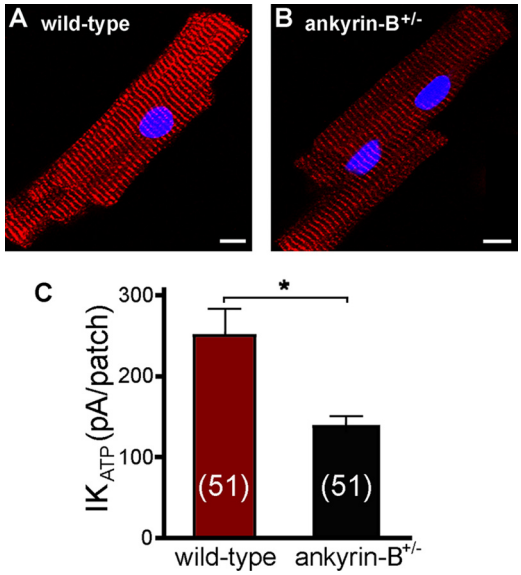

FIGURE 4. **Ankyrin-$B^{+/-}$ myocytes display decreased Kir6.2 membrane expression and reduced I_{KATP}.** *A* and *B*, Kir6.2 expression in wild-type and ankyrin-$B^{+/-}$ adult mouse cardiomyocytes, respectively. Note the reduced Kir6.2 expression in ankyrin-$B^{+/-}$ cardiomyocytes, particularly overlying the transverse tubule network. *Scale bars* = 10 μm. Nuclei stained (*purple*) with TO-PRO-3 AM 633. *C*, functional measurements of I_{KATP} in wild-type and ankyrin-$B^{+/-}$ adult cardiomyocytes. Measurements were made at 0 mM ATP to open all available membrane K_{ATP} channels. Note that I_{KATP}/patch was decreased by nearly 50% in ankyrin-$B^{+/-}$ cardiomyocytes (n = 51 myocytes/genotype; *, $p < 0.005$).

that this ternary complex is mediated by both ankyrin-B and SUR β-subunit interactions with Kir6.2.

Ankyrin-$B^{+/-}$ Cardiomyocytes Display Decreased Kir6.2 Membrane Expression and Reduced I_{KATP}—On the basis of reduced Kir6.2 expression in ankyrin-$B^{+/-}$ heart, we evaluated the role of ankyrin-B deficiency in 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. 4*A*). Loss of ankyrin-B resulted in decreased levels of Kir6.2 immunostaining throughout the cardiomyocyte and particularly across the transverse tubule network (Fig. 4*B*). Thus, loss of Kir6.2 by immunoblotting (Fig. 1*B*) was paralleled by decreased Kir6.2 immunostaining in primary ankyrin-$B^{+/-}$ isolated cardiomyocytes (Fig. 4*B*). Although ankyrin-B has previously been shown to affect the membrane localization of NCX and Na/K-ATPase, ankyrin-B loss did not affect the localization of other critical cardiac ion channels and transporters, including $Ca_v1.2$, $Na_v1.5$, and SERCA (6, 9).

We performed functional electrophysiological analysis of K_{ATP} channel function to quantitatively evaluate the effects of ankyrin-B on Kir6.2 membrane expression in heart. I_{KATP} was analyzed in excised membrane patches from wild-type and ankyrin-$B^{+/-}$ cardiomyocytes at 0 μM ATP to promote opening of all membrane-bound K_{ATP} channels (current inhibited by ATP). In agreement with immunoblotting and immunostaining results, we observed a nearly 50% decrease in membrane I_{KATP} density in ankyrin-$B^{+/-}$ cardiomyocytes compared with wild-type myocytes (Fig. 4*C*). In summary, ankyrin-B directly associates with Kir6.2 and is necessary for normal Kir6.2 membrane expression in cardiomyocytes.

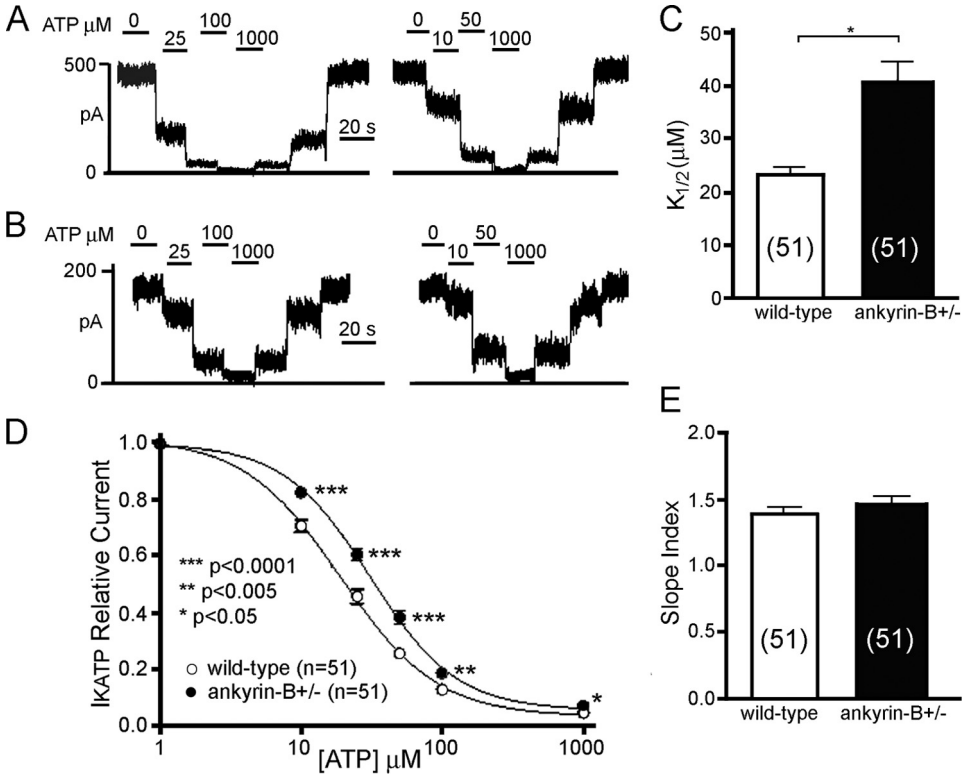

FIGURE 5. **Ankyrin-B regulates myocyte I_{KATP} ATP sensitivity.** *A–D*, in excised inside-out membrane patches, K_{ATP} channels in ankyrin-$B^{+/-}$ myocytes were less sensitive to inhibitory ATP than those in wild-type myocytes. *C*, mean K_{50} for ankyrin-$B^{+/-}$ = 41.47 μM and for WT = 24.38 μM (*, $p < 0.01$). Data in *A* and *B* depict I_{KATP} recorded from cell membrane patches excised from wild-type and ankyrin-$B^{+/-}$ 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 ($I_{KATP,rel}$). *Lines* correspond to least-squares fits of the Hill equation (see "Experimental Procedures"). At all corresponding ATP concentrations, I_{KATP} was significantly greater in ankyrin-$B^{+/-}$ than in wild-type ventricular myocytes, indicating that K_{ATP} channels of ankyrin-$B^{+/-}$ ventricular myocytes are less sensitive to inhibitory ATP than those of WT. *E*, the slope index is the same in both genotypes ($p > 0.05$).

Finally, we measured the single-channel open probability of K_{ATP} channels from WT and ankyrin-$B^{+/-}$ mouse cardiomyocytes. In the

Ankyrin-B Regulates K_{ATP} Channel ATP Sensitivity and P_o—We next analyzed the role of ankyrin-B in cardiac I_{KATP} membrane regulation. Notably, we observed differences in I_{KATP} ATP sensitivity in ankyrin-$B^{+/-}$ cardiomyocytes. Specifically, I_{KATP} (analyzed in excised inside-out membrane patches) in ankyrin-$B^{+/-}$ myocytes was less sensitive to inhibitory ATP compared with wild-type myocytes (K_{50} ([ATP] causing half-maximal inhibition) = 24.38 μM for WT and 41.47 μM for ankyrin-$B^{+/-}$; $p < 0.01$) (Fig. 5, *A–D*). The slope index was equivalent between cardiomyocyte genotypes ($p > 0.05$) (Fig. 5*E*).

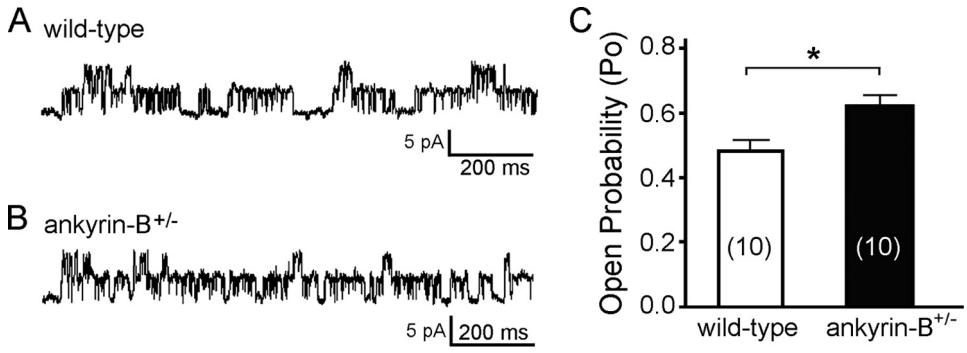

FIGURE 6. **K_{ATP} channels from wild-type and ankyrin-$B^{+/-}$ mice exhibit different open probability in the absence of ATP.** *A* and *B*, shown are examples of single-channel recordings from wild-type and ankyrin-$B^{+/-}$ cardiomyocytes. The *calibration bars* indicate 200 ms (*abscissa*) and 5 pA (*ordinate*). *C*, the bar graph represents calculated single-channel I_{KATP} open probability in wild-type and ankyrin-$B^{+/-}$ mice. P_o was significant greater in ankyrin-$B^{+/-}$ than in wild-type myocytes (0.62 ± 0.03 and 0.48 ± 0.05, respectively; $n = 10$; *, $p < 0.05$).

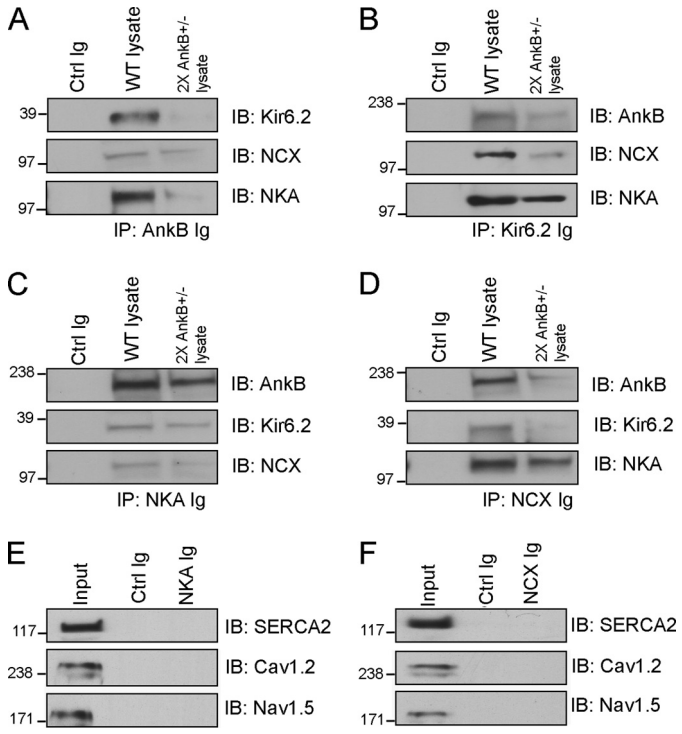

FIGURE 7. **Ankyrin-B forms a macromolecular complex with K_{ATP}, Na/K-ATPase, and NCX that is reduced in ankyrin-$B^{+/-}$ heart.** *A–D*, detergent-soluble lysates from wild-type and ankyrin-$B^{+/-}$ mouse hearts were used for co-immunoprecipitations with the indicated antibodies. *IB*, immunoblot; *IP*, immunoprecipitation. Co-immunoprecipitations of ankyrin-$B^{+/-}$ lysates used doubled amounts of input lysate to compensate for the reduction of ankyrin-B. *E* and *F*, detergent-soluble lysates from wild-type and ankyrin-$B^{+/-}$ mouse hearts were used for control co-immunoprecipitations, demonstrating no interaction of Na/K-ATPase (*NKA*) and NCX with SERCA2, $Ca_v1.2$, and $Na_v1.5$.

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

Ankyrin-B Organizes Complexes of Functionally Related Membrane Proteins—In heart and other tissues, K_{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 heterocomplexes between two ankyrin-binding proteins (9, 32), we tested whether ankyrin-B could form a multiprotein 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. Anti-ankyrin-B Ig co-immunoprecipitated Na/K-ATPase and Kir6.2, as well as cardiac NCX (also ankyrin-B partner (33)) (Fig. 7*A*). Anti-Na/K-ATPase Ig co-immunoprecipitated ankyrin-B, NCX, and Kir6.2 (Fig. 7*C*). Moreover, anti-Kir6.2 Ig co-immunoprecipitated ankyrin-B, Na/K-ATPase, and NCX from heart lysates (Fig. 7*B*). Finally, anti-NCX Ig co-immunoprecipitated 220-kDa ankyrin-B, Na/K-ATPase, and Kir6.2 (Fig. 7*D*). Notably, consistent with previous studies, we did not observe association of ankyrin-B-targeted proteins with other cardiac membrane or structural proteins, including $Ca_v1.2$, $Na_v1.5$, and SERCA2 (Fig. 7, *E* and *F*). These mutual co-immunoprecipitations provide evidence for a macromolecular complex in heart containing ankyrin-B associated with Na/K-ATPase, Kir6.2, and NCX.

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

DISCUSSION

Our new findings demonstrate a role for ankyrin-B in Kir6.2 membrane expression and regulation in heart. K_{ATP} channels have now been recognized to serve a cardioprotective role in ischemia via a K_{ATP} channel-mediated shortening of the cardiac action potential (34). Notably, acute global ischemia has a greater negative impact on heart function in Kir6.2 knock-out animals compared with wild-type animals (reviewed in Refs. 35 and 36). Additionally, K_{ATP} 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 Na/K-ATPase in the heart's response to ischemia reperfusion (39). Notably, a functional link between Na/K-ATPase and K_{ATP} has also been described (27). Haruna *et al.* (27) demonstrated that a coordinate interaction between the K_{ATP} channel and Na/K-ATPase modulates ischemic preconditioning. Here, we have identified a potential molecular link between the K_{ATP} channel and Na/K-ATPase. Specifically, we have demonstrated 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 K_{ATP} channel and Na/K-ATPase.

Our group has recently described a role for ankyrin-B in the membrane expression and metabolic regulation of the K_{ATP} channel in pancreatic beta cells (14). Whereas pancreatic beta cells express only Kir6.2 and SUR1 subunits, 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 K_{ATP} 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 heart. These data argue that Kir6.1-containing cardiac K_{ATP} channels are regulated by an ankyrin-B-independent mechanism. Furthermore, our data suggest that inhibitory gating regulation by ATP of Kir6.2-containing K_{ATP} 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.

Since 1995, the field has attempted to define the link between K_{ATP} channels and the cytoskeleton (40–44). A cytoskeletal complex was hypothesized based on experiments that demonstrated the strong stimulatory effect of actin-depolymerizing drugs on K_{ATP} channel opening (42). Specifically, actin depolymerization activates K_{ATP} channels by reducing the sensitivity of K_{ATP} channels to ATP-dependent closure (40–44). Decreased ATP sensitivity implies a pathway of inhibitory signals modulated by the cytoskeleton. Thus, the association of K_{ATP} channels with the cytoskeleton modulates ligand-dependent regulation. Because ankyrins scaffold voltage-gated sodium channels, Na/K-ATPase, the anion exchanger, and NCX to the cytoskeleton (22), our data may define the link between the cytoskeleton and K_{ATP} channels.

Finally, although our data demonstrate a requirement of ankyrin-B for Kir6.2 membrane expression, the specific cellular role(s) of 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.

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