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 sarcosomal $\kappa_{\text{ATP}}$ channels directly associate with ankyrin-B in heart via the $\kappa_{\text{ATP}}$ 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 for ankyrin-B in regulating $\kappa_{\text{ATP}}$ channel gating. Finally, we demonstrate that ankyrin-B forms a membrane complex with $\kappa_{\text{ATP}}$ 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-excitatory cells (1). Three genes ($\text{ANK1}$, $\text{ANK2}$, and $\text{ANK3}$) encode a host of structurally similar but functionally distinct ankyrin polypeptides ($\text{ankyrin-B}$, $\text{ankyrin-R}$, and $\text{ankyrin-G}$, 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 $\text{ANK2}$ 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 $\text{Ank2}$ (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, $\text{ANK2}$ 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 $\text{in vivo}$ 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 $\kappa_{\text{ATP}}$ channel regulation. Ankyrin-B associates with the cardiac $\kappa_{\text{ATP}}$ 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 $I_{\kappa_{\text{ATP}}}$. Moreover, we demonstrate that ankyrin-B regulates cardiac $\kappa_{\text{ATP}}$ channel gating. Finally, we demonstrate that ankyrin-B coordinates a complex of the $\kappa_{\text{ATP}}$ channel and Na/K-ATPase. Together, these data define new roles for ankyrin-B in cardiac membrane protein expression, identify a new $\text{in vivo}$ 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

* This work was supported, in whole or in part, by National Institutes of Health Grants HL084583 and HL083422 (to P. J. M.), HL079031, HL62494, and HL70250 (to M. E. A.), and HL096805 (to T. J. H.). This work was also supported by the Pew Scholars Trust (to P. J. M.) and the Fondation Leducq (to M. E. A. and P. J. Mohler). 1 Both authors contributed equally to this work. 2 To whom correspondence should be addressed: University of Iowa Carver College of Medicine, CB5B 2283, 285 Newton Rd., Iowa City, IA 52242. E-mail: peter-mohler@uiowa.edu.
isolated from Langendorff-perfused hearts not subjected to ischemia, as described previously (15). $I_{K_{\text{ATP}}}$ 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 mehms 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, 1 mM MgCl$_2$, 5 mM EGTA, and 5 mM HEPES-KOH (pH 7.3). Cardiac cells were superfused with intracellular solution containing 140 mM KCl, 10 mM K$^+$, 5 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 10 μM leupeptin, and 10 μM/ml pepstatin) and homogenized. 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 × $g$ 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$_{1.2}$ (polyclonal), and anti-Na$_{1.5}$ (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-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 (GQRFVIV-EEEDGR; 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; GHFFVSIIVTEEGV; 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$_2$.

**$K_{\text{ATP}}$ Constructs**—Human Kir6.2 (KCNJ11; NP_000516) and human SUR1 (ABCC8; NP_003432.2) 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-
Ankyrin-B Regulates \( K_{\text{ATP}} \) Channels in Heart

**RESULTS**

**K\(_{\text{ATP}}\) Channel Subunit Expression Is Decreased in Ankyrin-B\(^{-/-}\) Heart**—The ATP-sensitive potassium channel (K\(_{\text{ATP}}\)) 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\(_{\text{ATP}}\) channel subunit expression in ankyrin-B\(^{-/-}\) mouse heart lysates by immunoblotting. We first evaluated cardiac K\(_{\text{ATP}}\) channel \( \alpha \)-subunit (Kir6.1 and Kir6.2) and \( \beta \)-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. 1B). Notably, we also observed a parallel decrease in the expression of both K\(_{\text{ATP}}\) channel \( \beta \)-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. 1F). As expected, ankyrin-B expression was significantly reduced in ankyrin-B\(^{-/-}\) whole heart lysates (57% decrease; \( n = 4 \); \( p < 0.001 \)) (Fig. 1A), 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 (\( Na^+/Ca^{2+} \) exchanger) and Na/K-ATPase expression levels were reduced in ankyrin-B\(^{-/-}\) ventricle (\( n = 4 \); \( p < 0.05 \)) (Fig. 1, H and I).

**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 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 \textit{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.
Ankyrin-B Regulates $K_{ATP}$ Channels in Heart

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. 2F). 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. 2F). Conversely, identical assays demonstrated that AnkB 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 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. 2G, this peptide interacted with ankyrin-B from heart lysate, with no appreciable pulldown in the control reaction (streptavidin beads alone) (Fig. 2G). 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 of the cardiac $K_{ATP}$ channel complex.

Ankyrin-B Forms a Ternary Complex with Kir6.2 and SLUR1/SUR2A—Interactions between Kir6.2 and $K_{ATP}$ channel SUR subunits regulate $K_{ATP}$ channel function (23). In fact, mice harboring mutant $K_{ATP}$ $\alpha$- and $\beta$-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 $\beta$-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. 3A). Likewise, anti-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 (Fig. 3A), 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 $\beta$-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...
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. 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 immunoblotting (Fig. 1B) was paralleled by decreased Kir6.2 immunostaining in primary ankyrin-B**+/−**isolated cardiomyocytes (Fig. 4B). 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,1.2, Na,1.5, 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. 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_ATP Channel ATP Sensitivity and P_0:** 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. 5E).

Finally, we measured the single-channel open probability of K_ATP channels from WT and ankyrin-B**+/−**mouse cardiomyocytes. In the

---

**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 overlaying 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).

**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 ([K_ATP/patch]). 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).
Ankyrin-B Regulates K\textsubscript{ATP} Channels in Heart

![Figure 6](image_url)

**FIGURE 6.** K\textsubscript{ATP} channels from wild-type and ankyrin-B\textsuperscript{+/–} 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\textsuperscript{+/–} cardiomyocytes. The calibration bars indicate 200 ms (abscissa) and 5 pA (ordinate). C, the bar graph represents calculated single-channel I\textsubscript{KATP} open probability in wild-type and ankyrin-B\textsuperscript{+/–} mice. \( P \_o \) was significant greater in ankyrin-B\textsuperscript{+/–} than in wild-type myocytes (0.62 ± 0.03 and 0.48 ± 0.05, respectively; \( n = 10; ^* \ p < 0.05 \)).

Ankyrin-B Organizes Complexes of Functionally Related Membrane Proteins—In heart and other tissues, K\textsubscript{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. 7A). Anti-Na/K-ATPase Ig co-immunoprecipitated ankyrin-B, NCX, and Kir6.2 (Fig. 7C). Moreover, anti-Kir6.2 Ig co-immunoprecipitated ankyrin-B, Na/K-ATPase, and NCX from heart lysates (Fig. 7B). Finally, anti-NCX Ig co-immunoprecipitated 220-kDa ankyrin-B, Na/K-ATPase, and Kir6.2 (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 Ca\textsubscript{2+}, Na\textsubscript{1.5}, 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\textsuperscript{+/–} hearts (deficient in ankyrin-B) (see Fig. 2A). Ankyrin-B\textsuperscript{+/–} hearts expressed reduced levels of 220-kDa ankyrin-B, Na/K-ATPase, NCX, and Kir6.2 (Fig. 1). Strikingly, ankyrin-B\textsuperscript{+/–} 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. 7A). Moreover, a similar reduction in NCX co-immunoprecipitation of Na/K-ATPase and Kir6.2 occurred using doubled ankyrin-B\textsuperscript{+/–} lysates (Fig. 7D). Na/K-ATPase also failed to associate with a significant fraction of NCX or Kir6.2 from ankyrin-B\textsuperscript{+/–} doubled lysates (Fig. 7C). Finally, anti-Kir6.2 Ig immunoprecipitated minimal levels of Na/K-ATPase and NCX from ankyrin-B\textsuperscript{+/–} doubled lysates (Fig. 7B). These data suggest that a specialized population of ankyrin-B, which is decreased in the ankyrin-B\textsuperscript{+/–} mouse heart, is essential for ankyrin-B interactions with Na/K-ATPase, NCX, and Kir6.2.

absence of ATP, we observed a significant difference between \( P \_o \) in ankyrin-B\textsuperscript{+/–} 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\textsuperscript{+/–} 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\textsubscript{ATP} channels and alters the regulation of residual membrane K\textsubscript{ATP} channels.
Ankyrin-B Regulates $K_{\text{ATP}}$ Channels in Heart

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.

REFERENCES

1. Bennett, V., and Baines, A. J. (2001) Physiol. Rev. 81, 1353–1392
2. Mohler, P. J., and Anderson, M. E. (2008) J. Cardiovasc. Electrophysiol. 19, 516–518
3. Nilsson, K. R., Jr., and Bennett, V. (2009) J. Cardiovasc. Pharmacol. 54, 106–115
4. Mohler, P. J., Revolta, I., Napolitano, C., LeMaillet, G., Lambert, S., Priori, S. G., and Bennett, V. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 17533–17538
5. Lowe, J. S., Palygin, O., Bhasin, N., Hund, T. J., Boydent, P. A., Shibata, E., Anderson, M. E., and Mohler, P. J. (2008) J. Cell Biol. 180, 173–186
6. Mohler, P. J., Schott, J. J., Gramolini, A. O., Dilly, K. W., Guatimosim, S., duBell, W. H., Song, L. S., Haurognek, K., Kyndt, F., Ali, M. E., Rogers, T. B., Lederer, W. J., Escande, D., Le Marec, H., and Bennett, V. (2003) Nature 421, 634–639
7. Mohler, P. J., Splawski, I., Napolitano, C., Bottelli, G., Sharpe, L., Timothy, K., Priori, S. G., Keating, M. T., and Bennett, V. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 9137–9142
8. Mohler, P. J., Le Scouarnec, S., Denjoy, I., Lowe, J. S., Guicheney, P., Caron, L., Driskell, I. M., Schott, J. J., Norris, K., Leenhardt, A., Kim, R. B., Escande, D., and Roden, D. M. (2007) Circulation 115, 432–441
9. Mohler, P. J., Davis, J. Q., and Bennett, V. (2005) PLoS Biol. 3, e423
10. Le Scouarnec, S., Bhasin, N., Vlieyres, C., Hund, T. J., Cunha, S. R., Koval, O., Marionneau, C., Chen, B., Wu, Y., Demolome, S., Song, L. S., Le Marec, H., Probst, V., Schott, J. J., Anderson, M. E., and Mohler, P. J. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 15617–15622
11. Sledlacek, K., Stark, K., Cunha, S. R., Pfeuffer, A., Weber, S., Berger, I., Per, S., Kääb, S., Wichmann, H. E., Mohler, P. J., Hengstenberg, C., and Jeron, A. (2008) Circ. Cardiovasc. Genet. 1, 93–99
12. Hund, T. J., Wright, P. J., Dun, W., Snyder, J. S., Boydent, P. A., and Mohler, P. J. (2009) Cardiovasc. Res. 81, 742–749
13. Gudmundsson, H., Hund, T. J., Wright, P. J., Kline, C. F., Snyder, J. S., Qian, L., Koval, O. M., Cunha, S. R., George, M., Rainey, M. A., Kashiw, F. E., Dun, W., Boydent, P. A., Anderson, M. E., Band, H., and Mohler, P. J. (2010) Circ. Res. 107, 84–95
14. Kline, C. F., Kurata, H. T., Hund, T. J., Cunha, S. R., Koval, O. M., Wright, P. J., Christensen, M., Anderson, M. E., Nichols, C. G., and Mohler, P. J. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 16669–16674
15. Li, J., Marionneau, C., Koval, O., Zingman, L., Mohler, P. J., Nerbonne, J. M., and Anderson, M. E. (2007) Channels 1, 387–394
16. Bhasin, N., Cunha, S. R., Mudannayake, M., Gigena, M. S., Rogers, T. B., and Mohler, P. J. (2007) Am. J. Physiol. Heart Circ. Physiol. 293, H109–H119
17. Abdi, K. M., Mohler, P. J., Davis, J. Q., and Bennett, V. (2006) J. Biol. Chem. 281, 5741–5749
18. Mohler, P. J., Hoffman, J. A., Davis, J. Q., Abdi, K. M., Kim, C. R., Jones, S. K., Davis, I. H., Roberts, K. F., and Bennett, V. (2004) J. Biol. Chem. 279, 25798–25804
19. Morrissey, A., Rosner, E., Lanning, J., Parachuru, L., Dhar Chowdhury, P.,...
Ankyrin-B Regulates $K_{\text{ATP}}$ Channels in Heart

Han, S., Lopez, G., Tong, X., Yoshida, H., Nakamura, T. Y., Artman, M., Giblin, J. P., Tinker, A., and Coetzee, W. A. (2005) BMC Physiol. 5, 1

20. Suzuki, M., Sasaki, N., Miki, T., Sakamoto, N., Ohmoto-Sekine, Y., Tamagawa, M., Seino, S., Marban, E., and Nakaya, H. (2002) J. Clin. Invest. 109, 509–516

21. Gumina, R. J., Pucar, D., Bast, P., Hodgson, D. M., Kurtz, C. E., Dzeja, P. P., Miki, T., Seino, S., and Terzic, A. (2003) Am. J. Physiol. Heart Circ. Physiol. 284, H2106–H2113

22. Cunha, S. R., and Mohler, P. J. (2006) Cardiovasc. Res. 71, 22–29

23. Wheeler, A., Wang, C., Yang, K., Fang, K., Davis, K., Styer, A. M., Mirshahi, U., Moreau, C., Revilloud, J., Vivaudou, M., Liu, S., Mirshahi, T., and Chan, K. W. (2008) Mol. Pharmacol. 74, 1333–1344

24. Proks, P., Girard, C., Baevre, H., Njølstad, P. R., and Ashcroft, F. M. (2006) Diabetes 55, 1731–1737

25. Tammaro, P., Girard, C., Molnes, J., Njølstad, P. R., and Ashcroft, F. M. (2006) EMBO J. 24, 2318–2330

26. Tammaro, P., Proks, P., and Ashcroft, F. M. (2006) J. Physiol. 571, 3–14

27. Haruna, T., Horie, M., Kouchi, I., Nawada, R., Tsuchiya, K., Akao, M., Otani, H., Murakami, T., and Sasayama, S. (1998) Circulation 98, 2905–2910

28. Glavind-Kristensen, M., Matchkov, V., Hansen, V. B., Forman, A., Nilsson, H., and Aalkjaer, C. (2004) Br. J. Pharmacol. 143, 872–880

29. Lencesova, L., O’Neill, A., Resneck, W. G., Bloch, R. J., and Blaustein, M. P. (2004) J. Biol. Chem. 279, 2885–2893

30. Morrow, J. S., Cianci, C. D., Ardito, T., Mann, A. S., and Kashgarian, M. (1989) J. Cell Biol. 108, 455–465

31. Nelson, W. J., and Veshnock, P. J. (1987) Nature 328, 533–536

32. Michaely, P., and Bennett, V. (1995) J. Biol. Chem. 270, 31298–31302

33. Cunha, S. R., Bhasin, N., and Mohler, P. J. (2007) J. Biol. Chem. 282, 4875–4883

34. Suzuki, M., Li, R. A., Miki, T., Uemura, H., Sakamoto, N., Ohmoto-Sekine, Y., Tamagawa, M., Ogura, T., Seino, S., Marban, E., and Nakaya, H. (2001) Circ. Res. 88, 570–577

35. Kane, G. C., Liu, X. K., Yamada, S., Olson, T. M., and Terzic, A. (2005) J. Mol. Cell. Cardiol. 38, 937–943

36. Minami, K., Miki, T., Kadomaki, T., and Seino, S. (2004) Diabetes 53, Suppl. 3, S176–S180

37. Gross, G. J., and Peart, J. N. (2003) Am. J. Physiol. Heart Circ. Physiol. 285, H921–H930

38. Yellon, D. M., and Downey, J. M. (2003) Physiol. Rev. 83, 1113–1151

39. Nawada, R., Murakami, T., Iwae, T., Nagai, K., Morita, Y., Kouchi, I., Akao, M., and Sasayama, S. (1997) Circulation 96, 599–604

40. Furukawa, T., Yamane, T., Terai, T., Katayama, Y., and Hiraoka, M. (1996) Pflugers Arch. 431, 504–512

41. Hibino, H., Terzic, A., Inanobe, A., Horio, Y., and Kurachi, Y. (1999) Curr. Topics Membranes 46, 243–272

42. Brady, P. A., Alekseev, A. E., Aleksandrova, L. A., Gomez, L. A., and Terzic, A. (1996) Am. J. Physiol. Heart Circ. Physiol. 271, H2710–H2716

43. Yokoshiki, H., Katsube, Y., Sunugawa, M., Seki, T., and Sperelakis, N. (1997) Pflugers Arch. 434, 203–205

44. Terzic, A., and Kurachi, Y. (1996) J. Physiol. 492, 395–404

45. Bennett, V. (1982) J. Cell. Biochem. 18, 49–65

46. Bennett, V. (1985) Annu. Rev. Biochem. 54, 273–304

47. Bennett, V. (1992) J. Biol. Chem. 267, 8703–8706