β_{IV}-Spectrin and CaMKII facilitate Kir6.2 regulation in pancreatic beta cells

Crystal F. Kline[a], Patrick J. Wright[a], Olha M. Koval[b], Erik J. Zmuda[c], Benjamin L. Johnson[a], Mark E. Anderson[d,e], Tsonwin Hai[c], Thomas J. Hund[a,b], and Peter J. Mohler[a,f,g,1]

[a]Dorothy M. Davis Heart and Lung Research Institute, [c]Department of Molecular and Cellular Biochemistry, [f]Division of Cardiovascular Medicine, Department of Internal Medicine, and [g]Department of Physiology and Cell Biology, The Ohio State University Wexner Medical Center, Columbus, OH 43210; [b]Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus, OH 43210; and [d]Division of Cardiovascular Medicine, Department of Internal Medicine, and [e]Department of Molecular Physiology and Biophysics, University of Iowa Carver College of Medicine, Iowa City, IA 52242

Edited by Vann Bennett, Duke University Medical Center, Durham, NC, and approved September 13, 2013 (received for review July 29, 2013)

Identified over a dozen years ago in the brain and pancreatic islet, β_{IV}-spectrin is critical for the local organization of protein complexes throughout the nervous system. β_{IV}-Spectrin targets ion channels and adapter proteins to axon initial segments and nodes of Ranvier in neurons, and β_{IV}-spectrin dysfunction underlies ataxia and early death in mice. Despite advances in β_{IV}-spectrin research in the nervous system, its role in pancreatic islet biology is unknown. Here, we report that β_{IV}-spectrin serves as a multifunctional structural and signaling platform in the pancreatic islet. We report that β_{IV}-spectrin directly associates with and targets the calcium/calmodulin-dependent protein kinase II (CaMKII) in pancreatic islets. In parallel, β_{IV}-spectrin targets ankyrin-B and the ATP-sensitive potassium channel. Consistent with these findings, β_{IV}-spectrin mutant mice lacking CaMKII- or ankyrin-binding motifs display selective loss of expression and targeting of key protein components, including CaMKIIδ. β_{IV}-Spectrin–targeted CaMKII directly phosphorylates the inwardly-rectifying potassium channel, Kir6.2 (alpha subunit of K_{ATP} channel complex), and we identify the specific residue, Kir6.2 T224, responsible for CaMKII-dependent regulation of K_{ATP} channel function. CaMKII-dependent phosphorylation alters channel regulation resulting in K_{ATP} channel inhibition, a cellular phenotype consistent with aberrant insulin regulation. Finally, we demonstrate aberrant K_{ATP} channel phosphorylation in β_{IV}-spectrin mutant mice. In summary, our findings establish a broader role for β_{IV}-spectrin in regulation of cell membrane excitability in the pancreatic islet, define the pathway for CaMKII local control in pancreatic beta cells, and identify the mechanism for CaMKII-dependent regulation of K_{ATP} channels.

trafficking | local regulation

The coordinate expression, localization, and regulation of ion channels are critical for excitable cell biology, namely, neuronal transmission, cardiac excitation–contraction coupling, endocrine/exocrine secretory regulation, and skeletal muscle function. The spectrin family of polypeptides, originally discovered as a critical component of the erythrocyte plasma membrane over 30 y ago, is now known to play important structural roles in both non-excitable and excitable cells (1, 2). Spectrin alpha (α) and beta (β) subunits form antiparallel dimers that self-associate to form tetramers, creating a submembranous scaffolding network anchored to actin filaments via β-spectrin (2). Whereas two genes encode α-spectrin polypeptides (α_I-, α_{II}-spectrin), spectrin family diversity is created through assembly of α-spectrin products with five β-spectrin genes (encoding β_I–β_V-spectrin) (2). Over a dozen years ago, β_{IV}-spectrin was identified in brain and pancreatic islets (3). Since these findings, a host of studies have identified β_{IV}-spectrin as a critical player for defining local membrane domains in the nervous system (4–6). Notably, β_{IV}-spectrin complexes serve essential roles in targeting ankyrin and associated membrane proteins, to axon initial segments and nodes of Ranvier. Dysfunction in β_{IV}-spectrin leads to aberrant membrane protein targeting, cell and animal phenotypes, and early death (6, 7). Despite these striking findings in brain, the role of β_{IV}-spectrin in pancreatic islets is unknown.

Here, we define a role for β_{IV}-spectrin in regulating membrane excitability in islets. β_{IV}-spectrin directly associates with ankyrin-B and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). We define the structural requirements for these interactions and show that β_{IV}-spectrin associates with additional islet proteins, including actin and the ATP sensitive potassium channel (K_{ATP} channel). Notably, β_{IV}-spectrin mutant mice that lack ankyrin- or CaMKII-binding activity display coordinate loss of ankyrin, K_{ATP} channel, and CaMKII expression and targeting. Finally, we demonstrate that β_{IV}-spectrin is critical for CaMKII-dependent regulation of the K_{ATP} channel through Kir6.2 residue T224 and show that Kir6.2 T224 phosphorylation is altered in β_{IV}-spectrin mutant mice. In summary, our findings provide initial insight into the role of β_{IV}-spectrin in the expression, targeting, and regulation of critical membrane and signaling proteins in islets.

Results

β_{IV}-Spectrin Associates with CaMKII in Pancreas. β_{IV}-Spectrin contains an N-terminal actin-binding domain, a series of 17 spectrin repeats, and a C-terminal domain (Fig. 1*A*). The C-terminal domain contains a conserved motif harboring high homology to the autoregulatory domain of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a site necessary for CaMKII intramolecular interaction (Fig. 1*B*) (8). This motif is present in a handful of CaMKII-associated proteins including the L-type Ca^{2+} channel beta subunit (β_{2a}) and the NR2B receptor (9). Given the lack of data on β_{IV}-spectrin function in the islet, we hypothesized that β_{IV}-spectrin may function in the pancreas to control CaMKII targeting and expression, similar to observed roles in myocytes (10).

We confirmed β_{IV}-spectrin mRNA and protein expression in the murine pancreas. Indeed, consistent with the only published data on pancreatic β_{IV}-spectrin (3), β_{IV}-spectrin is found in pancreas (Fig. 1*C*) and expressed in islets by immunoblot and immunostaining (Fig. 1*D* and Fig. 2*A*). In contrast, we observed minimal β_{IV}-spectrin immunostaining in exocrine acinar cells surrounding the islet (Fig. 1*D*). Like β_{IV}-spectrin, we observed

Significance

This study defines a functional role for β_{IV}-spectrin in pancreas, expands the pathways for calcium/calmodulin-dependent protein kinase II (CaMKII) local control in excitable cells, and identifies a mechanism for CaMKII-dependent regulation of K_{ATP} channels.

Author contributions: C.F.K., P.J.W., O.M.K., E.J.Z., M.E.A., T.H., T.J.H., and P.J.M. designed research; C.F.K., P.J.W., O.M.K., E.J.Z., and B.L.J. performed research; C.F.K., P.J.W., O.M.K., E.J.Z., T.H., and T.J.H. contributed new reagents/analytic tools; C.F.K., P.J.W., O.M.K., E.J.Z., B.L.J., M.E.A., T.H., T.J.H., and P.J.M. analyzed data; and C.F.K., T.H., T.J.H., and P.J.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: peter.mohler@osumc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314195110/-/DCSupplemental.

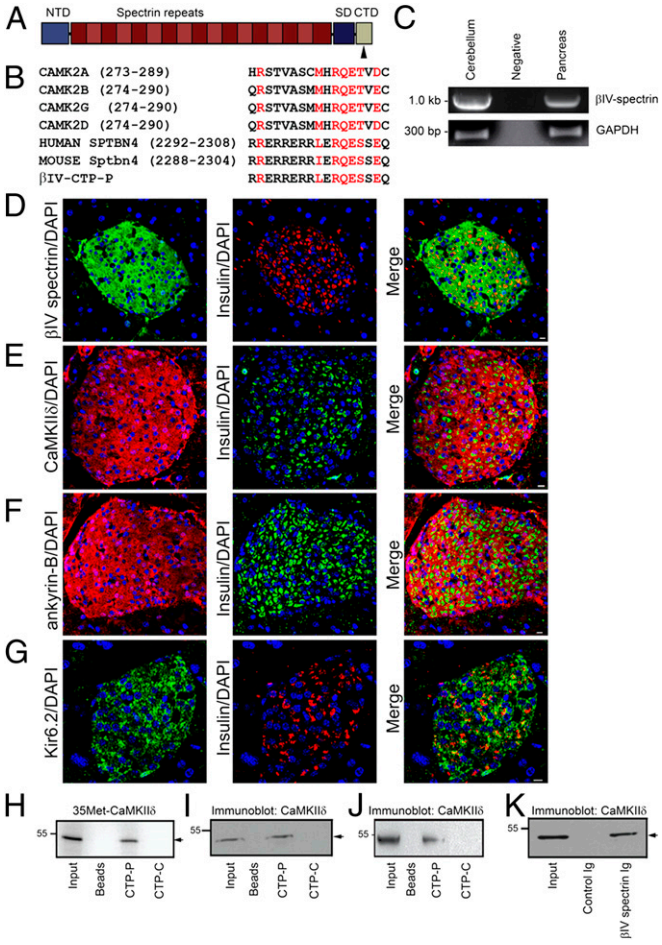

Fig. 1. β_{IV}-Spectrin is a CaMKII-binding partner in the pancreatic islet. (*A* and *B*) β_{IV}-Spectrin has an N-terminal actin-binding domain (NTD), 17 spectrin repeats, and a specific/C-terminal domain (SD/CTD). The CaMKII-binding site is denoted by an arrow that is homologous to the CaMKII autoregulatory domain motif in all CaMKII genes and conserved across species (red font). (*C*) β_{IV}-Spectrin mRNA expression in mouse cerebellum and pancreas. Immunostaining of mouse islets for (*D*) β_{IV}-spectrin, (*E*) CaMKIIδ, (*F*) AnkB, and (*G*) Kir6.2. Sections were colabeled to denote nuclei (DAPI) and beta cells (insulin Ig). (Scale bar, 10 μm.) (*H*) CTP-P (peptide homologous to the CaMKII-binding motif of β_{IV}-spectrin) directly binds radiolabeled CaMKIIδ (^{35}Met-CaMKIIδ) in pull-down experiments. No appreciable binding was observed with beads or control CTP-C (control scrambled) peptide. (*I* and *J*) CTP-P peptide associates with endogenous CaMKIIδ from whole pancreas (*I*) and pancreatic islet lysate (*J*) in pull-down experiments. We observed no binding with beads alone or the control CTP-C peptide. (*K*) β_{IV}-Spectrin Ig immunoprecipitates endogenous pancreatic CaMKIIδ, with no binding observed with control Ig or beads alone.

expression of the CaMKII isoform δ within the murine islet (Fig. 1 *E* and *J*).

We tested a potential β_{IV}-spectrin/CaMKII interaction in pancreas using radiolabeled protein and purified mouse pancreatic islet lysate. β_{IV}-Spectrin directly associates with CaMKIIδ in vitro, determined using a peptide that corresponds to the putative CaMKII-binding motif in the β_{IV}-spectrin C-terminal domain (CTP-P). CTP-P, but not beads alone nor CTP-C (β_{IV}-spectrin C-terminal scrambled control peptide), bound radiolabeled CaMKIIδ (Fig. 1*H*) and endogenous CaMKIIδ from isolated pancreatic islets or whole pancreas (Fig. 1 *I* and *J*). Furthermore, β_{IV}-spectrin Ig, but neither control Ig nor beads alone, was able to immunoprecipitate with CaMKIIδ from pancreas and pancreatic islet cell lysates (Figs. 1*K* and 2*A*). Together, these data support a β_{IV}-spectrin/CaMKIIδ interaction in pancreatic islets via a C-terminal domain in β_{IV}-spectrin.

Identification of β_{IV}-Spectrin– and Ankyrin-B–Associated Proteins in Islets. β-Spectrin polypeptides associate with ankyrin proteins through a conserved binding motif in the β-spectrin 15th spectrin repeat (11). Ankyrin-B (AnkB) is present within the islet and necessary for the targeting and expression of the ATP-sensitive potassium channel (K_{ATP}) (12) and inositol 1,4,5 trisphosphate (IP3) receptor (13). Human mutations in Kir6.2 (K_{ATP} channel α-subunit) that block binding with AnkB are associated with neonatal diabetes mellitus (12, 14), although these variants may also affect K_{ATP} Kir6.2/SUR1 subunit coupling (15). Consistent with β_{IV}-spectrin localization, AnkB and Kir6.2 are highly

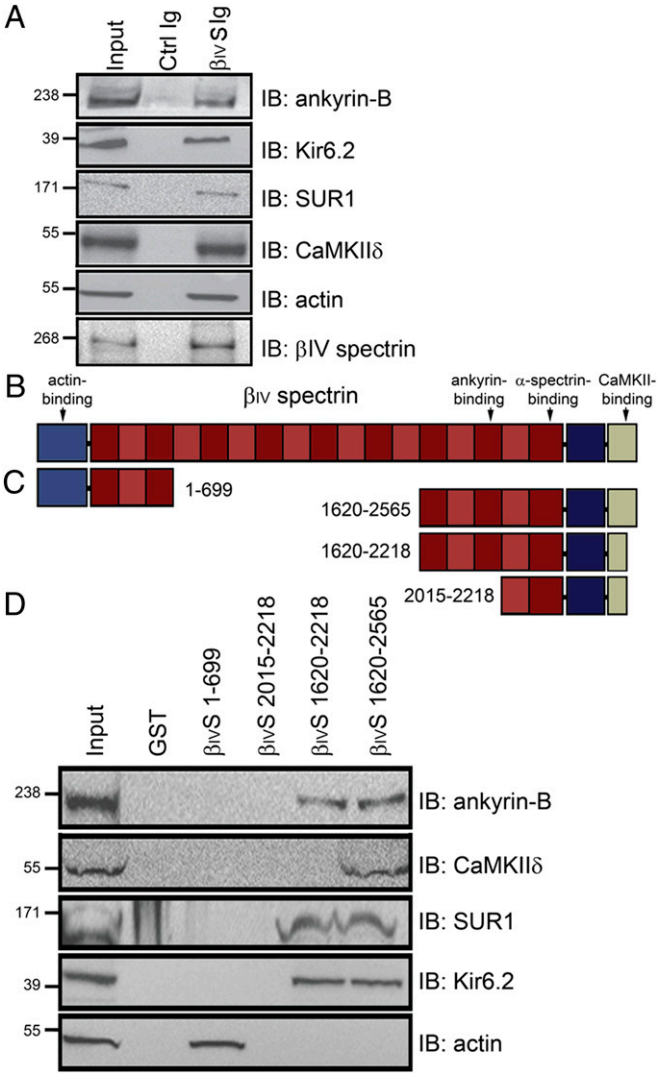

Fig. 2. β_{IV}-Spectrin–associated proteins in pancreatic islets. (*A*) β_{IV}-Spectrin associates with AnkB, Kir6.2, SUR1, CaMKIIδ, and actin from pancreatic islet lysate by coimmunoprecipitation. (*B* and *C*) Structure of β_{IV}-spectrin and β_{IV}-spectrin GST-fusion constructs used in pull-down experiments from detergent-soluble pancreas lysates. (*D*) Whereas CaMKIIδ associates only with β_{IV}-spectrin fusion constructs containing the distal C-terminal CaMKII binding motif in islet lysates, β_{IV}-spectrin–dependent association with AnkB, Kir6.2, and SUR1 requires residues 1620–2218 (includes 15th spectrin repeat). β_{IV}-Spectrin 1–699 was the only construct to copurify with actin from islet lysates. We observed no interaction of either CaMKIIδ or AnkB with N-terminal GST–β_{IV}-spectrin fusion construct.

PHYSIOLOGY

expressed in the islet where the K_{ATP} channel regulates cellular excitability and insulin secretion (Fig. 1 *F* and *G*). Moreover, AnkB and β_{IV}-spectrin associate from purified islet lysates by coimmunoprecipitation (Fig. 2*A*). In addition to ankyrin-B, we also observed CaMKIIδ, Kir6.2, and SUR1 (K_{ATP} channel subunit) in β_{IV}-spectrin coimmunoprecipitation experiments (Fig. 2*A*).

Defining the Structural Requirements for β_{IV}-Spectrin Binding for CaMKII and AnkB. We constructed a series of β_{IV}-spectrin GST-fusion proteins containing key regions of the molecule to define the structural requirements for β_{IV}-spectrin association with CaMKII and AnkB (Fig. 2 *B* and *C*). Consistent with peptide pull-down experiments, a β_{IV}-spectrin fusion protein containing the full C terminus (residues 1620–2565) associated with CaMKIIδ from islets (Fig. 2*D* and Fig. S1). In contrast, fusion proteins lacking the putative CaMKII-association motif (residues 1620–2218 and 2015–2218) did not bind islet CaMKIIδ (Fig. 2*D*). We observed no CaMKII-binding activity for a control β_{IV}-spectrin N-terminal fusion protein (residues 1–699; Fig. 2*D*). On the other hand, C-terminal β_{IV}-spectrin GST-fusion proteins containing residues 1620–2218 and 1620–2565 displayed robust AnkB-binding activity, whereas truncation of the fusion proximal to spectrin repeat 16 (residues 2015–2218) resulted in loss of AnkB-binding activity (Fig. 2*D*). We previously demonstrated that AnkB directly associates with Kir6.2 (12, 16) and AnkB/SUR1 interactions require Kir6.2 (Fig. S2). Consistent with these data, C-terminal β_{IV}-spectrin 1620–2218 and 1620–2565 displayed binding activity for Kir6.2 and SUR1, but residues 2015–2218 lacked ability to associate with Kir6.2 or SUR1 (Fig. 2*D*). We observed no binding of the β_{IV}-spectrin N terminus to CaMKII, AnkB, Kir6.2, or SUR1; however, as expected, this domain associated with actin (Fig. 2*D*). Our results support a model where β_{IV}-spectrin associates with actin, AnkB, and CaMKIIδ through its N terminus, central spectrin repeat domain, and C terminus, respectively.

β_{IV}-Spectrin Mutant Mice Display Select Loss of CaMKII and Ankyrin Binding. Nearly 60 y ago, a spontaneous line of "quivering" (*qv*) mice was identified that displayed severe ataxia, hind-limb paralysis, deafness, and tremor (7). Subsequent studies identified specific mutations across the murine β_{IV}-spectrin gene, resulting in the *qv* mouse phenotypes. These in vivo models have proved critical in defining the roles of β_{IV}-spectrin function in physiology and disease (7). To test the role of β_{IV}-spectrin in islet channel and signaling molecule expression and targeting, we used *qv* mouse lines harboring mutations that selectively alter AnkB (qv^{4J}) and CaMKII association (qv^{3J}; Fig. S3*A*). We coimmunoprecipitated CaMKIIδ, AnkB, Kir6.2, SUR1, and actin with β_{IV}-spectrin from wild-type (WT) mouse islet lysates (Fig. S3*B*). Likewise, parallel AnkB Ig coimmunoprecipitations revealed β_{IV}-spectrin, CaMKIIδ, Kir6.2, and SUR1 as AnkB-associated proteins (Fig. S3*C*). We observed loss of CaMKIIδ association, but not AnkB, Kir6.2, or SUR1, with β_{IV}-spectrin in islet lysates from the qv^{3J} mouse lacking spectrin/CaMKII interaction (Fig. S3 *B* and *C*); however, as noted in the next section, CaMKII protein levels are decreased in qv^{3J} and qv^{4J} islets, so coimmunoprecipitation inputs were adjusted to compensate for differences in protein expression (*SI Materials and Methods*). Additionally, CaMKIIδ immunoprecipitated with AnkB Ig in WT, but not qv^{3J} islets (Fig. S3*C*). In qv^{4J} mice lacking the ankyrin-binding domain, we observed loss of β_{IV}-spectrin association with AnkB, Kir6.2, and SUR1, as well as inability of AnkB to associate with CaMKIIδ (Fig. S3 *B* and *C*). Finally, AnkB interactions with Kir6.2 and SUR1 were independent of β_{IV}-spectrin or actin as AnkB Ig coimmunoprecipitated both Kir6.2 and SUR1 in qv^{4J} islet lysates (Fig. S3*C*).

β_{IV}-Spectrin Is Required for CaMKII Expression and Localization. Based on our data, we hypothesized that β_{IV}-spectrin regulates CaMKII posttranslational expression and targeting. We assessed the requirement of β_{IV}-spectrin for CaMKII expression and targeting in WT, qv^{3J}, and qv^{4J} beta cells. As expected, β_{IV}-spectrin was expressed throughout the cytosol of WT, qv^{3J}, and qv^{4J} beta cells (Fig. 3*A*). AnkB expression was present within both WT and qv^{3J} beta cells, but consistent with the qv^{4J} mutation lacking ankyrin-binding activity, we observed reduced and diffuse staining of AnkB in qv^{4J} beta cells (Fig. 3*B*). In parallel with its dependence on AnkB for targeting, Kir6.2 (and SUR1) expression (expressed throughout cytosol) was significantly reduced in qv^{4J} beta cells compared with WT, but not in qv^{3J} islets (Fig. 3 *C* and *E*). Finally we observed loss of CaMKIIδ expression in both qv^{3J} and qv^{4J} beta cells (Fig. 3*D*). Immunoblots confirmed significantly reduced CaMKIIδ expression in islet lysates from both qv^{3J} and qv^{4J} models (Fig. 3*F*). β_{IV}-spectrin may regulate the posttranscriptional stability of CaMKIIδ, as CaMKIIδ mRNA levels were unchanged between WT, qv^{3J}, and qv^{4J} mouse islets (Fig. S4). Alternatively, other cell or molecular mechanisms such as reduced CaMKII translation may underlie reduced expression of CaMKII in qv^{3J} and qv^{4J} islets.

CaMKII Regulates K_{ATP} Channel Function. CaMKII activity has been linked with regulation of K_{ATP} channel function, albeit indirectly (17). We hypothesized that CaMKII may directly phosphorylate K_{ATP} channels via Kir6.2. We analyzed the primary amino acid sequence of Kir6.2 for putative CaMKII phosphorylation sites (using consensus sequence [RXXS/T]). Three putative CaMKII phosphorylation sites were identified: S37, T224, and S372 (Fig. 4*A*) and site-directed mutagenesis was used to substitute an alanine at each site. To determine if these mutants altered Kir6.2 function, we analyzed the activity of WT Kir6.2 (with SUR1) and mutant channels in COSm6 cultured cells that lack significant I_{KATP}. Transfected cells were evaluated in the absence and presence of a constitutively active CaMKII (CaMKIIδ T286D). Cells expressing WT Kir6.2 showed significant decrease in I_{KATP} when expressed with active CaMKII, as did Kir6.2 S37A and Kir6.2 S372A (Fig. 4 *B*, *C*, and *E*). However, cells transfected with Kir6.2 T224A showed no significant decrease in I_{KATP} in the presence of active CaMKII (Fig. 4 *D* and *G*). Notably, when T224 was substituted with a glutamic acid to create a phosphomimetic (Kir6.2 T224E), we observed a significant decrease in K_{ATP} channel current at baseline (compared with WT Kir6.2), even in the absence of CaMKIIδ overexpression (Fig. 4 *F* and *G*).

To further analyze this site as a potential CaMKII target, we performed in vitro phosphorylation assays on WT and Kir6.2 T224 mutant proteins. Whereas β_{2a}, a known CaMKII phosphorylation target and WT Kir6.2 were phosphorylated in vitro by CaMKII, Kir6.2-T224A and Kir6.2-T224E showed no significant CaMKII-mediated phosphorylation (Fig. 4*H*). Together, these data strongly support the role of CaMKII direct phosphorylation, demonstrate that this phosphorylation is at least in part direct, and implicate Kir6.2 T224 as a CaMKII target site in this pathway.

CaMKII Regulates Kir6.2 by Phosphorylation of Kir6.2 T224. We created an affinity-purified phospho-specific antibody against Kir6.2 pT224 to evaluate the role of the β_{IV}-spectrin/CaMKII pathway for K_{ATP} regulation in pancreas. The specificity of the antibody for the Kir6.2 pT224 site was confirmed by immunoblots that detected GFP-Kir6.2 from transfected cells, but not Kir6.2 T224A or Kir6.2 T224E (Fig. S5).

We evaluated Kir6.2 pT224 Ig in INS-1 cells, a rat insulinoma cell line commonly used to evaluate Kir6.2 and insulin secretion. Cells were analyzed for Kir6.2 pT224 ± isoproterenol (1 μM) to stimulate CaMKII activity. Notably, we observed nearly a fourfold increase in Kir6.2 pT224 [and twofold increase in activated CaMKII (CaMKII pT286)] in isoproterenol (Iso)-treated cells compared with untreated cells, whereas we observed no difference in total Kir6.2 or SUR1 levels (Fig. 5 *A* and *B*). To determine whether this activation required CaMKII, we performed parallel experiments in the presence of the CaMKII inhibitor, autocamtide-2–related inhibitory peptide (AIP). In support of

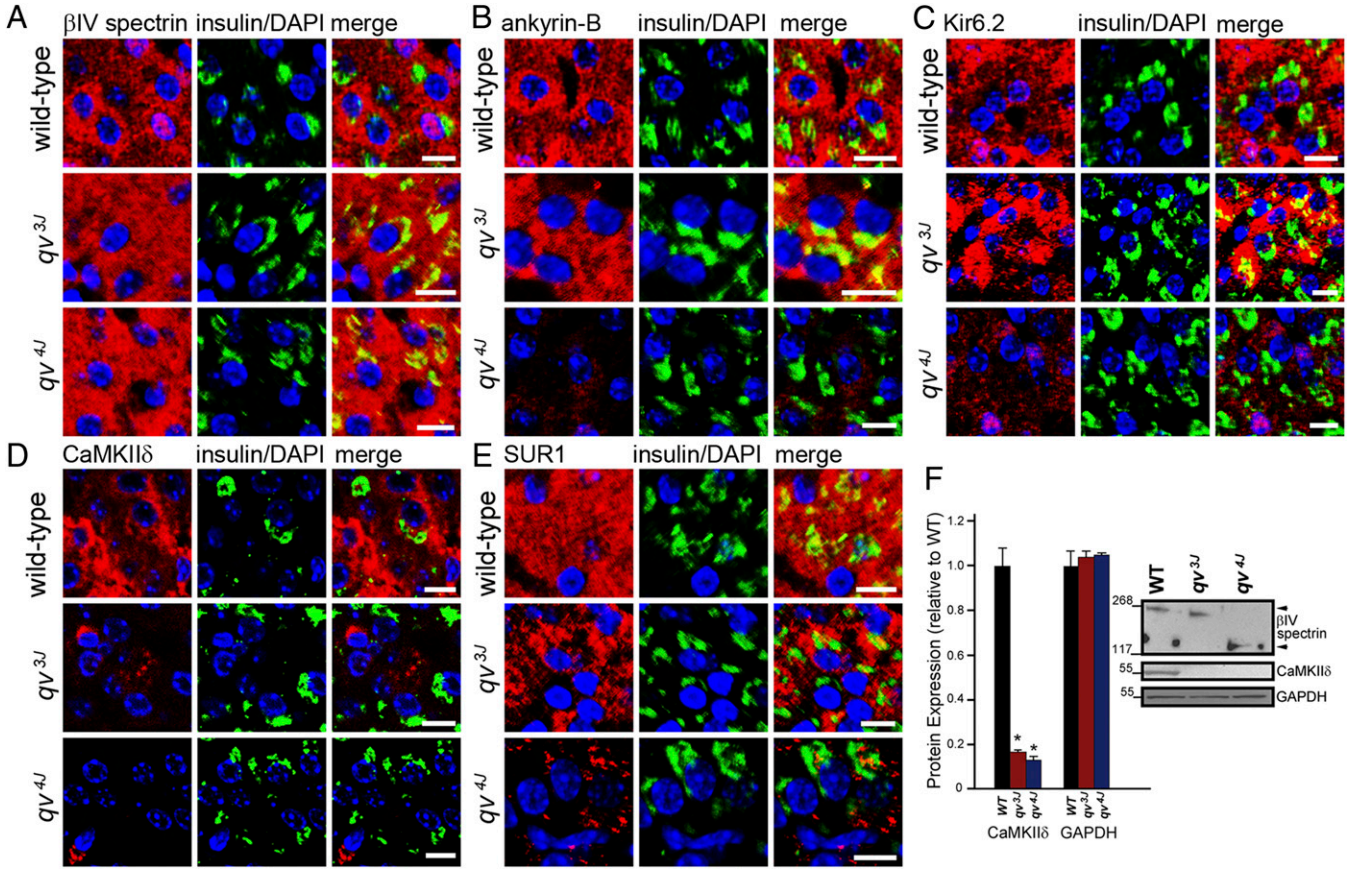

Fig. 3. β_{IV}-Spectrin is required for CaMKIIδ, AnkB, Kir6.2, and SUR1 expression in beta cells. (*A*) WT, qv^{3J}, and qv^{4J} beta cells express β_{IV}-spectrin [red; colabeled with DAPI (blue) and insulin (green)]. (*B*, *C*, and *E*) Whereas expressed in WT and qv^{3J} beta cells, AnkB, Kir6.2, and SUR1 expression is decreased in qv^{4J} islets. (*D*) Whereas concentrated in WT beta cells, CaMKIIδ expression is reduced in the qv^{3J} and qv^{4J} mouse beta cells. (Scale bar, 10 μm.) (*F*) CaMKIIδ protein levels are significantly decreased in qv^{3J} and qv^{4J} islet lysates ($n = 4$ mice, $P < 0.05$), whereas CaMKIIδ mRNA levels are unchanged (Fig. S4).

a role of CaMKII in regulation of Kir6.2 pT224, AIP blocked both CaMKII pT286 and Kir6.2 pT224 activation (Fig. 5 *A* and *B*).

Consistent with (*i*) a role for β_{IV}-spectrin in CaMKII regulation and (*ii*) a role for CaMKII in the direct regulation of Kir6.2 in vivo T224, Kir6.2 pT224 associated with β_{IV}-spectrin and AnkB in coimmunoprecipitation experiments from WT, but not qv^{3J} or qv^{4J}, pancreatic islet lysates (Fig. 5 *C* and *D*). Further, we observed decreased levels of Kir6.2 pT224 in qv^{3J} and qv^{4J} pancreatic islet lysates (Fig. 5 *E* and *F*) compared with WT islets. In agreement with immunostaining data, we observed decreased Kir6.2 and SUR1 levels in qv^{4J}, but not qv^{3J} islets (Fig. 5 *E* and *F*). Finally, staining of pancreatic islets revealed similar results, namely, a significant decrease in Kir6.2 pT224 expression in islets from qv^{3J} mice compared with WT mice (Fig. S6). These data support the hypothesis that β_{IV}-spectrin is critical for the regulation of K_{ATP} channel activity through direct CaMKII phosphorylation of Kir6.2.

Discussion

The K_{ATP} channel is a vital component in the regulation of insulin secretion, with mutations resulting in diabetes mellitus or hyperinsulinemia (18). However, little progress has been made regarding the posttranslational modification of the K_{ATP} channel. β_{IV}-spectrin is a multifunctional regulatory protein for K_{ATP} channels in the islet. Using mutant β_{IV}-spectrin mouse models, we determined that β_{IV}-spectrin is necessary for the expression and targeting of select structural and regulatory proteins in the islet. For example, our data show that β_{IV}-spectrin is critical for expression of the CaMKIIδ polypeptide in islets. Thus, β_{IV}-spectrin fails to coimmunoprecipitate with CaMKIIδ in qv^{3J} islets (Fig. S3) due to both its inability to associate with CaMKIIδ (Fig. 2 *C* and *D*) and the reduced steady-state levels of CaMKIIδ polypeptide in the qv^{3J} lysate (Fig. 3). Additionally, we identified a regulatory mechanism for K_{ATP} channels: direct phosphorylation of Kir6.2 T224 by CaMKII. Specifically, Kir6.2 T224 phosphorylation results in a significant decrease in I_{KATP}. Finally, using an antibody that specifically recognizes the phosphorylated form of Kir6.2 T224, we demonstrated that qv^{3J} and qv^{4J} islets lack phosphorylated Kir6.2 T224, as well as CaMKIIδ. Collectively, our data define CaMKII as a membrane skeleton component in the pancreatic islet and β_{IV}-spectrin/AnkB as a necessary molecular platform for K_{ATP} channel regulation. As discussed below, future experiments to evaluate Kir6.2 T224 phosphorylation status in human and animal models of diabetes/metabolic disease will be important to assess the potential of this target site as a biomarker in the early progression of disease. Further, whereas our data demonstrate that β_{IV}-spectrin associates with CaMKII, ankyrin-B, actin, Kir6.2, and SUR1 in islets, these results do not necessarily support that these proteins reside in a single macromolecular complex. Future experiments to validate a single macromolecular complex would include expanded coimmunoprecipitation studies that (*i*) verify that antibodies against each of the proteins in the putative complex recovers each of the other components and (*ii*) demonstrate that recovery of one of the components of the putative complex is prevented in the absence of one of the other molecules.

Whereas this study addresses the specific role of Kir6.2/SUR1 in the islet, K_{ATP} channels are widely distributed in the central and peripheral nervous system, pituitary, cardiac muscle, smooth muscle, and skeletal muscle (18, 19) where they regulate such diverse physiological functions as neuronal excitability, hormone

PHYSIOLOGY

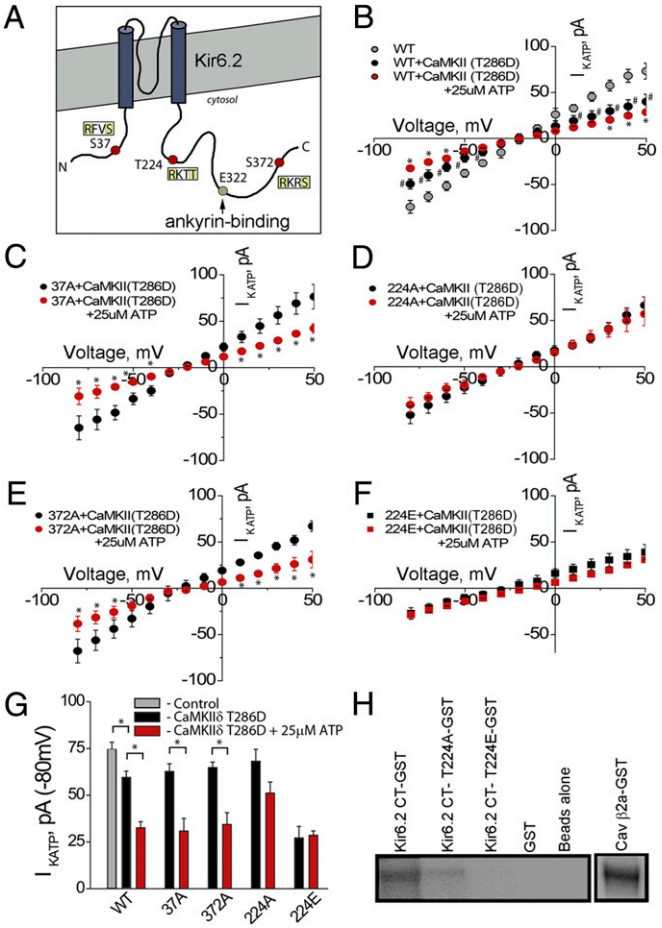

Fig. 4. CaMKII regulates K_{ATP} channel function. (*A*) Kir6.2 with AnkB-binding site denoted at Kir6.2 E322. Image denotes three putative CaMKII-phosphorylation sites: S37, T224, and S372. (*B*) COSm6 cells transfected with Kir6.2/CaMKII T286D demonstrate a significant reduction in I_{KATP}, compared with control, ±ATP (25 μM). Likewise, we observed significant reductions in I_{KATP} in cells transfected with CaMKII T286D and Kir6.2 S37A (*C*) and Kir6.2 S372A (*E*), ±25 μM ATP. We observed no significant change in I_{KATP} in cells transfected with CaMKII T286D/Kir6.2 T224A, ±25 μM ATP (*D*). (*F*) Finally, transfection with CaMKII T286D/Kir6.2 T224E (phosphomimetic) demonstrates a significant reduction in I_{KATP} compared with control, but no significant difference with and without ATP administration. (*G*) Summary of functional I_{KATP} data ($n > 7$ cells per treatment; $P < 0.05$). (*H*) Purified and immobilized Kir6.2, Kir6.2 T224A, and Kir6.2 T224E were assessed for CaMKII-mediated phosphorylation in vitro. Control (β_{2a}) and Kir6.2 showed robust CaMKII-mediated phosphorylation. There was significantly reduced phosphorylation of Kir6.2 T224A and Kir6.2 T224E.

secretion, vascular tone, heart rate, and protection of cells against metabolic stress (18). Notably, human *KCNJ11* gene variants result in developmental delay, epilepsy, and neonatal diabetes (DEND) due to overactive K_{ATP} channels in muscle, peripheral nerves, and brain.

In heart, K_{ATP} channels serve a protective role by activating under metabolic stress and leading to a shortening of the action potential. To this end, K_{ATP} channels serve as protectors against cardiac maladaptation to stress, as indicated by hearts deficient in K_{ATP} channels being more susceptible to Ca^{2+}-dependent maladaptive remodeling, progressing to organ failure and death. Whereas *KCNJ11* polymorphisms have been associated with cardiac disease, no clear gene mutations have been directly linked with severe forms of arrhythmia or myopathy to date.

In addition to regulation by intracellular nucleotides, K_{ATP} channel activity, and consequently its physiological function, can be modified by protein phosphorylation. For example, CaMKII activity has been linked with K_{ATP} channel regulation in heart and brain (17, 20). K_{ATP} channels are also modulated by cAMP-dependent protein kinase (PKA) (21), Ca^{2+}/phospholipid-dependent protein kinase (PKC) (22), extracellular signal-regulated kinase (ERK) (23), and cGMP-dependent protein kinase (PKG) (24). Prior work from Jan and coworkers identified Kir6.2 T224 as a site for PKA-dependent phosphorylation in HEK293 cells (25). Likely, both CaMKII and PKA will have functional roles on Kir6.2 regulation in vivo, although our data demonstrate that CaMKII inhibition is sufficient to ameliorate Kir6.2 pT224 phosphorylation in isoproterenol-treated INS1 cells.

Whereas our findings identify β_{IV}-spectrin as a component in the regulation of K_{ATP} channel activity in the islet, they also raise many questions regarding the specific role of CaMKII-mediated phosphorylation. There is a growing body of evidence from enzyme activation studies in support of a role for CaMKII in insulin secretion. Specifically, there is a correlation between CaMKII activation with glucose-stimulated insulin secretion in rat islets (26). CaMKII activation in the beta cell has been linked to phosphorylation of proteins that are involved in secretory events, namely, microtubule-associated protein-2 and synapsin I. Whereas the phosphorylation of proteins involved in granule exocytosis has been identified, little work has been performed to define a role for CaMKII in the regulation of beta cell ion channel function. Previous studies have demonstrated that suppression of cardiac CaMKII, via the transgenic expression of a CaMKII inhibitor, results in an augmentation of K_{ATP}-dependent current (17). We have found that expression of an active form of CaMKII produces a significant decrease in I_{KATP} in a heterologous expression system, suggesting that CaMKII-mediated phosphorylation of the K_{ATP} channel results in a decrease in

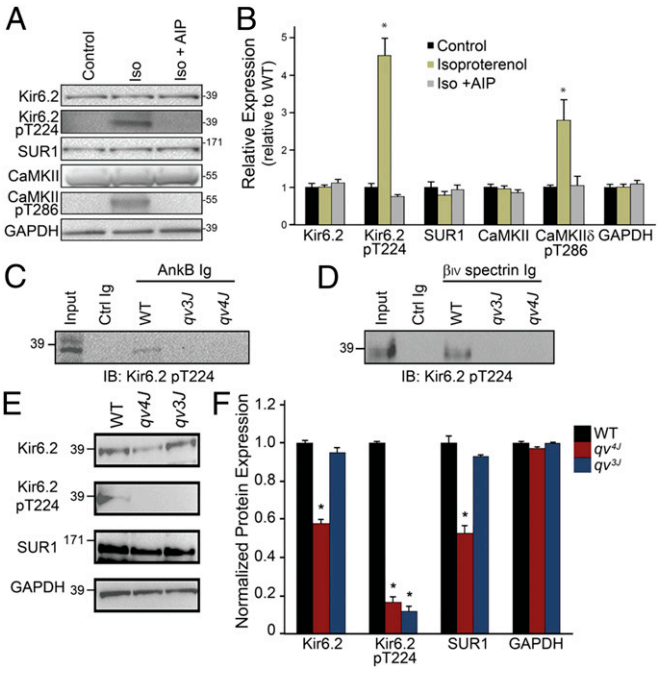

Fig. 5. CaMKII regulates Kir6.2 by direct phosphorylation of Kir6.2 T224. (*A* and *B*) INS-1 display an increase in Kir6.2 pT224 and CaMKII pT286 expression when stimulated with isoproterenol ($n = 3$ per treatment, $P < 0.05$). CaMKII inhibitor AIP (Iso + AIP) blocked CaMKII T286 and Kir6.2 T224 phosphorylation ($n = 3$ per treatment, NS vs. control). (*C* and *D*) AnkB and β_{IV}-spectrin associate with Kir6.2 pT224 in WT, but not qv^{3J} or qv^{4J} pancreatic islet lysates. (*E* and *F*) Kir6.2 pT224 levels are decreased in qv^{3J} and qv^{4J} pancreatic islet lysates. Expression presented relative to GAPDH expression ($n = 3$ per treatment, $P < 0.05$). In *F*, expression is normalized to GAPDH and compared with WT levels.

I_{KATP}, whereas inhibition of CaMKII results in an increase in I_{KATP}. Two interesting questions for future evaluation are raised based on this finding. First, what is the physiological consequence of CaMKII-mediated phosphorylation of pancreatic K_{ATP} channels? CaMKII-dependent regulation of K_{ATP} channels may play multiple roles in K_{ATP} channel targeting, K_{ATP} channel plasma membrane density, and K_{ATP} channel biophysical regulation. Studies in heart have demonstrated that suppression of CaMKII activity results in an augmentation of I_{KATP} due to a change in sarcolemmal K_{ATP} channel density (17). Future studies to discern specific consequences of CaMKII-mediated K_{ATP} channel phosphorylation (i.e., single channel kinetics, ATP sensitivity, and/or plasma membrane density/stability) will be important to provide insight into the specific roles of CaMKII in beta cell K_{ATP} channel modulation and potential regulation of insulin secretion. As noted above, CaMKII-mediated phosphorylation of the K_{ATP} channel results in a decrease in I_{KATP}, therefore we hypothesize CaMKII activation to decrease glucose-stimulated insulin secretion. Considering that previous studies have shown a direct relationship between the activation of pancreatic CaMKII and glucose-stimulated insulin secretion, it will be important to eventually evaluate glucose-stimulated insulin secretion in qv^{3J} and qv^{4J} mice that have disrupted β_{IV}-spectrin–mediated CaMKII expression and localization. However, for these studies, it will be important to consider the inherent physiological differences for insulin regulation in mice vs. humans (i.e., Kir6.2 null mice do not display hypoglycemia, but display increased insulin sensitivity and low insulin levels). It will be interesting to evaluate the effect of CaMKII inhibition on K_{ATP} channel ATP sensitivity, which represents a critical factor in K_{ATP} channel regulation. Second, what is the localization of K_{ATP} channel populations targeted by CaMKII? Whereas we and others have observed Kir6.2 and SUR1 across the beta cell (immunostaining throughout cytosol and membrane, Figs. 1 and 3), K_{ATP} channel subunits have also been observed at the insulin granule (27). Compared with the HEK cell or cardiomyocyte, the pancreatic beta cell is small with few well-defined membrane features. Therefore, future experiments using high-resolution double immunogold labeling of the beta cell for CaMKII and K_{ATP} channel subunits will be necessary to resolve this issue. In fact, beyond CaMKII and K_{ATP} channel subunits, our data demonstrate multiple heterogeneous subpopulations of spectrin and ankyrin in beta cells (with or without colocalization with insulin). It will be interesting for future studies to define the specific localization, molecular identities, and functions of these subpopulations.

In summary, our findings demonstrate a role for β_{IV}-spectrin in the targeting and expression of CaMKII in the pancreatic islet. Further, the direct coupling of β_{IV}-spectrin to AnkB potentially localizes K_{ATP} channel subunits in immediate proximity with CaMKII for direct Kir6.2 phosphorylation (Kir6.2 T224). We predict that these proteins play key roles in vertebrate excitable cell physiology, although this regulation is likely complex. For example, we previously demonstrated that a human Kir6.2 E322K permanent neonatal diabetes mutation that blocks AnkB association is associated with both defects in membrane trafficking and Kir6.2 membrane biophysical activity (12). However, Tarasov et al. found that this site may also be involved in coupling Kir6.2 regulation with SUR1 (15), and individuals with the *KCNJ11* E322K mutation respond to sulfonylurea treatment. Whereas additional data will be necessary to define the mechanisms underlying the regulation of these proteins at baseline and in disease, these findings ultimately support the clear in vivo roles of the cytoskeleton and cytoskeletal-associated proteins in the regulation of excitable cell function.

Materials and Methods

Kir6.2 mutants (S37A, S37E, T224A, T224E, S372A, and S372E) were generated using the Stratagene QuikChange site-directed mutagenesis kit, manufacturer's protocols, and unique primers.

Additional materials and methods are found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was funded by National Institutes of Health Grants HL096805 and HL114893 (to T.J.H.); HL084583 and HL083422 (to P.J.M.); HL079031, HL096652, and HL070250 (to M.E.A.); and the Gilead Scholars Program (T.J.H.), the American Heart Association (P.J.M.), the Saving Tiny Hearts Society (P.J.M.), and the Fondation Leducq (08CVD01) (to M.E.A. and P.J.M.).

1. Machnicka B, Grochowalska R, Bogusławska DM, Sikorski AF, Lecomte MC (2012) Spectrin-based skeleton as an actor in cell signaling. *Cell Mol Life Sci* 69(2):191–201.
2. Bennett V, Healy J (2009) Membrane domains based on ankyrin and spectrin associated with cell-cell interactions. *Cold Spring Harb Perspect Biol* 1(6):a003012.
3. Berghs S, et al. (2000) betaIV spectrin, a new spectrin localized at axon initial segments and nodes of ranvier in the central and peripheral nervous system. *J Cell Biol* 151(5):985–1002.
4. Schafer DP, et al. (2009) Disruption of the axon initial segment cytoskeleton is a new mechanism for neuronal injury. *J Neurosci* 29(42):13242–13254.
5. Lacas-Gervais S, et al. (2004) BetaIVSigma1 spectrin stabilizes the nodes of Ranvier and axon initial segments. *J Cell Biol* 166(7):983–990.
6. Yang Y, Lacas-Gervais S, Morest DK, Solimena M, Rasband MN (2004) BetaIV spectrins are essential for membrane stability and the molecular organization of nodes of Ranvier. *J Neurosci* 24(33):7230–7240.
7. Parkinson NJ, et al. (2001) Mutant beta-spectrin 4 causes auditory and motor neuropathies in quivering mice. *Nat Genet* 29(1):61–65.
8. Yang E, Schulman H (1999) Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 274(37):26199–26208.
9. Strack S, McNeill RB, Colbran RJ (2000) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 275(31):23798–23806.
10. Hund TJ, et al. (2010) A β(IV)-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. *J Clin Invest* 120(10):3508–3519.
11. Kennedy SP, Warren SL, Forget BG, Morrow JS (1991) Ankyrin binds to the 15th repetitive unit of erythroid and nonerythroid beta-spectrin. *J Cell Biol* 115(1):267–277.
12. Kline CF, et al. (2009) Dual role of K ATP channel C-terminal motif in membrane targeting and metabolic regulation. *Proc Natl Acad Sci USA* 106(39):16669–16674.
13. Healy JA, et al. (2010) Cholinergic augmentation of insulin release requires ankyrin-B. *Sci Signal* 3(113):ra19.
14. Kline CF, Hund TJ, Mohler PJ (2010) Ankyrin regulates KATP channel membrane trafficking and gating in excitable cells. *Channels (Austin)* 4(1):55–57.
15. Tarasov AI, et al. (2007) Functional analysis of two Kir6.2 (KCNJ11) mutations, K170T and E322K, causing neonatal diabetes. *Diabetes Obes Metab* 9(Suppl 2):46–55.
16. Li J, Kline CF, Hund TJ, Anderson ME, Mohler PJ (2010) Ankyrin-B regulates Kir6.2 membrane expression and function in heart. *J Biol Chem* 285(37):28723–28730.
17. Li J, et al. (2007) Calmodulin kinase II inhibition enhances ischemic preconditioning by augmenting ATP-sensitive K+ current. *Channels (Austin)* 1(5):387–394.
18. Ashcroft FM (2007) The Walter B. Cannon Physiology in Perspective Lecture, 2007. ATP-sensitive K+ channels and disease: From molecule to malady. *Am J Physiol Endocrinol Metab* 293(4):E880–E889.
19. Babenko AP, Aguilar-Bryan L, Bryan J (1998) A view of sur/KIR6.X, KATP channels. *Annu Rev Physiol* 60:667–687.
20. Yan XS, Ma JH, Zhang PH (2009) Modulation of K(ATP) currents in rat ventricular myocytes by hypoxia and a redox reaction. *Acta Pharmacol Sin* 30(10):1399–1414.
21. Wellman GC, Quayle JM, Standen NB (1998) ATP-sensitive K+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. *J Physiol* 507(Pt 1):117–129.
22. Light PE, Allen BG, Walsh MP, French RJ (1995) Regulation of adenosine triphosphate-sensitive potassium channels from rabbit ventricular myocytes by protein kinase C and type 2A protein phosphatase. *Biochemistry* 34(21):7252–7257.
23. Lin YF, Chai Y (2008) Functional modulation of the ATP-sensitive potassium channel by extracellular signal-regulated kinase-mediated phosphorylation. *Neuroscience* 152(2):371–380.
24. Ropero AB, et al. (1999) Non-genomic actions of 17beta-oestradiol in mouse pancreatic beta-cells are mediated by a cGMP-dependent protein kinase. *J Physiol* 521(Pt 2): 397–407.
25. Lin YF, Jan YN, Jan LY (2000) Regulation of ATP-sensitive potassium channel function by protein kinase A-mediated phosphorylation in transfected HEK293 cells. *EMBO J* 19(5):942–955.
26. Wenham RM, Landt M, Easom RA (1994) Glucose activates the multifunctional Ca2+ /calmodulin-dependent protein kinase II in isolated rat pancreatic islets. *J Biol Chem* 269(7):4947–4952.
27. Geng X, Li L, Watkins S, Robbins PD, Drain P (2003) The insulin secretory granule is the major site of K(ATP) channels of the endocrine pancreas. *Diabetes* 52(3):767–776.