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The Potassium Channel Kir4.1 Associates with the Dystrophin-Glycoprotein Complex via α-Syntrophin in Glia*

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One of the major physiological roles of potassium channels in glial cells is to promote "potassium spatial buffering" in the central nervous system, a process necessary to maintain an optimal potassium concentration in the extracellular environment. This process requires the precise distribution of potassium channels accumulated at high density in discrete subdomains of glial cell membranes. To obtain a better understanding of how glial cells selectively target potassium channels to discrete membrane subdomains, we addressed the question of whether the glial inwardly rectifying potassium channel Kir4.1 associates with the dystrophin-glycoprotein complex (DGC). Immunoprecipitation experiments revealed that Kir4.1 is associated with the DGC in mouse brain and cultured cortical astrocytes. *In vitro* immunoprecipitation and pull-down assays demonstrated that Kir4.1 can bind directly to α-syntrophin, requiring the presence of the last three amino acids of the channel (SNV), a consensus PDZ domain-binding motif. Furthermore, Kir4.1 failed to associate with the DGC in brains from α-syntrophin knockout mice. These results suggest that Kir4.1 is localized in glial cells by its association with the DGC through a PDZ domain-mediated interaction with α-syntrophin and suggest an important role for the DGC in central nervous system physiology.

The inwardly rectifying potassium channel Kir4.1 is a tetrameric transmembrane protein expressed in the central nervous system. Within the central nervous system, this channel is found primarily in glial cells, including astrocytes within the brain (1) and Müller cells of the retina (2). Glial Kir channels are involved in the process known as potassium spatial buffering, which helps to regulate the extracellular K^+ concentration. This process ensues when K^+ released from neurons is absorbed from the extracellular space by glial cells and redistributed to areas where the extracellular K^+ concentration is low (3, 4). Kir4.1 channels are highly enriched in astrocytic processes that surround blood vessels (5) as well as in the end-feet and perivascular processes in Müller cells (6). It has been hypothesized that this unique distribution within glial cells helps to facilitate the potassium buffering process by producing subcellular domains of high K^+ conductance (7, 8). The water channel aquaporin-4 (AQP4)[1] can co-localize with Kir4.1 in glial cells (9) and was also recently shown to play a significant role in potassium buffering in brain (10).

The subcellular distribution of proteins such as Kir4.1 in glial cells implies an underlying mechanism for these discrete localization patterns. One possible mechanism includes the binding to proteins that contain PDZ (PSD-95/Discs large/ZO-1) domains. These domains are responsible for a wide array of protein-protein interactions in the central nervous system and elsewhere (11). Kir4.1 harbors a consensus type I PDZ domain-binding region at its C terminus, and *in vitro* studies have shown that Kir4.1 can interact with proteins that possess these domains (12). One specific group of proteins shown to include this motif are the syntrophins, which are found as part of a multiprotein complex known as the dystrophin-glycoprotein complex (DGC) (13).

The DGC is a membrane-spanning group of proteins (14) most recognized for its role in muscle, where it may take part in the maintenance of myofiber structure and/or calcium homeostasis (15). Mutations in these proteins are responsible for a variety of muscular dystrophies (16, 17). A notable proportion of muscular dystrophy patients exhibit non-progressive cognitive deficits, suggesting a link between the DGC and brain function (18).

A DGC assembly was recently characterized in Müller glial cells of the retina (19), and the subcellular distribution of both AQP4 (20) and Kir4.1 (21) is dependent on the presence of the short dystrophin isoform Dp71. Within the brain, astrocytes have been shown to include DGC components α-dystrobrevin and β-dystroglycan (22), α-syntrophin (23), α-dystroglycan (24), utrophin (24, 25), dystrobrevins (26), and Dp71 (27), implying the presence of a DGC in this cell type as well. The expression and localization of AQP4 in astrocytes are dependent on α-syntrophin (23, 28, 29), establishing at least one role for an astrocytic DGC. Recent evidence also suggests that the DGC confers subcellular clustering and localization of signaling molecules and transmembrane proteins in the brain (30–32).

Based on the preceding evidence, we hypothesized that Kir4.1 is associated with the DGC in brain and astrocytes. Moreover, we propose that the interaction between Kir4.1 and the DGC may be mediated by a direct PDZ domain-dependent interaction with α-syntrophin. Based on our results and other recent data, we propose a model in which Kir4.1 and AQP4 may be co-associated via their respective interactions with the DGC.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal antibodies against Kir4.1 were as characterized and described previously (6). Goat polyclonal antibodies

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[1] The abbreviations used are: AQP4, aquaporin-4; DGC, dystrophin-glycoprotein complex; DSP, dithiobis(succinimidyl propionate); GST, glutathione *S*-transferase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HA, hemagglutinin.

against Kir4.1 were generated against the peptide sequence CLREQAEKEGSALSVRISNV by Bethyl Laboratories, Inc. (Montgomery, TX). Guinea pig anti-α-syntrophin antibodies were generated against the peptide sequence CRQPSSPGPQPRNLSEA by Affinity Bioreagents (Golden, CO). Rabbit anti-α-syntrophin polyclonal antibody was obtained from Sigma. Mouse anti-β-dystroglycan monoclonal antibody (clone 43DAG1/8D5) was from Novacastra (Newcastle upon Tyne, United Kingdom). Rabbit polyclonal antibody HA.11 was obtained from Babco (Richmond, CA). Mouse anti-dystrophin C terminus monoclonal antibody (clone 6C5) and mouse anti-glial fibrillary acidic protein monoclonal antibody (clone GA5) were from Chemicon International, Inc. (Temecula, CA). Mouse anti-c-Myc monoclonal antibody (clone 9E10) was obtained from Roche Diagnostics. Secondary antibodies used for Western blotting were peroxidase-conjugated anti-mouse, anti-rabbit, anti-guinea pig, or anti-goat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Cell Cultures—Mouse cortical astrocyte cultures were prepared as described previously (33). Astrocytes were allowed to grow in culture for 2 weeks, during which time they were split twice. Cells were grown to confluence prior to use in biochemical experiments. These cells were verified to be astrocytes by immunostaining with antibodies against glial fibrillary acidic protein, which revealed positive staining in >95% of all cells. COS-7 cells were grown in 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin in Dulbecco's modified Eagle's medium and transfected using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions.

Cross-linking and Lysis—Cross-linking solution was prepared by first dissolving 2 mM dithiobis(succinimidyl propionate) (DSP; Pierce) in Me_2SO, which was then added at a 10-fold dilution (final [DSP] = 200 μM) to buffer consisting of 250 mM sucrose, 7.5 mM Na_2HPO_4, 5 mM EGTA, 5 mM EDTA (pH 7.4), and Complete protease inhibitor mixture (Roche Diagnostics). For astrocyte cultures, plates were washed with phosphate-buffered saline and incubated in cross-linking solution with gentle agitation for 30 min at room temperature. The cross-linking reaction was then quenched by the addition of 50 mM Tris (pH 7.4) for 15 min at room temperature. The supernatant was removed, and cells were lysed for 30 min at 4 °C in 500 μl of lysis buffer consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 50 mM EGTA, and 0.1% Triton X-100 plus Complete protease inhibitor mixture. Cell lysates were spun at top speed in a microcentrifuge at 4 °C for 10 min, and supernatants were recovered and utilized immediately. Brain tissue lysates were prepared from adult C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) or from α-syntrophin null mice as described previously (5). Mice were killed, and brains were removed and homogenized using a mechanical tissue homogenizer in buffer consisting of 250 mM sucrose, 7.5 mM Na_2HPO_4, 5 mM EGTA, 5 mM EDTA (pH 7.4), and Complete protease inhibitor mixture. The homogenate was then supplemented with 200 μM DSP predissolved in Me_2SO and agitated at room temperature for 30 min. The cross-linking reaction was quenched with 50 mM Tris (pH 7.4) for 15 min, and proteins were extracted by the addition of 150 mM NaCl and 1% Triton X-100 under gentle agitation for 1 h at 4 °C. Lysates were then spun at top speed in a microcentrifuge at 4 °C for 10 min, and supernatants collected and spun again. The final supernatant was collected and used immediately. For COS-7 cells, 24 h after transfection, each plate was washed once with phosphate-buffered saline and harvested in 500 μl of lysis buffer. Lysates were then centrifuged at 1000 × *g*, and supernatants were collected and utilized immediately. It should be noted that the cross-linking reagent was not used for co-immunoprecipitations in COS-7 cells or for any of the glutathione *S*-transferase (GST) pull-down assays.

Immunoprecipitation—Mouse brain lysates were precleared of immunoglobulin by incubation for 1–3 h at 4 °C with protein A- or G-Sepharose beads (Zymed Laboratories Inc.), which were then removed by centrifugation. Brain and cell lysates were incubated with 1–15 μg of the pull-down antibody overnight at 4 °C on a mechanical rotator. Recombinant protein G- or A-Sepharose beads preblocked in 2% bovine serum albumin were then added for 1 h at 4 °C. The beads were washed five times with lysis buffer and resuspended in SDS-PAGE loading buffer, and samples were subjected to gel electrophoresis and Western blotting either as described previously (21) or using NuPAGE BisTris 4–12% gradient gels (Invitrogen), followed by subsequent transfer to polyvinylidene difluoride membranes according to the manufacturer's instructions.

DNA Constructs—GST fusion constructs were engineered by PCR. Template DNA consisted of rat Kir4.1 cDNA in a pcDNA3.1 vector previously modified to contain an EcoRI restriction site immediately downstream of the stop codon. An internal PCR primer containing a 5′-EcoRI restriction site was synthesized and used concurrently with the bovine growth hormone pcDNA3.1 reverse primer to amplify a DNA fragment to encode the last 87 amino acids of the channel protein. For the truncation mutant (GST-Kir4.1Δ), prior to the PCR described above, a stop codon was introduced at Ser^{377} using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting PCR products were digested with EcoRI and ligated to the pGEX-4T-2 vector (Amersham Biosciences). Myc-α-syntrophin was constructed using mouse α-syntrophin cDNA in pBluescript SK, which was digested with BssHII and SmaI to extract a fragment containing all except for the first five codons of the open reading frame. The resulting sticky ends were filled in using T4 DNA polymerase, and the fragment was ligated to the pCMV-Tag3B vector (Stratagene) predigested with EcoRV, resulting in a Myc tag at the N terminus of the protein. For the α-syn$^-$ construct, pCMV-Tag3B/Myc-α-syntrophin was used as a template for PCR using a forward primer complementary to a sequence immediately downstream of the α-syntrophin PDZ domain and tagged at the 5′-end with an Eco47III restriction site, used concurrently with a 3′-T7 primer. The resulting PCR fragment and full-length construct were digested with Eco47III and SmaI, and the PCR fragment was ligated to the vector. The resulting cDNA encoded a Myc-tagged α-syntrophin construct lacking the PDZ domain.

Synthesis of GST Proteins—GST fusion constructs were transformed into BL21 Codon Plus-RP cells (Stratagene) according to the manufacturer's instructions. Single colonies were used to inoculate 10 ml of 2XYT medium + 50 μg/ml ampicillin and grown overnight in a 37 °C shaking water bath. The next morning, 1 ml of the overnight culture was used to inoculate a fresh 100-ml culture of 2XYT medium + 50 μg/ml ampicillin and grown to A_{600} = 0.7, and then 0.5 mM isopropyl-β-D-thiogalactopyranoside was added to induce protein expression. After 2 h, cultures were harvested by centrifugation, resuspended in 5 ml of phosphate-buffered saline, and then sonicated for 4 × 15 s at 1-min intervals. The sonicates were removed by centrifugation, and the supernatant was added to 100 μl of a 50% slurry of glutathione-Sepharose beads (Amersham Biosciences) and incubated at 4 °C for 1 h. Following incubation, the beads were washed extensively, aliquotted, and stored at −80 °C until needed.

GST Pull-down Assay—Cell lysates were incubated in 20 μl of glutathione-Sepharose beads previously conjugated to the GST fusion protein of interest. Samples were incubated on a mechanical rotator overnight at 4 °C. The next morning, the samples were washed extensively with lysis buffer, resuspended in 1× SDS-PAGE loading buffer, and then subjected to SDS-PAGE and Western blotting as described (21).

RESULTS

The Brain-specific DGC Forms a Complex with Kir4.1—To date, the mechanisms that underlie the targeting and subcellular localization of Kir4.1 in astrocytes remain unknown. Our recent data showing the essential role of the short dystrophin isoform Dp71 in the localization of Kir4.1 channels in Müller cells of the retina (21) suggest an interaction between these proteins in glial cells. To verify whether proteins of the DGC interact with Kir4.1, we performed a series of immunoprecipitation experiments using mouse brain lysates. The cross-linking reagent DSP has been previously used to stabilize interactions involving the DGC (23, 34) and was used to stabilize protein-protein interactions in our studies (see "Materials and Methods"). The results of these experiments are shown in Fig. 1. We first asked whether the glial dystrophin isoform Dp71 and the Kir4.1 channel proteins co-associate in brain. We used rabbit polyclonal antibodies to immunoprecipitate Kir4.1 from brain and recovered the immunocomplexes with protein A-Sepharose beads, which were then used for Western blotting. Fig. 1*A* (*IP:Kir4.1 lane*) shows that we were able to detect a band at ~71 kDa when we probed the resulting Western blot with anti-dystrophin antibodies. These data indicate that Kir4.1 channels and the dystrophin isoform Dp71 form a macromolecular complex in brain. Specificity of the immunoprecipitation was verified by running a mock immunoprecipitation lacking the anti-Kir4.1 antibody, which did not bring down the Dp71 protein (Fig. 1*A*, *pA lane*). We also tested the interactions of Kir4.1 channels with other components of the DGC, *viz.* α-syntrophin and β-dystroglycan. Within the DGC, α-syntrophin is localized to the intracellular compartment and binds directly to dystrophin (35). When we performed immunoprecipitation assays with anti-α-syntrophin antibody and probed

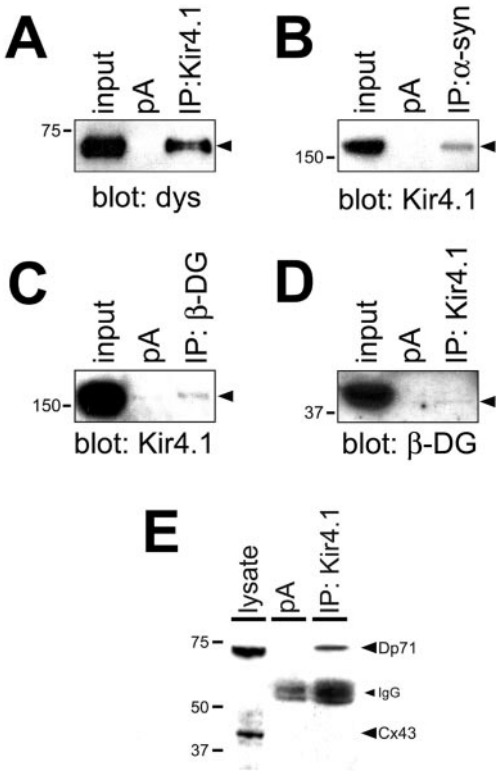

FIG. 1. **Kir4.1 and DGC components co-associate whole mouse brain.** *A*, brain lysates were immunoprecipitated with antibodies against the potassium channel Kir4.1, and the resulting Western blot was probed with antibodies against dystrophin (*dys*). The *input lane* reveals a band at ~71 kDa, indicating the presence of the short dystrophin isoform, Dp71, in brain. The *IP:Kir4.1 lane* shows the recovery of Dp71 (*arrowhead*) in samples that were incubated with anti-Kir4.1 antibodies and then recovered by protein A-Sepharose, and the *pA lane* shows that the Sepharose matrix alone did not result in the recovery of Dp71. *B*, Kir4.1 from mouse brain migrated in its tetrameric form at ~200 kDa (*input lane*) and was recovered when lysates were precipitated with anti-α-syntrophin antibody (*IP:α-syn lane*, *arrowhead*). Protein A-Sepharose alone did not result in the recovery of Kir4.1 (*pA lane*). *C*, antibodies against β-dystroglycan were used for immunoprecipitation, and the blot was probed for Kir4.1. As shown in the *IP:β-DG lane*, Kir4.1 was recovered when anti-β-dystroglycan antibodies were used for precipitation (*arrowhead*), but not with protein A alone (*pA lane*). *D*, when the converse experiment of the one shown in *C* was performed, immunoprecipitation with anti-Kir4.1 antibody and detection with anti-β-dystroglycan antibody (*β-DG*) revealed a band at ~43 kDa (*lane IP:Kir4.1 lane*, *arrowhead*), but not with protein A alone (*pA lane*), suggesting that β-dystroglycan was recovered by the precipitation of Kir4.1. *E*, to address whether DSP can introduce artifactual interactions, we first repeated the experiment shown in *A*, and Dp71 was recovered by the precipitation of Kir4.1. We then incubated the membrane with monoclonal antibodies against connexin-43, a gap junction hemichannel protein prominent in astrocytic membranes, resulting in the presence of a band at ~43 kDa in the *input lane*, but not in the immunoprecipitation lane (*IP:Kir4.1 lane*), suggesting that DSP does not introduce artifactual interactions. Whole wild-type mouse brains were homogenized, cross-linked in 200 μM DSP, and then lysed. The lysate was divided equally into samples (minus 5 μl for input) and incubated either with the pull-down antibody and recovered with protein A-Sepharose beads (*IP:Kir4.1 lane*) or without antibody and with protein A-Sepharose only (*pA lane*). Recovered immunocomplexes were processed by SDS-PAGE and Western blotting. Approximate molecular masses are given on the left of each panel in kilodaltons.

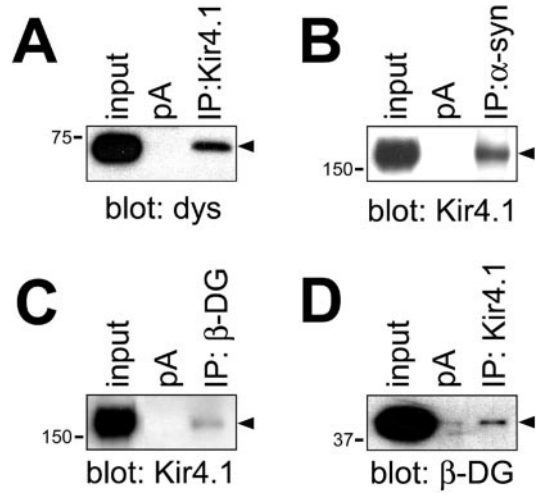

FIG. 2. **Kir4.1 and DGC components co-associate in cultured cortical astrocytes.** *A*, the short isoform of dystrophin, Dp71, was apparent in astrocytes as a band at ~71 kDa when probed with anti-dystrophin antibodies (*dys*; *input lane*) and was recovered when lysates were precipitated with anti-Kir4.1 antibody (*IP:Kir4.1 lane*, *arrowhead*). Incubation with protein A-Sepharose alone did not result in the recovery of Dp71 (*pA lane*). *B*, lysates were immunoprecipitated with antibodies against α-syntrophin, and the Western blot was incubated with antibodies against Kir4.1. The *input lane* shows the 200-kDa tetrameric form of Kir4.1 in these lysates, and the *IP:α-syn lane* shows that when α-syntrophin was precipitated, Kir4.1 was recovered (*arrowhead*). Protein A-Sepharose alone did not pull down Kir4.1 (*pA lane*). *C*, antibodies against β-dystroglycan were used for immunoprecipitation from astrocyte lysates and then probed for Kir4.1. As shown in the *IP:β-DG lane* (*arrowhead*), precipitation of β-dystroglycan resulted in the recovery of Kir4.1, whereas protein A-Sepharose alone did not (*pA lane*). *D*, we then performed the reverse experiment of the one in *C* by precipitating with anti-Kir4.1 antibody and probing for β-dystroglycan (*β-DG*). A band at ~43 kDa in the *IP:Kir4.1 lane* (*arrowhead*) indicates that β-dystroglycan was recovered by the precipitation of Kir4.1, but not with protein A-Sepharose alone (*pA lane*). Astrocyte cultures from neonatal mice were grown to confluence, cross-linked in culture in 200 μM DSP, and then lysed. Lysates were divided equally into samples (minus input control sample) and incubated either with the pull-down antibody and recovered with protein A-Sepharose beads (*IP:Kir4.1 lane*) or without antibody and with protein A-Sepharose only (*pA lane*). Recovered complexes were subjected to SDS-PAGE and Western blotting. Approximate molecular masses are given on the left of each panel in kilodaltons.

the precipitate for the presence of Kir4.1, we detected a band at ~200 kDa, the expected size for the tetrameric form of Kir4.1 (Fig. 1*B*). Once again, our blotting antibody did not show a positive result when the pull-down antibody was excluded from the reaction. We next attempted to demonstrate an interaction between Kir4.1 and β-dystroglycan. This component of the DGC spans the cell membrane and binds directly to Dp71, but not to α-syntrophin (36, 37). Upon immunoprecipitation with monoclonal antibodies against β-dystroglycan and subsequent probing for Kir4.1, we detected the expected band of ~200 kDa (Fig. 1*C*). To verify the specificity of the coprecipitation, we performed the reciprocal experiments in which Kir4.1 and β-dystroglycan were coprecipitated using an antibody against Kir4.1 (Fig. 1*D*). In both experiments, the identified bands were quite faint, although there appears to be a band present in the immunoprecipitation lane (*IP:Kir4.1 lane*) that is not present in the protein A-Sepharose-only lane (*pA lane*), demonstrating the ability of Kir4.1 (Fig. 1*C*) and β-dystroglycan (Fig. 1*D*) to co-immunoprecipitate. A major concern in using cross-linking reagents is the chance that artifactual interactions are formed in the cross-linking reaction. When we tested for this possibility, we failed to detect interactions between Kir4.1 channels and other glial transmembrane proteins such as the gap junction protein connexin-43, as shown in Fig. 1*E*.

The DGC in Cultured Cortical Astrocytes Forms a Complex with Kir4.1—To verify whether interaction between Kir4.1 and the DGC can occur specifically in astrocytes, we cultured cortical astrocytes from neonatal mice (postnatal days 1–3) and conducted a series of co-immunoprecipitation assays using these cells. Fig. 2*A* (*IP:Kir4.1 lane*) shows that when we immunoprecipitated Kir4.1 from cultured astrocytes, we detected a positive band at ~71 kDa in the immunoprecipitated sample

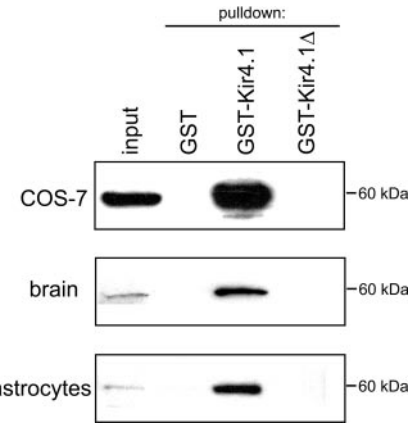

FIG. 3. **The C terminus of Kir4.1 is required for the binding to α-syntrophin *in vitro*.** We constructed GST fusion proteins containing either the last 87 amino acids of Kir4.1 with the intact C terminus (GST-Kir4.1) or the last 84 amino acids of the channel without the terminal PDZ domain-binding region (GST-Kir4.1Δ). Each of these constructs or GST alone was incubated with the tissue lysates indicated. *Upper panel*, COS-7 cells were transfected with Myc-α-syntrophin. Cells were lysed and incubated with GST, GST-Kir4.1, or GST-Kir4.1Δ. The *first lane* (*input*) shows the expected size for Myc-α-syntrophin from cell lysates. Lysates incubated with GST-Kir4.1 resulted in the recovery of Myc-α-syntrophin (*third lane*), whereas GST (*second lane*) and GST-Kir4.1Δ (*fourth lane*) did not. *Middle panel*, fusion proteins were incubated with lysates from whole mouse brain, and recovered complexes were probed for α-syntrophin. The sample incubated with GST-Kir4.1 resulted in a positive band at ~60 kDa when probed with antibodies against α-syntrophin, whereas incubation with GST or GST-Kir4.1Δ did not. *Lower panel*, fusion proteins were incubated with lysates from cultured cortical astrocytes. The sample incubated with GST-Kir4.1 resulted in the recovery of α-syntrophin, whereas neither GST nor GST-Kir4.1Δ recovered α-syntrophin from these cells.

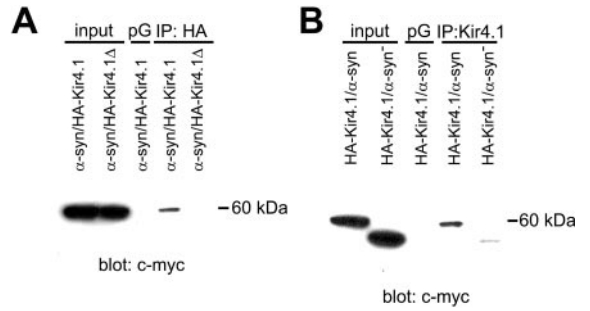

FIG. 4. **Kir4.1 and α-syntrophin interact in a PDZ domain-dependent manner *in situ*.** *A*, COS-7 cells were cotransfected with Myc-α-syntrophin (*α-syn*) and either full-length HA-Kir4.1 or a truncated version lacking the last three amino acids (HA-Kir4.1Δ). After protein expression, the cells were lysed and incubated with antibodies against HA. Recovered complexes were processed by Western blotting and probed with anti-c-Myc antibody. The *first two lanes* were loaded with lysates from Myc-α-syntrophin/HA-Kir4.1 or Myc-α-syntrophin/HA-Kir4.1Δ, as indicated, to demonstrate that Myc-α-syntrophin was expressed under both conditions. The *last two lanes* show that when samples were immunoprecipitated (*IP*) with anti-HA antibody, the lysates expressing full-length HA-Kir4.1 resulted in the recovery of Myc-α-syntrophin, whereas the lysates expressing HA-Kir4.1Δ did not. When Myc-α-syntrophin/HA-Kir4.1-expressing cells were incubated with protein G-Sepharose beads only (*pG*; *third lane*), Myc-α-syntrophin was not recovered. *B*, COS-7 cells were cotransfected with HA-Kir4.1 and either full-length Myc-α-syntrophin (*α-syn*) or a Myc-α-syntrophin construct from which the PDZ domain had been removed (*α-syn*$^-$). Cell lysates were immunoprecipitated with anti-HA antibodies and recovered with protein G-Sepharose, and the Western blot was probed for c-Myc. The *first two lanes* show that when lysates from each of these conditions were directly loaded onto the gel, they both expressed their respective Myc-α-syntrophin constructs; the full-length construct migrated at 59 kDa, whereas the removal of the PDZ domain is reflected by a lower molecular mass band (*second lane*). The *fourth lane* shows that when a sample cotransfected with HA-Kir4.1 and Myc-α-syntrophin was immunoprecipitated with anti-HA antibody, Myc-α-syntrophin was recovered. The *fifth lane* shows that when the immunoprecipitation was performed with cells cotransfected with HA-Kir4.1 and Myc-α-syn$^-$, the recovery of the latter protein was significantly reduced in comparison with the full-length protein, suggesting that the PDZ domain in α-syntrophin plays a major role in the binding to Kir4.1. Protein G-Sepharose alone did not recover any Myc-α-syntrophin (*third lane*).

when probed with anti-dystrophin antibodies. This result indicates the macromolecular assembly of Dp71 and Kir4.1 channels in astrocytes. Interactions of Kir4.1 channels with other proteins of the DGC were also detected in this cell type. For example, when α-syntrophin was immunoprecipitated and the resulting Western blot was probed with anti-Kir4.1 antibodies, a positive band at ~200 kDa was seen (Fig. 2*B*, *IP:α-syn lane*), indicating the recovery of Kir4.1 in the immunocomplex, and the same result was seen when we used anti-β-dystroglycan antibodies for immunoprecipitation (Fig. 2*C*). Furthermore, when Kir4.1 was immunoprecipitated from astrocytes, an immunopositive band of 43 kDa was revealed after probing the blot with anti-β-dystroglycan antibodies (Fig. 2*D*). Together, these results indicate that Kir4.1 interacts with the DGC components Dp71, α-syntrophin, and β-dystroglycan in astrocytes.

Kir4.1 Associates with α-Syntrophin in a PDZ Domain-dependent Manner—Based on our data demonstrating that Kir4.1 can associate with proteins of the DGC, our next goal was to identify which of the DGC components can directly associate with the channel. Because α-syntrophin harbors a type I PDZ domain and because the C-terminal sequence of Kir4.1 (-SNV-COOH) has a consensus motif for binding to this domain, we sought to determine whether these proteins can directly associate by this mechanism. To this end, three approaches were used. First, we performed a pull-down assay using fusion proteins of GST and the C terminus of Kir4.1 incubated with lysates from COS-7 cells transiently transfected with Myc-α-syntrophin. The results from this experiment revealed an anti-c-Myc immunoreactive band when lysates were incubated in the presence of the recombinant GST-Kir4.1 protein, but not with GST alone or recombinant GST-Kir4.1Δ, which lacks the extreme C terminus of the channel (Fig. 3, *upper panel*). Second, we performed an *in vitro* binding assay using the same immobilized fusion proteins incubated with lysates from either whole brain or astrocyte cultures as sources for α-syntrophin. The results in both circumstances were similar and are summarized in Fig. 3, confirming direct *in vitro* PDZ domain-dependent binding between Kir4.1 and α-syntrophin. The latter interactions were also confirmed to be highly specific, as recombinant GST-Kir4.1Δ or GST alone failed to bring down the endogenous α-syntrophin. Third, we performed immunoprecipitation assays in COS-7 cells transfected with Kir4.1 and α-syntrophin cDNAs to show that these proteins can interact *in situ*. In our first experiment using this approach, Myc-α-syntrophin was cotransfected with either hemagglutinin (HA)-Kir4.1 or HA-Kir4.1Δ in COS-7 cells, and lysates were immunoprecipitated with anti-HA antibodies. When these samples were then probed with anti-Myc antibodies, an immunopositive band at ~60 kDa, corresponding to Myc-α-syntrophin, was present in cells cotransfected with HA-Kir4.1, but not with HA-Kir4.1Δ (Fig. 4*A*). We then cotransfected HA-Kir4.1 with either full-length Myc-α-syntrophin or a truncated version lacking the PDZ domain (Myc-α-syn$^-$). When HA-Kir4.1 was immunoprecipitated, we were able to recover the full-length Myc-α-syntrophin in the precipitated complex, and although we were also able to detect the Myc-α-syn$^-$ construct, it was to a drastically reduced degree (Fig. 4*B*). Together, these results indicate that Kir4.1 is able to bind directly to α-syntrophin and that this interaction is PDZ domain-dependent.

Kir4.1 Fails to Associate with the DGC in the Absence of α-Syntrophin—Based on the preceding evidence that Kir4.1

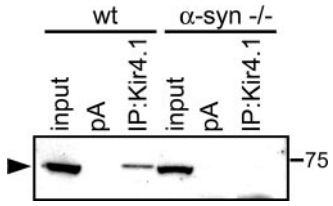

FIG. 5. **The brains from both wild-type and α-syntrophin knockout (α-syn$^{-/-}$) mice were homogenized, chemically cross-linked, and then incubated with antibodies against Kir4.1.** Shown here is the Western blot that was probed with antibodies against dystrophin. The lysate-only (*input*) lanes show a prominent band at ~71 kDa, demonstrating the expected size for Dp71 (*arrowhead*). In the wild-type (*wt*) brain, immunoprecipitation with anti-Kir4.1 antibody (*IP:Kir4.1*) resulted in the recovery of Dp71 (*third lane*); however, when brains from α-syntrophin null mice were used, Dp71 was not detected (*sixth lane*). Protein A-Sepharose controls (*pA*) did not result in the recovery of Dp71, demonstrating that this interaction was specific. The molecular mass is indicated on the right in kilodaltons.

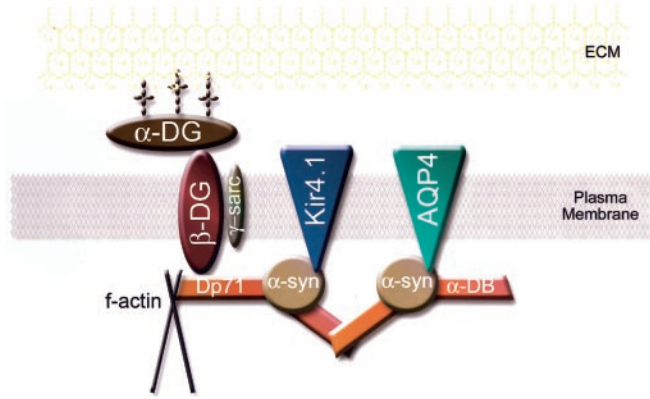

FIG. 6. **Proposed model for the binding of Kir4.1 and AQP4 to the DGC.** Kir4.1 and AQP4 may each bind an α-syntrophin syntrophin molecule (*α-syn*), allowing for both channels to be part of the same complex. Syntrophin can bind either α-dystrobrevin (*α-DB*) (50) or Dp71 (51). Dp71 is also associated with the membrane-spanning protein β-dystroglycan (*β-DG*) (19), which is itself bound to α-dystroglycan (*α-DG*) (52) in the extracellular space. γ-Sarcoglycan (*γ-sarc*) was also suggested to be a member of the glial DGC (19). *ECM*, extracellular matrix.

can bind to α-syntrophin in a PDZ domain-dependent manner, we wanted to determine whether α-syntrophin is the direct binding partner for Kir4.1, mediating the interaction between Kir4.1 and the DGC *in vivo*. To do so, we utilized brains from α-syntrophin knockout mice (previously described (38)) to determine whether Kir4.1 can still bind Dp71 in the absence of α-syntrophin. Brain tissues were chemically cross-linked; and as expected, in wild-type brains, the immunoprecipitation of Kir4.1 resulted in the recovery of Dp71 (Fig. 5). However, when the same experiment was performed using brains from α-syntrophin null mice, we were no longer able to detect the presence of Dp71 in our immunoprecipitates. These results suggest that α-syntrophin is required for the interaction between Kir4.1 and the DGC *in vivo*.

DISCUSSION

In this study, we have begun to investigate the molecular determinants of the interaction between the inwardly rectifying potassium channel Kir4.1 and proteins of the DGC. By examining co-immunoprecipitations between Kir4.1 and DGC proteins such as Dp71, α-syntrophin, and β-dystroglycan in mouse brain and cultured astrocytes, we found compelling evidence for such associations. Furthermore, we showed that α-syntrophin may be the direct link between Kir4.1 and the DGC since the C terminus of Kir4.1 can associate with α-syntrophin in a PDZ domain-dependent manner, and when α-syntrophin is absent, Kir4.1 no longer associates with other proteins of the DGC. These data suggest, for the first time, a direct association between the DGC and a potassium channel. Furthermore, our data presented herein extend previous work in the field to suggest a molecular model in which AQP4 and Kir4.1 could physically co-associate through their interactions with the DGC (Fig. 6).

Recent studies have revealed an apparent interdependence between α-syntrophin, Kir4.1, and AQP4. First, it was shown that Kir4.1, AQP4, and α-syntrophin all have a tendency to increase expression in a parallel fashion in human astrocytes in a number of pathological brain states (28). Second, studies have also shown that Kir4.1 and AQP4 (9) and AQP4 and α-syntrophin (39) are tightly co-localized in the end-feet of glial cells. Experiments using α-syntrophin knockout mice also revealed that the localization of AQP4 to these subcellular regions is dependent α-syntrophin (10, 23, 29). Third, potassium buffering is coupled to changes in the volume of the extracellular space (40, 41), suggesting a functional relationship between Kir4.1 and AQP4. Last, studies have shown that Kir4.1 is dependent on the presence of Dp71 for its distinctive subcellular localization pattern in Müller cells of the mammalian retina (20, 21). Together, these results indicate that DGC components are required for the proper localization of AQP4 and Kir4.1 and that α-syntrophin is the intermediary between the DGC and AQP4. Our results extend these findings to suggest that Kir4.1 is also linked directly to α-syntrophin.

Despite these findings, there is recent evidence suggesting that there may be some differences in the way that Kir4.1 and AQP4 interact with the DGC. For example, immunoelectron microscopy revealed that the absence of α-syntrophin dramatically alters the localization pattern of AQP4 (but not Kir4.1) in astrocytic end-feet (10). One possible explanation to reconcile these data is that other PDZ domain-containing proteins may also be present and could compensate by localizing Kir4.1 in the absence of α-syntrophin. The membrane-associated guanylate kinase protein SAP97 was shown to be present in Bergmann glia of the cerebellum (42) as well as in Müller cells of the retina (12) and can interact with Kir4.1 (12). As well, another membrane-associated guanylate kinase protein, PSD-93, was suggested to be associated with the Müller cell-specific arrangement of the DGC (19). Both SAP97 and PSD-93 can interact with Kir4.1 in a PDZ domain-dependent manner,[2] suggesting that the presence of either of these proteins *in vivo* could provide feasible compensation for α-syntrophin. Differences in the C termini of Kir4.1 (-RISNV-COOH) and AQP4 (-VLSSV-COOH) may result in differential affinities for particular PDZ domains and may facilitate the preferential binding of Kir4.1 (but not AQP4) to such compensatory proteins.

There are multiple functional implications for the association between Kir4.1 and the DGC in glial cells. Like neurons, glial cells of the central nervous system display distinct patterns of subcellular specialization, including the localization of Kir channels in Müller cells of the retina (2, 6), in astrocytes (5), and in Bergmann glia of the cerebellum (42). The strategic localization of Kir channels is believed to facilitate the process of potassium spatial buffering, where the increase in the extracellular K^+ concentration from depolarizing neurons is absorbed through Kir channels by adjacent astrocytes and then shunted through a network of these cells to regions of the brain where $[K^+]$ is low (3). The radial Müller glial cells of the retina perform a modified, single-cell version of this process termed "potassium siphoning," where potassium is absorbed from "K^+ sources" (*e.g.* areas of high neuronal activity), moved through the cell, and released into "K^+ sinks" such as the vitreous

[2] N. C. Connors and P. Kofuji, unpublished data.

humor (8). Recent studies showing the unique subcellular distribution patterns of Kir4.1 in the end-feet and perivascular processes of Müller cells (2, 6) and in perisynaptic and perivascular processes in astrocytes of the olfactory bulb (5) suggest that this distribution of Kir4.1 in glial cells is critical for the potassium buffering function. This hypothesis is supported by modeling studies (7). Until now, the mechanisms for the subcellular localization of Kir4.1 and the resulting facilitation of the potassium buffering function were unknown. The results presented herein, along with our prior results (21), introduce one such molecular mechanism.

The possible interaction between Kir4.1, AQP4, and DGC proteins also suggests a model for the consolidation of the channels in glial cell membrane domains where there is a high likelihood of a basal lamina, such as the inner limiting membrane of Müller cells and around blood vessels in both astrocytes and Müller cells. Given that α-dystroglycan serves as a receptor to extracellular matrix molecules such laminin and agrin (see Ref. 43), then a critical step in the clustering and localization of Kir4.1 and AQP4 channels in glial cells would occur upon apposition of the DGC proteins to the basal lamina. Deficiencies in the post-translational modification of α-dystroglycan are a common feature of muscular dystrophies such as Walker-Warburg syndrome and muscle-eye-brain disease (14), and these anomalies in α-dystroglycan seem to affect the binding to extracellular matrix ligands, including laminin and agrin (44). It will be interesting to verify whether the distribution and expression of Kir4.1 and AQP4 channels are altered in these diseased states.

Another important implication for the binding to DGC proteins is the possible effects of Kir4.1 and AQP4 channels on glial function by forming complexes that also include signaling molecules. The biophysical properties and activity of Kir and AQP channels are modified by diverse protein kinases and regulatory molecules (45–48). The association of the DGC with signaling molecules, including neuronal nitric-oxide synthase (30), syntrophin-associated serine/threonine kinase, and Grb2 (31, 35, 49), has been described. It is also notable that α-syntrophin can bind directly to phosphatidylinositol 4,5-bisphosphate, a potent activator of Kir channels (45). These observations suggest that the DGC may facilitate Kir4.1 and AQP4 channel activity both by localizing the channel to particular subcellular compartments and by having influence over the activation state of the channel by the presentation of modulatory molecules to the channels. Considering the importance of potassium buffering for neuronal homeostasis, the ramifications of these possibilities suggest that the DGC may have a multidimensional influence over cellular and systemic physiology in the central nervous system.

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