Identification of Native Atrial G-protein-regulated Inwardly Rectifying K\textsuperscript{+} (GIRK) Channel Homomultimers*

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G-protein-regulated inwardly rectifying K\textsuperscript{+} (GIRK) channels play critical inhibitory roles throughout the nervous system, heart, and pancreas. They are believed to be heterotetramers consisting of GIRK1 (Kir3.1) and either GIRK2 (Kir3.2), GIRK3 (Kir3.3), or GIRK4 (Kir3.4) subunits. The GIRK1 subunit is hypothesized to be critical to form GIRK channels with normal channel kinetics based on heterologous expression studies. However, GIRK2 and GIRK3 proteins are present in areas of the brain where no GIRK1 has been detected. Here we demonstrate that GIRK tetramers lacking GIRK1 can be purified from bovine heart atria. We have found that only half of GIRK4 is purified as the GIRK1-GIRK4 heterotetramer, whereas the remaining GIRK4 forms a high molecular weight, SDS-resistant complex that does not contain GIRK1. These GIRK4 complexes, most likely GIRK4 homotetramers, were previously not seen because of their aberrant migration on SDS-polyacrylamide gels. We propose that all of GIRK1 and half of GIRK4 proteins in atria combine to form the heterotetramer I_{K_{AC}}\textsubscript{GIRK}, whereas the remaining GIRK4 forms a novel tetrameric complex. GIRK4 homotetramers form channels with unusual single channel behavior, and their contribution to native currents requires further investigation.

EXPRESS PROCEDEUS

Isolation and Solubilization of Atrial Membrane Proteins—Bovine atrial plasma membranes were isolated as described (31). Membranes were solubilized in 1.0% CHAPS-HEDN buffer, pH 7.5 (in mM: 10 HEPES, 1 EDTA, 1 dithiothreitol, and 100 NaCl). The protease inhibitors leupeptin (50 μg/ml), phenylmethylsulfonyl fluoride (100 μg/ml), aprotinin (1 μg/ml), and pepstatin (2 μg/ml) (all from Sigma) were used in all experiments.

Expression and Purification of GIRK1 and GIRK4 Homotetramers from COS-7 Cells—Plasma membrane proteins containing epitope-tagged GIRK1-AU5 and GIRK4-AU5 were isolated from COS-7 cells (32). Solubilization was performed as described above for the native atrial membrane proteins. For the GIRK4 immunoprecipitation, protein A-Sepharose (Amersham Pharmacia Biotech) was pre-incubated with 3 μg of anti-GIRK4 antibodies (anti-CIRN2) (15). After 30 min of pre-incubation, solubilized proteins from a single 100-mm GIRK4-transfected dish of cells were added and incubated at 4 °C for 150 min. The protein A-Sepharose-antibody-GIRK4 complexes were washed 5 × 1 ml with immunoprecipitation buffer (1% CHAPS, 10 mM HEPES, 100 mM NaCl, 5 mM EDTA, pH 7.5), and the GIRK4 was eluted with 1 mg/ml CIRN2 antigenic peptide for 150 min at 22 °C with 3 eluate exchanges. For the anti-GIRK1 immunoprecipitation, solubilized membrane proteins from COS-7 cells transfected with GIRK1 were added to protein A-Sepharose and a 1:50 dilution of asacit fluid containing AU5 monoclonal antibodies (BabCO, Berkeley Antibody Co., Richmond, CA) and incubated at 4 °C for 150 min. The protein A-Sepharose-antibody-GIRK1 complexes were washed 5 × 1 ml with immunoprecipitation buffer. GIRK1 was eluted with 0.25 mg/ml AU5 antigenic peptide at 22 °C with 3 × 100 μl eluate exchanges for 1 h each.

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‡ The abbreviations used are: GIRK, G-protein-regulated inwardly rectifying K\textsuperscript{+} channel; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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recombinant protein. Increasing the reaction volumes >10-fold had no effect on the adducts formed. Approximately 10 μg of crude solubilized atrial protein or 10 μg of solubilized COS-7 membrane protein was used in each reaction.

For cross-linking studies, a solution containing 5 mM 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP, Pierce) was prepared as a 10× stock solution immediately before use. The reactions were allowed to proceed for 1 h on ice and then terminated for 30 min with 50 mM Tris, pH 7.5.

Immunodepletion—For anti-GIRK4 antibody immunodepletion, between 0.025 and 0.4 μg of anti-GIRK4 peptide antibody (anti-CIRN2, generated against amino acids 19–32) was used to immunoprecipitate an approximately 10 μg of atrial membrane proteins for 150 min at 4 °C. 10-μl aliquots of the supernatants were analyzed by SDS-PAGE and immunoblotting. For anti-GIRK1 immunoprecipitation, between 0.1 and 2.0 μl of anti-GIRK1 fusion protein antibodies (anti-Csh serum, generated against the last 156 amino acids of GIRK1) were used to immunoprecipitate approximately 10 μg of atrial membrane proteins overnight at 4 °C. Before immunoprecipitation, anti-GIRK1 antibodies were preincubated with protein A-Sepharose (Amersham Pharmacia Biotech) for 120 min and washed 4 times with 1-mL volumes of immunoprecipitation buffer. 15-μl (of 90 μL total immunoprecipitation volumes) aliquots of supernatants were analyzed by SDS-PAGE and immunoblotted.

Size Exclusion Chromatography—Size exclusion chromatography was performed as described previously (27).

SDS-PAGE Analysis and Immunoblotting—Unless specified otherwise, atrial membrane proteins or recombinant GIRK protein was resuspended in Laemmli sample buffer containing 100 mM dithiothreitol for 15 min at 50 °C, 30 min at room temperature, and 15 min at 50 °C. 3–10% separating, 3% stacking, and precast 2–15% gels were utilized. Samples were analyzed by immunoblotting with anti-GIRK1 antibodies (generated against amino acids 1–22) and/or anti-GIRK4 antibodies. Transfer times for Western blot analysis were extended to >2 h at 15 V to improve transfer of the complexes. When sequential probing with antibodies was necessary, polyvinylidene fluoride membranes (Millipore, Bedford, MA) were stripped with 62.5 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol for 15 min at 50 °C. A GS-700 imaging densitometer (Bio-Rad) was used to analyze the protein gels and immunoblots. Molecular weights were calculated using densitometry profiles from a combination of prestained high molecular weight markers (Bio-Rad) and low and high molecular weight markers (Amersham Pharmacia Biotech). In a portion of the gels, thyroglobulin (Amersham Pharmacia Biotech) was added to ensure linearity through at least 330 kDa.

Special Laemmli sample buffer conditions described in (Fig. 1 A) were as follows. For urea-treated samples, urea was added directly to the sample buffer to a final concentration of 8M. For NaCl-treated samples, the sample buffer was adjusted to contain a final concentration of 400 mM NaCl. Alternatively, the sample buffer was adjusted to contain a final concentration of 400 mM dithiothreitol (DTT), or 10% 2-mercaptoethanol, as follows. For urea-treated samples, urea was added directly to the sample buffer for 30 min at 50 °C followed by 30 min at room temperature. In addition to Laemmli sample buffer, the indicated samples were treated with either 8 M urea, 10% trichloroacetic acid, or 400 mM NaCl. The blot was probed with anti-GIRK4 (CIRN2) antibodies (αGIRK4(CIRN2))(A), stripped and reprobed with anti-GIRK1 antibodies (αGIRK1) (B), and stripped and reprobed with anti-GIRK4 (fusion) antibodies (αGIRK4(fusion))(C). A high molecular weight band (asterisk) was recognized by both the anti-GIRK4(CIRN2) and anti-GIRK4(fusion) protein antibodies.

RESULTS

A High Molecular Weight, SDS-resistant, Complex in Heart Atria Contains GIRK4—Native atrial membrane proteins were analyzed by 2–15% SDS-PAGE and immunoblotted (Fig. 1). Antibodies generated against amino acids 19–32 of GIRK4 (anti-CIRN2) (9, 33) recognized a 45- and 200-kDa protein (or protein complex). In control experiments, neither the 45-kDa protein nor the 200-kDa protein was recognized by anti-GIRK4 (anti-CIRN2) antibodies if the antibodies were preincubated with antigenic peptide (data not shown). The 45-kDa protein is GIRK4 (15), whereas the 200-kDa protein band has not been previously described. In addition, the 200-kDa protein (or protein complex) was detected by a second anti-GIRK4 antibody generated against amino acids 337–420 (fusion), verifying that the ~200-kDa band contained GIRK4 (Fig. 1C). Because of its high molecular weight, it is likely that the ~200-kDa protein band was lost during traditional SDS-PAGE analysis of heart membranes. In previous studies it is possible that the ~200-kDa protein band may have failed to migrate out of stacking gels, which are normally removed before blotting. Alternatively, the ~200-kDa protein band may have transferred poorly during immunoblotting or been mistaken for antibody. After stripping and reprobing the immunoblot with anti-GIRK1 antibodies, we observed bands at ~55- and ~60–75-kDa, which represent GIRK1 and glycosylated GIRK1, respectively (15) (Fig. 1B). The ~200-kDa band was not recognized by anti-GIRK1 antibodies, indicating that it did not contain GIRK1 protein.

We considered the possibility that the ~200-kDa band was the result of unknown protein (of ~155 kDa) associating with GIRK4 via a disulfide bond. However, the mobility of the ~200-kDa band was not altered by high concentrations of reducing reagents (100 mM dithiothreitol, or 10% 2-mercaptoethanol, data not shown). In addition, the mobility of the ~200-kDa band was not altered by treatment with high salt concentrations or urea, which reduces aggregation of membrane proteins (34) (Fig. 1, A and B). Trichloroacetic acid precipitation was the only method that altered the ~200-kDa band. Trichloroacetic acid treatment either shifted the ~200-kDa band to ~45 kDa or failed to precipitate its components. We could not distinguish between these two possibilities because the GIRK4 monomer band is always observed at ~45 kDa.

The High Molecular Weight, SDS-resistant, GIRK4 Is Not Associated with GIRK1, Whereas All of the Low Molecular Weight GIRK4 Exists as GIRK1-GIRK4 Heterotetramers—It has previously been reported that all GIRK4 is associated with GIRK1 in heart to form the heterotetramer I_{ACh}. Because the ~200-kDa GIRK4-containing complex was previously not seen, we examined whether GIRK4 in this complex was associated with GIRK1. To address this question, atrial sarcolemmal membranes were solubilized in 1% CHAPS and immunodepleted with increasing concentrations of anti-GIRK1 antibo-
We carried out immunodepletion experiments using ramers—germ agglutinin except by its association with GIRK1 (27). GIRK4, which is not glycosylated (15), does not bind wheat germ agglutinin affinity column, whereas the 200-kDa GIRK4-containing complex did not (27) (data not shown).

By trichloroacetic acid-precipitating the GIRK1-immunodepleted aliquots before SDS-PAGE analysis, we were able to shift at least a portion of the ~200-kDa complex to 45 kDa (compare Fig. 2, C and D), consistent with a disruption of the complex into individual components. In addition to the immunodepletion experiments, partial purification of I_{KACH} confirmed that the ~200-kDa complex was not associated with GIRK1. The ~45-kDa GIRK4 and all of the GIRK1 bound a wheat germ agglutinin affinity column, whereas the ~200-kDa GIRK4-containing complex did not (27) (data not shown). GIRK4, which is not glycosylated (15), does not bind wheat germ agglutinin except by its association with GIRK1 (27).

**All the GIRK1 in Atria Exists as GIRK1-GIRK4 Heterotetramers**—We carried out immunodepletion experiments using anti-GIRK4 antibodies to assess whether all of GIRK1 was associated with the 45-kDa GIRK4 protein. Immunodepletion was carried out with increasing concentrations of anti-GIRK4 antibodies. The immunodepleted supernatants were analyzed by 10% SDS-PAGE and immunoblotted simultaneously with anti-GIRK1 and anti-GIRK4 antibodies (Fig. 3). More than 90% of GIRK1 communoprecipitated with GIRK4. Taken together, the anti-GIRK1 and anti-GIRK4 immunodepletion experiments indicate that GIRK1 and the 45-kDa GIRK4 are associated with each other in a heterotetrameric GIRK1-GIRK4 complex. Consequently, the ~200-kDa GIRK4 is part of a novel complex that does not contain GIRK1.

**Native Atrial SDS-resistant GIRK4-containing Complex Is Completely and Specifically Immunoprecipitated with Anti-GIRK4 Antibodies**—Three aliquots of solubilized atrial sarcolemmal membranes were analyzed by 2–15% SDS-PAGE and immunoblotting. The first aliquot was left untreated and served as a loading control (Fig. 4, control). The second aliquot was immunodepleted with anti-GIRK1 antibodies alone (Fig. 4, aGIRK1 super). The third aliquot was immunodepleted with anti-GIRK1 antibodies followed by anti-GIRK4 antibodies (Fig. 4, aGIRK4 super). Finally, the remaining anti-GIRK4 antibody-antigen complex from the third aliquot was eluted with anti-GIRK4 antitope (Fig. 4, aGIRK4 eluate). As demonstrated by ProA/serum-only controls, the band labeled antibody (Ab) was derived from the crude anti-GIRK1 serum (data not shown).

**SDS-resistant GIRK4 Complexes Form in COS-7 Cells Transfected with GIRK4, as Well as in Native Heart Atria**—We tested whether a complex similar to the ~200-kDa complex in native heart cells could be isolated from the COS-7 cell expression system. COS-7 cells were transfected with either GIRK1 or GIRK4 and their membranes were isolated and solubilized in buffer containing 1% CHAPS. The solubilized membrane protein was immunoprecipitated with its corresponding antibody (anti-GIRK1 antibodies for the GIRK1-transfected cells or anti-GIRK4 antibodies for the GIRK4-transfected cells) and eluted with antigenic peptide. The eluates containing purified GIRK1 (Fig. 5, lane 1) or GIRK4 (Fig. 5, lane 2) were analyzed by 3–10% SDS-PAGE and immunoblotted with anti-GIRK1 antibodies (Fig. 5, lanes 1 and 2). The same blot was then stripped and reprobed with anti-GIRK4 antibodies (Fig. 5, lanes 3 and 4). A complex similar to the native atrial ~200-kDa GIRK4-containing complex formed in COS-7 cells transfected with GIRK4, but not in cells transfected with GIRK1. The recombinant GIRK4 complex was less stable than the native complex and did not require trichloroacetic acid precipitation for partial disruption. The ratio of the ~200-kDa GIRK4-containing complex to the ~45-kDa GIRK4 monomer varied between trials. Cotransfection with GIRK1 and GIRK4 reduced the proportion of ~200-kDa GIRK4 (data not shown). Taken together, this data indicates that the native ~200-kDa GIRK4-containing complex may be composed solely of GIRK4.
solely with GIRK4, and had a molecular weight appropriate for a GIRK4 homotetramer. To test this hypothesis, we chemically cross-linked recombinant GIRK4 into tetramers. A similar procedure was used to demonstrate that expression of GIRK1 alone, GIRK4 alone, or GIRK4-GIRK1 together in COS-7 cells resulted in tetramer formation (27). By SDS-PAGE analysis, we compared the migration of the chemically cross-linked GIRK4 homotetramers to the migration of the native ~200-kDa GIRK4-containing complex. COS-7 cells were transfected with GIRK4, and their membranes were isolated. An aliquot of COS-7 cell membranes was chemically cross-linked with DTSSP and analyzed by SDS-PAGE alongside native atrial membrane proteins. The gel was immunoblotted and probed with anti-GIRK4 antibodies (Fig. 6). The ~200-kDa GIRK4-containing complex from native atrial cells and the chemically cross-linked GIRK4 homotetramers migrated identically. The simplest interpretation of this data is that the native ~200-kDa GIRK4 complex is a GIRK4 homotetramer.

To ensure that the ~200-kDa GIRK4-containing complex was a homotetramer and not an SDS-PAGE artifact, we analyzed freshly solubilized atrial membranes by size exclusion chromatography (Fig. 7). The putative GIRK4 homotetramers eluted slightly after the GIRK1-GIRK4 heterotetramers. The computed molecular weight difference between the two complexes was 35-kDa. A GIRK4 homotetramer is ~30-kDa smaller than a GIRK1-GIRK4 heterotetramer. Thus this result is consistent with our hypothesis. However, caution must be taken when interpreting this data, because detergent binding can alter the perceived molecular weight of the GIRK complexes. Unlike the GIRK4 homotetramers, a small portion of the GIRK1-GIRK4 heterotetramers eluted just after the void volume of the size exclusion chromatography column. This portion of GIRK1-GIRK4 heterotetramers may be associated with a novel protein or proteins.

**DISCUSSION**

Here we describe purification of an SDS-resistant, GIRK4-containing tetrameric protein complex from heart membranes. The complex could not be dissociated with high reducing agent concentrations, urea, mild heating, or high salt concentrations. Trichloroacetic acid precipitation, however, consistently disrupted the complex. High thermal and detergent stability is not uncommon for membrane proteins (36–39). A number of ion channels with structures similar to GIRK4 are heat and SDS-resistant (37, 38). This heat and detergent resistance is because of the extreme stability of α-helices in nonpolar environments (36, 39, 40). Based on structure prediction algorithms, a search of the GIRK4 primary sequence revealed no tendency to form coiled-coil interactions (41). Interestingly, the KcsA K⁺ channel of *Streptomyces lividans* has similar detergent stability, which was reported to aid in its crystallization (37, 42).

Further study of the SDS-resistant complex confirmed that it contained GIRK4. The complex was recognized by two different antibodies generated against separate regions of GIRK4, and the GIRK4-containing complex was immunoprecipitated with anti-GIRK4 antibodies. In contrast, neither anti-GIRK4 anti-
bodies that were blocked with antigenic peptide, nor anti-GIRK1 antibodies recognized the SDS-resistant complex. These results indicate that this complex does not contain GIRK1 and that the anti-GIRK4 antibody binding is specific. Also, the trichloroacetic acid-disruptible, GIRK4-containing complex is absent in GIRK4 knockout mice, again indicating that the SDS-resistant complex contains GIRK4 (6).

The GIRK4 contained in this SDS-resistant complex was not associated with GIRK1. The GIRK4-containing complex did not coimmunoprecipitate with anti-GIRK1 antibodies and did not bind a wheat germ agglutinin affinity column. Also, we demonstrated that all of the GIRK1 and the ~45-kDa GIRK4 were associated with each other. This indicates that the SDS-resistant GIRK4-containing complex is novel, whereas the GIRK1 and the low molecular weight GIRK4 together form $I_{KCaC}$. $I_{KCaC}$ is a GIRK1-GIRK4 heterotetramer, most likely composed of two GIRK1 subunits and two GIRK4 subunits (27, 35, 43).

An SDS-resistant GIRK4-containing complex, similar to that found in native atrial cells, could be isolated from COS-7 cells transfected with GIRK4. We have shown that recombinant chemically cross-linked GIRK4 homotetramers and the native GIRK4-containing complex migrated identically on SDS-PAGE gels. Also, the GIRK4-containing complex eluted as a GIRK4 homotetramer from a size exclusion chromatography column. The simplest interpretation of our data is that the native ~200-kDa GIRK4-containing complex is a GIRK4 homotetramer.

A GIRK subunit, other than GIRK4, could make up a portion of this putative GIRK4 tetramer. However, this would require the unknown GIRK subunit to be the same molecular weight as GIRK4. Northern blots consistently indicate that GIRK1 and GIRK4 are the only known GIRK subunits expressed in atria (6, 44). Alternatively, the putative GIRK4 homomultimer could contain a combination of GIRK4 splice variants, although such GIRK4 splice variants have not been described.

It seems unlikely that the putative GIRK4 homotetramers are an artifact of the protein isolation procedure. GIRK4 homotetramers were observed whether atrial membranes were analyzed directly by SDS-PAGE or first solubilized in CHAPS-containing buffer. In addition, GIRK4 subunits from native GIRK1-GIRK4 heterotetramers did not dissociate to form GIRK4 homotetramers, because this would require an equivalent number of GIRK1 subunits to exist as dissociated monomers or GIRK1 homotetramers. Immunodepletion experiments indicated that ~90% of GIRK1 is associated with GIRK4. Also, we never observed GIRK4 homotetramers in our purified GIRK1-GIRK4 heterotetramer preparations, even after long incubation times (27).

Overall, the data presented here indicates that approximately half of GIRK4 in atria exists as homomultimers, whereas the other half is a component of the GIRK1-GIRK4 heterotetramer, $I_{KCaC}$ (Fig. 8). Also, our size exclusion chromatography experiments indicate that the GIRK1-GIRK4 heterotetramer, unlike the GIRK4 homomultimer, may be associated with an additional protein(s). Because this additional protein(s) is only associated with the GIRK1-GIRK4 heterotetramer, this association is most likely conferred by the GIRK1 subunit of the GIRK1-GIRK4 heterotetramer.

Our findings raise a number of questions. How do GIRK4 homomultimers coexist with GIRK4-GIRK1 heterotetramers in the same tissue? It will be interesting to determine if these two populations of channels occur in the same atrial myocytes or if they occur in separate regions of the atria. The GIRK4 homomultimers may even be derived from the nervous tissue innervating heart (45). If GIRK1-GIRK4 heterotetramers and GIRK4 homomultimers occur in the same cells, do they localize to the same subcellular compartments? GIRK homomultimers and heterotetramers could be activated by different G-protein-coupled receptors. Most importantly, what is the physiologic function of GIRK4 homomultimers? Do they function as channels or have they been adapted for some alternative use? Generally, the heterologous expression of GIRK4 homomultimers results in currents that do not resemble previously described myocardial currents. Large whole cell currents with long single channel open times were reported for recombinant GIRK4 homomultimers (14), but the long single channel open times differ...
from those described previously by others (15, 19, 20, 33).

In heterologous expression systems, a factor or an accessory subunit normally present in native tissue may be absent. GIRK channels are modulated by phosphatidylinositol 4,5-diphosphate (10, 11), internal Na⁺ (12, 13), mechanical stretch (14), and potentially by phosphorylation. Conceivably, the GIRK4 homomultimeric channel may require cell-to-cell contact or an extracellular matrix component for activation, a condition seldom tested when recording from patches. GIRKs, unlike other inwardly rectifying K⁺ channels, contain a sequence in their extracellular domains that is associated with integrin binding (RGD) (1). This sequence is usually found in extracellular matrix proteins. Alternatively, GIRK4 homomultimers may be inhibited by traditional patch clamp configurations (14). In any case, these findings should renew interest in the expression and existence of GIRK tetramers that do not contain GIRK1. Purkinje cells and basket cells of cerebellum, the substantia nigra, the inferior olivary nuclei, and solitary nucleus of the brainstem contain GIRK subunits but may not contain GIRK1 (21–26, 46, 47). Also, because of the anomalous migration of GIRK4 homomultimers on Western blots, the GIRK4 content of many tissues may have been underestimated. Thus, GIRK tetramers without GIRK1 may be physiologically relevant, but proof of their function will require further experimentation.

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