Tetrameric Subunit Structure of the Native Brain Inwardly Rectifying Potassium Channel K\textsubscript{ir} 2.2*

Kimberly F. Raab-Graham and Carol A. Vandenberg‡

From the Department of Molecular, Cellular, and Developmental Biology, and Neuroscience Research Institute, University of California, Santa Barbara, California 93106

Strongly inwardly rectifying potassium channels of the K\textsubscript{ir} 2 subfamily (IRK1, IRK2, and IRK3) are involved in maintenance and modulation of cell excitability in brain and heart. Electrophysiological studies of channels expressed in heterologous systems have suggested that the pore-conducting pathway contains four subunits. However, inferences from electrophysiological studies have not been tested on native channels and do not address the possibility of nonconducting auxiliary subunits. Here, we investigate the subunit stoichiometry of endogenous inwardly rectifying potassium channel K\textsubscript{ir} 2.2 (IRK2) from rat brain. Using chemical cross-linking, immunoprecipitation, and velocity sedimentation, we report physical evidence demonstrating the tetrameric organization of the native channel. K\textsubscript{ir} 2.2 was sequentially cross-linked to produce bands on SDS-polyacrylamide gel electrophoresis corresponding in size to monomer, dimer, trimer, and three forms of tetramer. Fully cross-linked channel was present as a single band corresponding to K\textsubscript{ir} 2.2, suggesting that the channel is composed of a single type of subunit. Hydrodynamic properties of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonic acid-solubilized channel were used to calculate the molecular mass of the channel. Velocity sedimentation in H\textsubscript{2}O or D\textsubscript{2}O gave a sharp peak with a sedimentation coefficient of 17.3 S. Gel filtration yielded a Stokes radius of 5.92 nm. These data indicate a multisubunit protein with a molecular mass of 193 kDa, calculated to contain 3.98 subunits. Together, these results demonstrate that K\textsubscript{ir} 2.2 channels are formed by the homotetrameric association of K\textsubscript{ir} 2.2 subunits and do not contain tightly associated auxiliary subunits. These studies suggest that K\textsubscript{ir} 2.2 channels differ in structure from related heterotetrameric ATP-sensitive K channels and heterotetrameric G-protein-regulated inward rectifier K channels.

Inwardly rectifying potassium channels (IR channels)\textsuperscript{1} are a specialized class of potassium channels that are characterized by their ability to conduct large inward currents at potentials negative to the potassium equilibrium potential and small outward currents at more positive potentials (1–5). The degree of rectification and the second messengers that regulate the IR channel have served as the criteria to divide this family into several classes, notably the classical strong inward rectifiers, the G protein-regulated inward rectifiers, and the ATP-sensitive inward rectifiers (1–5).

In this study, we are interested in the classical strong inward rectifiers, first described in skeletal muscle as “anomalous” rectifiers, and typified by the I\textsubscript{K1} current of cardiac myocytes. These channels also are abundant in brain, macrophages, osteoelasts, and placenta, as well as other tissues (1–3).

These channels are physiologically important for the maintenance and modulation of cell excitability, repolarization of action potentials, and determination of cell resting potential. The shape of an action potential is created by the coordinated conduction of different ions through their respective channels. The contribution of inward rectifier channels to action potential waveform is well illustrated in cardiac cells, where the strong rectification properties of I\textsubscript{K1} promote the long duration of the ventricular action potential by conducting only small outward currents during the plateau and contribute to rapid termination of the action potential with large currents during the repolarization phase (1, 6). In addition, these channels are essential for establishment of the resting potential. In neuronal cells, classical inward rectifiers are thought to participate in electrical signaling and information processing by setting cell resting potential and modulating cell excitability (7–10).

To date, three cDNAs (K\textsubscript{ir} 2.1, K\textsubscript{ir} 2.2, and K\textsubscript{ir} 2.3) encoding members of the classical inward rectifier family have been isolated (11–15). All are found in heart and brain, as well as showing subtype-specific distributions in other tissues. It has been suggested that another strong, and predominantly glial, inward rectifier is encoded by the K\textsubscript{ir} 4.1 subtype (16). Heterologous expression of eRNA, transcribed from K\textsubscript{ir} 2.1, K\textsubscript{ir} 2.2, or K\textsubscript{ir} 2.3 cDNA, in Xenopus oocytes encode proteins that display the steep rectification properties of classic inward rectifiers in native cells (11–15). Although the physiology of these channels is beginning to be understood, the native molecular structure and composition of the classical inward rectifier channels have yet to be determined.

The stoichiometries of pore-conducting pathways of several other ion channels and neurotransmitter receptors have been characterized. Studies have demonstrated that the channel “core” is formed by the co-assembly of four homologous subunits for voltage-gated K+ channels, a single polypeptide composed of four internal repeats for Na+ and Ca++ channels, two homologous subunits for Cl− chloride channels, or five homologous subunits for ACh, glycine, and GABA\textsubscript{A} neurotransmitter receptor channels (4, 17–19). In addition to the core pore-
forming subunits, studies of endogenous channels from native tissues have demonstrated the presence of auxiliary subunits for all classes of voltage-dependent cation channels (20).

The subunit stoichiometry of classical inward rectifier potassium channels has been approached recently with physiological experiments using heterologously expressed cRNAs encoding a combination of tandem wild type and mutant K$_{\text{ir}}$ 2.1 subunits in *Xenopus* oocytes (21). This and a similar study suggest that the pore-conducting pathway is formed by the contribution of four subunits for K$_{\text{ir}}$ 2.1 (21) and K$_{\text{ir}}$ 4.1 (22). Although elegant in their design and able to describe the minimal composition of a heterologously expressed channel, electrophysiological studies of expressed channel constructs are not able to detect the presence of auxiliary nonconducting subunits and do not address the question of the composition and size of endogenous channels from native tissues.

Subunit stoichiometry and quaternary structure is particularly interesting among inward rectifier channels, since recent studies indicate that the channel subfamilies may differ from one another. Expression studies of ATP-sensitive inward rectifier channel subunits have demonstrated the necessity for auxiliary subunits to form ion-conducting channels. Consequently, these channels are thought to contain a total of eight subunits consisting of four K$_{\text{ir}}$ 6.2 subunits and four sulfonylurea receptor subunits (23–25). The molecular composition of the G protein-coupled inward rectifiers (K$_{\text{ir}}$ 3.x/GIRK) is also beginning to be dissected. Members of the K$_{\text{ir}}$ 3.0 subfamily have been shown to function primarily as heteromultimeric tetramers (26–30). Furthermore, it has been suggested that positional effects of individual subunits in heteromultimeric channels dictate the physiological properties of the pore (28, 29, 31).

In this study, we examine the stoichiometry of K$_{\text{ir}}$ 2.2 (IRK2) channels from rat cortex plasma membranes. This is the first study to report the native stoichiometry of a member of the classical strong inward rectifier family. We chose a biochemical approach in order to utilize physical methods for detecting the presence or the absence of nonconducting, auxiliary subunits that otherwise could not be detected by physiological means. We report here, by cross-linking, immunoprecipitation, and velocity sedimentation, that rat cortex K$_{\text{ir}}$ 2.2 channels are homotetrameric.

**MATERIALS AND METHODS**

**Preparation and Characterization of K$_{\text{ir}}$ 2.2 Antibodies—** Rabbit polyclonal antibodies were made against a synthetic peptide (DEVAT-GIRK) and used in immunoblot analysis. Antibodies were tested for specificity by immunoblot analysis of in vitro translated K$_{\text{ir}}$ 2.2, K$_{\text{ir}}$ 2.3, and K$_{\text{ir}}$ 3.2 protein (11, 12, 15). To increase their translation efficiency, K$_{\text{ir}}$ 2.2 cDNAs used for in vitro transcription/translation were tagged at their N termini with five amino acids (MATTH in pCITE-4a (Novagen), K$_{\text{ir}}$ 2.1) or S-Tag (36 amino acids in pCITE-4a (Novagen), K$_{\text{ir}}$ 2.2), or T7-Tag (11 amino acids in pET-23a (Novagen), K$_{\text{ir}}$ 2.3). The antibody was further characterized by immunoblotting rat cortex plasma membranes to assess specificity in tissue and abundance of the channel.

In Vivo Transcription and Translation of IR Channel cDNAs—cDNAs encoding K$_{\text{ir}}$ 2.1, K$_{\text{ir}}$ 2.2, and K$_{\text{ir}}$ 2.3 were transcribed and translated in the presence of [$^{35}$S]methionine and canine pancreas microsomal membranes (Promega). Ten µl of the reaction was separated on a 10% SDS-PAGE followed by either autoradiography to assess relative amounts of protein or immunoblotted with anti-K$_{\text{ir}}$ 2.2 channel antibodies.

**Isolation of Rat Cortex Plasma Membranes—** Membranes were isolated by a modification of the method described by Sharp et al. (32). In brief, 5 g of rat cerebral cortices were homogenized in 50 ml of buffer A (320 mM sucrose, 20 mM Hepes, pH 7.4, 1 mM EDTA, 1× Complete™ protease inhibitor mixture (Boehringer Mannheim)). Homogenate was centrifuged at 16,000 × g for 20 min. The resulting pellet was resuspended and layered on a two-step sucrose cushion consisting of equal volumes of 0.8 M sucrose and 1.2 M sucrose. SDS-polyacrylamide gel, as specified in the figure legends. Proteins were transferred to nitrocellulose (Hybond, Amersham Pharmacia Biotech), and blots were blocked in Trans-buff ered saline (TBS) containing 5% nonfat milk (TBS/MBF) for 1 h at room temperature. Incubation with primary antibody was carried out in TBS containing either K$_{\text{ir}}$ 2.2 antiseraum (1:200) or affinity-purified anti-K$_{\text{ir}}$ 2.2 (1:100) for 1 h. Blots were subsequently washed twice in TBS and once in TBS for a total of 30 min. Secondary antibody incubation was performed using horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000) for 40 min in TBS. Blots were washed for at least 45 min, first in TBS, followed by TBS. Signal was detected by incubating the blot in substrate for chemiluminescent detection (Super Signal; Pierce) for 1 min and exposing to preflashed film.

**Cross-linking of K$_{\text{ir}}$ 2.2 Channel Protein—** Cross-linking was performed essentially as described by Lai et al. (33). Rat cortex plasma membranes (1.5 mg) were solubilized in buffer B (150 mM NaCl, 5 mM EDTA, 20 mM Hepes, pH 7.0, 1% CHAPS, and 1× Complete™ protease inhibitor mixture (Boehringer Mannheim)) and centrifuged at 100,000 × g for 30 min at 4 °C to remove insoluble material. Solubilized membranes were loaded on a 5–30% (w/v) sucrose gradient constructed in buffer B and centrifuged for 20 h at 36,000 rpm in a Beckman SW41 rotor at 4 °C. Fractions (250 µl each) were collected from the top of the gradient and assayed for K$_{\text{ir}}$ 2.2 protein by immunoblot analysis. Peak immunoreactive fractions were pooled, the volume was adjusted to 800 µl with phosphate-buffered saline, and then 100 µl of the covalent cross-linker glutaraldehyde (12 mM final concentration, EM Sciences) was added to initiate the reaction. Intermediate cross-linked products were observed by removing aliquots from the reaction at various times and quenching by the addition of 1 M Tris, pH 8.0 (166 mM final concentration). Cross-linked products were added to reducing SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

**Biotinylation of Rat Cortex Plasma Membranes—** Membranes (1 mg) were solubilized in 1 ml of a biotinylation buffer (50 mM NaHCO$_3$, pH 8.5, 0.26 mM NHS-LC-biotin (Pierce)), and incubated at room temperature, rotating for 15 min. The reaction was quenched by adding an equal volume of 0.5 M glycine and incubated at room temperature for 15 min followed by an additional incubation on ice for 30 min. The membranes were pelleted at 100,000 × g for 15 min at 4 °C. Membranes were resuspended in buffer B without detergent at a final concentration of 1 mg/ml and either used directly for immunoprecipitation or stored at 70 °C.

**Immunoprecipitation—** Immunoprecipitation was performed by a modification of the method described by Burgess et al. (34). In brief, 300 µg of biotinylated membranes/reaction was solubilized in buffer C (1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM Hepes, pH 7.4, and 1× Complete™ protease inhibitor mixture (Boehringer Mannheim)) and incubated at room temperature with preimmune serum and protein A-agarose (Pierce) for 5 h. K$_{\text{ir}}$ 2.2 antibody was prebound to protein A-agarose in the presence or the absence of antigenic peptide in buffer C for 5 h at room temperature. Preabsorbed antibody-protein A complex was added to preimmune treated samples and incubated overnight at 4 °C. Immune complex was washed once in buffer C, layered on a 35% sucrose cushion, and pelleted in the microcentrifuge. Two additional washes with buffer C followed by one wash with H$_2$O were performed before elution of immune complex from protein A-agarose by boiling in the presence of 1% SDS, 10 mM Tris, pH 8.0, for 5 min. The supernatant was removed from the protein A-agarose beads and diluted 10-fold with buffer C. 20 µl of streptavidin-agarose beads (Sigma) was added to the sample and allowed to incubate at room temperature for 30 min. Biotinylated protein-streptavidin-agarose complex was washed four times with buffer C. Protein was eluted with SDS sample buffer, electrophoresed through an 8.75% polyacrylamide gel, and transferred to nitrocellulose. The blot was incubated with streptavidin conjugated to horseradish peroxidase (1:100) (Amersham Pharmacia Biotech) to detect biotinylated proteins.

**H$_2$O/D$_2$O Sucrose Gradient Analysis of K$_{\text{ir}}$ 2.2 Channel Protein—**
Membranes were prepared and solubilized as described above. Solubilized membrane protein (1.5 mg) was loaded on a 11.5-mL linear 3–30% sucrose gradient constructed in either H2O or D2O and centrifuged in a Beckman SW41 rotor at 36,000 rpm, at 4°C, for 20 or 30 h, respectively. Standard proteins were run in parallel gradients under identical conditions. The 3–30% sucrose gradients were prepared at room temperature and allowed to equilibrate at 4°C for 2 h.

Fractions (250 μL) were collected from the top of the gradient. Each fraction was separated by SDS-PAGE and Western blotting with affinity-purified Kir 2.2 antibodies. The position of the Kir 2.2 peak used to calculate the sedimentation properties of the complex was determined by the intensity of the immunoblotting signal, and interactions between fractions was based on the peak shape.

**Ge Filtration of Kir 2.2 Protein—CHAPS-solubilized membrane protein (2 mg) was applied to a 0.5 × 37.5-cm Sephacryl 400 HR column equilibrated with buffer B. Proteins were eluted at a rate of 0.15 mL/min for a total of 35 mL. Dextran Blue 2000 (Amersham Pharmacia Biotech) and phenol red (Sigma) were included in each sample to determine the void volume (V0) and the total volume (VT) of the column matrix. Standard proteins with well-characterized Stokes radii were applied to the same column and eluted under identical conditions. The elution profile of the calibration standards was determined by measurement of absorbance at 280 nm. One hundred eighty 250-μL fractions or fifty-four 500-μL fractions were collected between V0 and VT to determine the elution profile of Kir 2.2 protein. Fractions were analyzed by SDS-PAGE and Western blotting as described above. Values for the partition coefficient, Kre, used in the determination of the Stokes radius, Ros, of Kir 2.2 were calculated and plotted as described by Ackers (35).

Calibration curves for both the sedimentation coefficient (sucesse gradients) and Stokes radii (gel filtration) were determined using the following standard proteins: bovine serum albumin (4.4 S), ovalbumin (3.6 S, 3.05 nm), aldolase (7.7 S, 4.81 nm), catalase (11.3 S), apoferritin (17.7 S, 5.1 nm), and thyroglobulin (8.5 nm) (36–39).

**Calculation of the Molecular Mass of Kir 2.2 Channels**—The molecular mass of the protein-detergent complex was calculated by the method of Clarke (36, 37). In brief, this involves determining the apparent sedimentation coefficient by carefully measuring the average sedimentation position and the corresponding density within the gradient for each protein. From these values, and from the known sedimentation coefficients of standard proteins, the viscosity of the gradient at the average sedimentation positions could be calculated. The partial specific volume of the complex was then determined from the sedimentation coefficients, densities, and viscosities at the average sedimentation position for gradients performed in H2O and D2O. Finally, the detergent contribution to the complex could be determined from the partial specific volume, and these values combined with the Stokes radius were used to calculate the molecular mass of the protein.

The sedimentation coefficient under experimental conditions, sucesse exp, was calculated for each protein using Equation 1.

\[
s_\text{exp} = (v - r_0)j\omega^2
\]

The point of origin, r0, is the distance from the center of rotation to the midpoint of the applied sample. The radius, r, specifies the distance traveled from the axis of rotation to the final migration position of the protein. Time, t, and omega, ω, denote the duration of centrifugation and the angular velocity, respectively. Values of r0 and r were determined from the dimensions of the rotor and tube and from the peak fraction number and volumes of collected fractions. The average position of each protein, ravg, during the time of centrifugation, is equal to \( (r_0 + r)/2 \).

The density, ρ, at ravg of each standard protein was determined by interpolation of the measured densities of the 3 and 30% sucrose/H2O and sucrose/D2O solutions and using the linear relationship between radius and density. The linearity of the gradient was confirmed by measuring the index of refraction for typical gradients.

The viscosity, η, at the average position for each standard protein, sucesse exp, was calculated using Equation 2, where sρavg and v are the known sedimentation coefficients under standard conditions and partial specific volumes of each standard protein, respectively. The densities and viscosities for water, ρwater, and ηwater, under standard conditions of 20°C are 0.99823 g/cm3 and 0.01002 g cm−1 s−1, respectively (40).

\[
s_\text{exp} = (s_\rho \rho_\text{avg}) v_\text{avg} \left[ \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{avg}} \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{water}} \right) \right]
\]

The viscosity at the ravg for Kir 2.2 was determined from interpolation of the plot of viscosity as a function of ravg for the standard proteins.

The partial specific volume of the Kir 2.2 complex, v, was calculated using Equation 3 after measurement of sedimentation parameters for the protein in H2O and D2O sucrose gradients. The sedimentation coefficients, densities, and viscosities measured under experimental conditions for both H2O and D2O sucrose gradients are denoted by the corresponding subscript H or D.

\[
v = \left( s_\rho \rho_\text{avg} / \left( \rho_\text{avg} - \rho_\text{water} \right) \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{avg}} \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{water}} \right) \rho_\text{water}
\]

Equation 4 was used to determine the detergent contribution to the molecular mass of the complex, M, where P and D denote the protein and detergent fractional contributions to the complex, and P + D = 1. The bound detergent was calculated using vCHAPS of 0.81 ml/g and an average partial specific volume for protein of 0.735 ml/g (40, 41).

\[
v = (\rho_\text{CHAPS} / \left( \rho_\text{CHAPS} - \rho_\text{water} \right)) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{avg}} \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{water}} \right) \rho_\text{water}
\]

Based on these calculations, Equation 5 was used to determine the sedimentation coefficient (sρ20,5) of the Kir 2.2 complex. The values used for sρ20,5 and pρ20,5 are the experimentally determined values for H2O/sucrose gradients.

\[
s_\rho^{20,5} = \left( s_\rho \rho_\text{avg} / \left( \rho_\text{avg} - \rho_\text{water} \right) \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{avg}} \right) \left( 1 - \frac{v_\text{avg}}{v_\rho_\text{water}} \right) \rho_\text{water}
\]

The molecular mass of the protein-detergent complex (M) was then determined from the sedimentation coefficient (sρ20,5), Stokes radius (Rb), and partial specific volume (v) of the complex using Equation 6. Avogadro’s number is denoted by N. The molecular mass of Kir 2.2 channel protein (M) was determined by Equation 7.

\[
M = (6 \pi D s_\rho R_b / v_\rho_\text{avg}) / (1 - v_\rho_\text{avg})
\]

\[
M = (M - nD)
\]

**RESULTS**

**Specificity of Kir 2.2 Antibodies—**To study the stoichiometry of Kir 2.2 channels in detergent-solubilized rat cortex plasma membranes, channel-specific antibodies were made to a short nonhomologous peptide corresponding to the primary sequence of Kir 2.2. Antibodies were purified from antiserum by affinity chromatography using the antigenic peptide as the affinity matrix. In characterizing the Kir 2.2 antibodies, we wanted to ensure that 1) the antibodies recognize Kir 2.2 protein, 2) the antibodies would not cross-react with other inwardly rectifying potassium channels, and 3) the antibodies specifically recognize a single Kir 2.2 protein band from native tissue. Since members of the Kir 2 subfamily are approximately the same molecular weight and are the most similar to each other in sequence, Kir 2.2, Kir 2.1, and Kir 2.3 in vitro translated proteins were used to assess the specificity of Kir 2.2 channel antibodies. In vitro translated protein was separated through a SDS-polyacrylamide gel followed by autoradiography of [35S]methionine-labeled protein and immunoblotting. Fig. 1A (left) shows [35S]autoradiography indicating the relative translation efficiencies for each channel protein (containing 17, 12, and 20 methionines, respectively, in Kir 2.2, Kir 2.1, and Kir 2.3). The additional bands below the full-length channel protein are probably truncated products of the protein, as is commonly reported with in vitro translation (42). Western blot analysis of the same three proteins demonstrated that Kir 2.2 channel antibodies only recognized Kir 2.2 protein and did not cross-react with any other member of the Kir 2 subfamily (Fig. 1A, right). To assess channel specificity in native tissue, rat cortex plasma membranes were separated by SDS-PAGE and Western blotted (Fig. 1B). Kir 2.2 channel antibodies recognized a single band at an apparent molecular mass of approximately 60 kDa (Fig. 1B), consistent with a molecular mass of 55 kDa for 6% SDS-PAGE to 64 kDa for 10% SDS-PAGE. This is close to the predicted molecular mass of 48.4 kDa based on the primary sequence of rat Kir 2.2 (12). The rat cortex Kir 2.2 protein migrated at a comparable molecular weight as the in vitro translated Kir 2.2 protein. Recognition of this band could be specifically blocked by preincubation of the antibody with the antigenic peptide (Fig. 1B).

**Cross-linking of Kir 2.2 Channel Protein—**To determine
channel stoichiometry, we treated detergent-solubilized Kir 2.2 channels, partially purified from rat cortex plasma membranes, with the covalent cross-linker glutaraldehyde. Progressive exposure of a cross-linking reagent to a multisubunit protein will result in the gradual formation of dimers, trimers, etc., up to the maximal number of subunits that constitute the native protein. Cross-linked products from a native tissue can then be detected by Western blotting with a specific antibody.

Rat cortex plasma membranes were first solubilized in a CHAPS-containing medium and layered on a 3–30% sucrose gradient, and the solubilized Kir 2.2 was enriched by sedimentation as specified under “Materials and Methods.” Fractions were collected and assayed for Kir 2.2 channel protein by immunoblot analysis. The peak fractions, demonstrating the maximal intensity of Kir 2.2 channel protein, were pooled and treated with glutaraldehyde for various amounts of time followed by denaturing gel electrophoresis. A time course of cross-linking produced a series of bands representing monomer, dimer, trimer, and a single diffuse band of tetramer on 6% SDS-polyacrylamide gels (not shown). In order to optimize the resolution of cross-linked products, the samples were separated on a 2–12% gradient gel (Fig. 2). The single diffuse band representing tetramer on the linear gel was resolved into three distinct bands on the gradient gel. We speculate that these bands represent three forms of covalently cross-linked tetramer with different intermolecular subunit conformations. Products larger than tetramer were not observed even after extended exposure to cross-linker (data not shown). Possible intermembrane subunit conformations are diagrammed adjacent to the proposed number of cross-linked subunits.

Initial assignment of number of cross-linked subunits was determined by apparent molecular mass of each band. Apparent molecular mass from the 2–12% gradient gels of Fig. 2 were 56, 122, and 188 kDa for monomer, dimer, and trimer, respectively, and approximately 228, 251, and 276 kDa for the tetramer bands.

To test our proposed multimeric state of the cross-linked products, the logarithm of the estimated number of cross-linked subunits was plotted against the relative mobility of each band on a 6% SDS-polyacrylamide gel. A linear relationship in this analysis would indicate that the cross-linked products are integral multimers of the monomeric subunit. Plot analysis of the cross-linked subunits produced a linear relationship (r = 0.985), indicating a direct stoichiometric relationship (Fig. 3). Cross-linking results thus are consistent with the hypothesis that the Kir 2.2 channel is composed of a tetrameric assembly of subunits.

Kir 2.2 Does Not Contain an Auxiliary β-Subunit—The presence of higher molecular weight cross-linked bands also might be explained by a tightly associated auxiliary subunit(s). To address this possibility, we biotinylated membranes and immunoprecipitated them with Kir 2.2 antibodies. Utilizing the biotin-streptavidin technology, we were able to visualize any biotinylated protein(s) that immunoprecipitated with Kir 2.2. A single biotinylated band of ~67–69 kDa was detected when the immunoprecipitate was blotted with streptavidin. This band ran at somewhat higher apparent molecular mass than native Kir 2.2, presumably due to the addition of biotin to multiple lysine residues of Kir 2.2. To confirm that this band was in fact

**Fig. 1.** Characterization of Kir 2.2 channel-specific antibodies. A, channel-specific antibodies were made against a synthetic peptide corresponding to a 21-amino acid region (390–410) near the C terminus of rat Kir 2.2 protein and purified by affinity chromatography. Antibody specificity for Kir 2.2 was assessed by immunoblotting of Kir 2.2, Kir 2.1, and Kir 2.3 proteins that had been translated in vitro (A, right). Translation efficiency was evaluated in parallel by autoradiography of aliquots of the translated proteins that had been labeled with [35S]methionine (A, left). In vitro translated Kir 2.2 migrated at a molecular mass of 67 kDa, which includes 3.9 kDa of the N-terminal S-Tag™. B, immunoblot analysis of rat cortex Kir 2.2. Aliquots of 50 µg/lane of rat cortex plasma membranes were electrophoresed through a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with Kir 2.2 antibodies. Affinity-purified Kir 2.2 antibodies recognized a single band, migrating at a molecular mass of ~64 kDa. Preincubation of antibody with excess antigenic peptide prior to blotting with anti-Kir 2.2 specifically blocked the signal.

**Fig. 2.** Immunoblot of Kir 2.2 subunits cross-linked with glutaraldehyde. Upon the addition of glutaraldehyde, a covalent cross-linking reagent, to partially purified Kir 2.2 protein, six bands representing integral multiples of the monomeric subunit were sequentially formed. Protein bands were resolved by electrophoresis through a 2–12% SDS-polyacrylamide gradient gel and detected by immunoblotting with affinity-purified Kir 2.2 antibody. Increased time of exposure to glutaraldehyde resulted in the formation of a single high molecular weight band. Possible intermembrane subunit conformations are diagrammed adjacent to the proposed number of cross-linked subunits.
**Hydrodynamic Properties of Kir 2.2**—A complementary approach to determine channel stoichiometry is to calculate the molecular mass of the channel by measuring the hydrodynamic properties of the native protein by velocity sedimentation and gel filtration. The rate at which a molecule sediments through a gel filtration column is dependent both on molecular mass and shape. The molecular mass of a solubilized membrane protein is the sum of the mass of the protein and the mass of the detergent bound to the protein. Therefore, one must calculate the contribution of the detergent to the complex in order to determine the apparent molecular mass of the protein. This can be accomplished by determining the apparent sedimentation coefficients of the protein-detergent complex in H2O and D2O and using these values in the equations outlined under “Materials and Methods.” The shape of a protein also influences its sedimentation velocity, and thus the Stokes radius is required to calculate molecular mass from sedimentation data. The Stokes radius can be determined experimentally by comparing its elution profile through a gel filtration column with a set of standard proteins with known Stokes radii.

In order to retain the channel’s membrane-associated conformation, rat cortex plasma membranes were solubilized in a 1% CHAPS buffer. CHAPS was chosen over other nonionizing detergents based on its low micellar molecular weight, its low aggregation number, its UV-visible absorbance properties, and its ability to solubilize the channel without artificial aggregation. With a low micellar molecular weight, resolution in hydrodynamic studies is enhanced due to the small size of the protein-detergent micelle, resulting in a sharp peak for Kir 2.2 in all of the hydrodynamic experiments. Furthermore, in gel filtration experiments where ultraviolet detection is necessary, CHAPS is advantageous, because it does not contain aromatic groups that will interfere with protein detection in the UV range as is the case for Triton X-100 (43, 44). Although CHAPS has many advantages for this application, the partial specific volume of CHAPS (0.81 ml/g) is close to the partial specific volume for an average protein (0.735 ml/g) (40, 41). As a result, the shift in measured sedimentation coefficients between gradients constructed in H2O versus D2O is less marked for CHAPS-solubilized proteins than for another common detergent, Triton X-100, for equal detergent/protein ratios \((T_{\text{Triton X-100}} = 0.91 \text{ ml/g})\). To compensate for this disadvantage, we accentuated the difference in sedimentation coefficients between the two mediums by collecting many fractions of a small sample size.

CHAPS-solubilized rat cortex plasma membranes were layered on 3–30% sucrose gradients constructed in either H2O or D2O. Immunoblot analysis of fractions collected after the completion of sedimentation demonstrated that Kir 2.2 channel protein, in both H2O/sucrose and D2O/sucrose, sedimented in a sharp peak with sedimentation values under experimental conditions, \(s_{T,m}\), of 5.15 and 2.96 S, respectively (Fig. 5). Each gradient was calibrated by running, in parallel, a set of soluble sedimentation standards with well characterized sedimentation coefficients and partial specific volumes. The sedimentation coefficients \(s_{T,m}\), corresponding densities \(\rho\), and viscosities \(\eta\) at the average sedimentation positions \(r_{\text{avg}}\) were determined for each protein. The average sedimentation position \(r_{\text{avg}}\) for Kir 2.2 and each standard protein is plotted versus the apparent sedimentation coefficients, densities, and viscosities for a typical experiment in Fig. 6. The apparent sedimentation coefficient and the density of Kir 2.2 at \(r_{\text{avg}}\) were calculated independently using the parameters of the gradient. First, the distance traveled through the gradient by the protein...
under the experimental conditions was determined to calculate $s_{T,m}$ (Fig. 6A). Second, the density was plotted as a linear function of sucrose concentration. The density at the average sedimentation position of Kir 2.2 was determined by interpolation of the densities of the initial 3% and final 30% sucrose solutions (Fig. 6B). The viscosity of Kir 2.2 at $r_{avg}$ was interpolated from the plot of $r_{avg}$ versus the calculated viscosities for the standard proteins (Fig. 6C). The different sedimentation coefficients for Kir 2.2 in H$_2$O/sucrose versus D$_2$O/sucrose indicate that Kir 2.2 has a different partial specific volume than the soluble protein standards, reflecting the detergent bound to the Kir 2.2 protein. Analysis of the sedimentation data, using the equations outlined under “Materials and Methods,” yielded a calculated partial specific volume of the protein-detergent complex of 0.783 ± 0.001 ml/g. From this information, it was determined that 64 ± 2% of the molecular mass of the complex was due to detergent bound to the protein (Table I).

The Stokes radius of Kir 2.2 native protein was determined by gel filtration. Calibration standards and CHAPS-solubilized rat cortex plasma membranes were applied to a Sephacryl 400HR column equilibrated with a 1% CHAPS buffer. Chromatographic separation of the standards and rat cortex plasma membranes were carried out under the conditions outlined under “Materials and Methods.” The partition coefficient ($K_D$)
for each calibration protein was determined by the equation \( V_e - V_o = (V_t - V_o) \). The inverse error function \( (erf^{-1}) \) for the measured \( (1 - K_D) \) for each protein was plotted against the established Stokes radius to yield a calibration curve for the column as seen in Fig. 7B (35). Fractions were collected between the void volume \( (V_o) \) and total volume \( (V_t) \) and assayed for \( K_r \). Channel protein by Western blot analysis. The elution profile of \( K_r \) channel protein contained a single sharp peak with a Stokes radius of 5.92 nm (Fig. 7).

Analysis of the sedimentation and gel filtration data together (Table I) gave a calculated molecular mass of the Kir 2.2 protein-detergent complex of 533,000 ± 7,000 Da. The predicted molecular mass of the protein, after the molecular mass of the detergent was subtracted, was 193,000 ± 8,000 Da. The predicted molecular mass based on the primary sequence of rat Kir 2.2 (12) was used to estimate the number of subunits (45). The calculated number of \( K_r \) channel subunits with a predicted monomeric molecular mass of 48,399 Da is 3.98 ± 0.17. Therefore, both our cross-linking studies and hydrodynamic studies show a tetrameric organization of the \( K_r \) channel.

**DISCUSSION**

The goal of this study was to gain structural information on the overall subunit stoichiometry and composition of classical strong inward rectifier \( K^{+} \) channels from native membranes. Our approach was 2-fold; we first cross-linked endogenous brain \( K_r \) subunits to determine the number of subunits of the native oligomer. We then calculated the molecular mass of the native protein by subjecting the same population of channels to velocity sedimentation and gel filtration. Using complementary approaches, we provide direct biochemical evidence that \( K_r \) channels are homotetrameric complexes of \( K_r \) subunits and do not contain auxiliary \( \beta \)-subunits.

**Cross-linking of Kir 2.2 Channel Proteins—Immunodetection of \( K_r \)**

Cross-linked products from partially purified rat cortex plasma membranes revealed the sequential formation of protein bands representing integral molecular weights of the monomer up to a final stoichiometry of tetramer (Fig. 2). Upon further separation of cross-linked products, three bands in the size range of tetrameric product were resolved. We hypothesize that these may possess varying numbers of intermolecular cross-links, possibly representing linear tetramer, circular trimer, or circular tetramer. Similar results have been observed with other channels. For example, Schulteis et al. (46) reported that cross-linking of Shaker \( K^{+} \) channels produced linear tetramer, circular tetramer, and, with a more reactive cross-linker, a third product that migrated between the two. These observations are consistent with the view that multisubunit proteins with multiple reaction sites will produce different cross-linked conformations, and these would be expected to migrate with different apparent molecular weights (47).

Given that circular tetramer will be the most extensively cross-linked product, we tentatively assigned the highest apparent molecular weight tetrameric band as circular tetramer. This assignment was based on our data indicating it to be the final product obtained after extensive cross-linking. This observation is also in agreement with the relative mobilities of cross-linked Shaker channels (46).

**Kir 2.2 Does Not Contain an Auxiliary Subunit—In interpreting the cross-linking data, another possibility that needed to be considered was that the band of highest apparent molecular weight between the uppermost two bands in cross-linking experiments would indicate that any additional subunit would be ~25 kDa. However, cross-linking experiments did not seem to support this interpretation, because there was no evidence for cross-linking of a small subunit with monomer, dimer, or trimer, suggesting that it was a unique tetramer conformation (Fig. 2).**

Further studies with biotinylated channel proteins gave

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**Table I**

<table>
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<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Partial specific volume of protein-detergent complex</td>
<td>0.783 ± 0.001 mlg complexity, b</td>
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strong support to the suggestion that the K<sub>ir</sub> 2.2 channel does not contain auxiliary subunits. When cortex membranes were biotinylated and then immunoprecipitated with anti-K<sub>ir</sub> 2.2 antibody, only a single band was subsequently detected with streptavidin following SDS-PAGE. This band was identified as K<sub>ir</sub> 2.2 by immunoblotting (Fig. 4). Since the biotinylation was via an amine-reactive reagent, most proteins would be expected to incorporate biotin due to the characteristic high incidence of exposed lysines in proteins (48). Therefore, the absence of additional bands provides convincing evidence that the channel is composed of a single subunit species.

From these data, we conclude that the native K<sub>ir</sub> 2.2 channel is a tetramer, composed of subunits of K<sub>ir</sub> 2.2 and possibly including other pore-forming subunits of identical molecular weight. Based on the molecular weights of known inward rectifier subunits, these data suggest that brain K<sub>ir</sub> 2.2 does not co-assemble with K<sub>ir</sub> 1.1/ROMK1, K<sub>ir</sub> 3.2/GIRK2 or K<sub>ir</sub> 3.4/GIRK4, since these are reported to coassemble preferentially with members of the K<sub>ir</sub> 3 subfamily of G-protein-regulated subunits, and K<sub>ir</sub> 3.4 is not abundant in the brain (49–51). These data do not exclude the possibility that the K<sub>ir</sub> 2.2 channels exist as heterotetramers with K<sub>ir</sub> 2.1, since the molecular mass of K<sub>ir</sub> 2.1 is similar to that of K<sub>ir</sub> 2.2 (both 48 kDa). Thus, our data suggest that K<sub>ir</sub> 2.2 channels may be homotetramers, and restrict any heterotetrameric assembly to within the K<sub>ir</sub> 2 subfamily to include only K<sub>ir</sub> 2.1. However, heterologous expression studies have shown that K<sub>ir</sub> 2.1 and K<sub>ir</sub> 2.2 are incompatible for coassembly (52), thus supporting the hypothesis that the K<sub>ir</sub> 2.2 channels are homomultimeric.

**Molecular Mass Determination of K<sub>ir</sub> 2.2**—As a complimentary approach to the physical characterization of the native channel, we measured the hydrodynamic properties of brain K<sub>ir</sub> 2.2. The physical parameters of CHAPS-solubilized K<sub>ir</sub> 2.2 channel proteins are summarized in Table I.

Estimation of molecular mass for a membrane protein is inherently more complicated than for a soluble protein. One can easily approximate the molecular mass of soluble protein by comparing it with the hydrodynamic properties and molecular mass of well characterized sedimentation standards (53). However, membrane proteins differ in that they bind detergent to replace the stabilizing hydrophobic interactions of the phospholipid bilayer. Therefore, the amount of detergent bound to the protein will skew the apparent molecular mass when determined by the above method.

To circumvent this problem, Shen et al. (54) proposed an approach for examining the stoichiometry of the voltage-dependent K<sup>+</sup> channel using a more denaturing detergent, Zwittergent 3–12 or 3–14, to disrupt the multimeric form of the channel down to its monomeric form. Their analysis was based on the supposition that the monomer and multimer would possess the same physiochemical properties. Therefore, they treated the monomer as an internal standard to approximate the number of subunits in the intact complex. This method was not applicable to K<sub>ir</sub> 2.2 because K<sub>ir</sub> 2.2 channels were not dissociated to monomeric form by a broad range of Zwittergent detergents (Zwittergent 3–08 through 3–16). In addition, we did not favor the underlying assumption that monomer and tetramer would bind equal amounts of detergent, since we expect that different hydrophobic regions of the protein would be exposed in the monomeric and the tetrameric state.

To avoid these complications, we established the molecular mass of K<sub>ir</sub> 2.2 by velocity sedimentation and gel filtration of detergent-solubilized K<sub>ir</sub> 2.2 channel protein (36, 37). We used a method that utilizes the difference in density between linear sucrose gradients constructed in D<sub>2</sub>O and H<sub>2</sub>O to measure apparent sedimentation coefficients. Soluble globular proteins would have the same hydrodynamic properties in both mediums. Therefore, the variability in sedimentation coefficients is a result of the detergent component of the complex. To determine the percentage of detergent bound to the protein, we calculated the partial specific volume (v) of the complex. Assuming that the binding of detergent to the protein is the same in both D<sub>2</sub>O and H<sub>2</sub>O, the percentage of detergent bound to the protein can be calculated by knowing the v for the detergent and the average v value for protein. These data and the measurement of the Stokes radius by gel filtration yielded a molecular mass of the protein as 193,000 ± 8,000 Da. Taking into account the cross-linking and immunoprecipitation results that show that the channel is composed of a single population of K<sub>ir</sub> 2.2 subunits, we calculate that the channel is made up of 3.98 ± 0.17 subunits. Thus, we demonstrate the homotetrameric organization of K<sub>ir</sub> 2.2 channels.

Similar biochemical approaches have been taken to determine the multimeric nature of other K<sup>+</sup> channels composed of their pore-forming α-subunits and auxiliary subunits. Inanobe et al. (27) measured the hydrodynamic properties of I<sub>KACh</sub>. They concluded that the native channel consisted of 3–5 subunits. Recently, Corey et al. (30) further defined the stoichiometry of I<sub>KACh</sub> by cross-linking purified atrial and recombinant heteromultimeric GIRK channels. They proposed that the native I<sub>KACh</sub> is composed of two GIRK1 subunits and two GIRK4 subunits. Several laboratories have determined the molecular mass of detergent-solubilized voltage-dependent K<sup>+</sup> channel/dendrotoxin receptor from synaptic membranes. From these data, a molecular mass of ~400 kDa was calculated consisting of 4 α-subunits and four β-subunits (55, 56).

**Conclusion**—We conclude that K<sub>ir</sub> 2.2 lacks auxiliary β-subunits, thus making this channel different from ATP-sensitive inward rectifier potassium channels, which coassemble with auxiliary sulfonylurea receptor subunits of ~140–170 kDa (23–25). K<sub>ir</sub> 2.2 also differs from the voltage-gated potassium channels of the Shaker subfamily (Kv 1.x), which may assemble with β-subunits of 35–42 kDa (57, 58), and from the mammalian large conductance calcium-activated potassium channel, which contains β-subunits of ~31 kDa (59).

The homotetrameric nature of K<sub>ir</sub> 2.2 channels further suggests that the K<sub>ir</sub> 2 family of classic strong inward rectifiers differs from the heterotetrameric K<sub>ir</sub> 3 family of G-protein-regulated inward rectifiers. Thus, each of the known subfamilies of inward rectifier channels displays a unique subunit structure.

Until now, the native stoichiometry of classic inward rectifier channels has not been investigated. We utilized two independent methods to demonstrate that brain K<sub>ir</sub> 2.2 channels exist as tetramers in native cells, thus providing the first characterization of this class of inward rectifier channels. These data make unlikely the possibility that K<sub>ir</sub> 2.2 channels contain auxiliary β-subunits and instead show that they are composed of a tetrameric organization of K<sub>ir</sub> 2.2 subunits. In light of our results, we conclude that K<sub>ir</sub> 2.2 functions as a homotetramer and that any additional proteins that may interact with the channel may serve to fine tune its regulation and hence be transient in their interaction with the channel. This work lays the foundation for a biochemical understanding of the function of strong inward rectifiers in the brain and heart.

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