

Biochemical characterization of the native Kv2.1 potassium channel

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Keywords

channels; oligomerization; potassium; proteomics; purification

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(Received 8 January 2005, revised 17 May 2005, accepted 2 June 2005)

doi:10.1111/j.1742-4658.2005.04802.x

Functional diversity of potassium channels in both prokaryotic and eukaryotic cells suggests multiple levels of regulation. Posttranslational regulation includes differential subunit assembly of homologous pore-forming subunits. In addition, a variety of modulatory subunits may interact with the pore complex either statically or dynamically. Kv2.1 is a delayed rectifier potassium channel isolated by expression cloning. The native polypeptide has not been purified, hence composition of the Kv2.1 channel complexes was not well understood. Here we report a biochemical characterization of Kv2.1 channel complexes from both recombinant cell lines and native rat brain. The channel complexes behave as large macromolecular complexes with an apparent oligomeric size of 650 kDa as judged by gel filtration chromatography. The molecular complexes have distinct biochemical populations detectable by a panel of antibodies. This is indicative of functional heterogeneity. Despite mRNA distribution in a variety of tissues, the native Kv2.1 polypeptides are more abundantly found in brain and have predominantly Kv2.1 subunits but not homologous Kv2.2 subunits. The proteins precipitated by anti-Kv2.1 and their physiological relevance are of interest for further investigation.

Potassium (K^+) channel pore-forming (α) subunits are by far one of the most diverse groups of channel proteins responsible for controlling membrane excitability, with 164 potassium channel genes in the human genome [1]. The diversity of potassium channels arises from several levels including the large number of genes coding for K^+ channel α subunits, alternative splicing, differential expression, combinatorial assembly of different α subunits, post-translational modification, as well as association with auxiliary subunits [2]. In addition, many K^+ channels interact with additional proteins such as regulatory enzymes and elements of the cytoskeleton [3]. Therefore, selective combinatorial assembly further contributes functional diversity. However, it is not known how much of this potential diversity is actually used in native cells [1,2]. Hence, understanding the molecular composition of native

channels is important for functional characterization *in vivo*.

There are several types of K^+ channels, including voltage-gated and Ca^{2+} -activated K^+ channels, inward rectifiers, 'leak' K^+ channels, and Na^+ -activated K^+ channels. Among these, all α subunits of the Shaker superfamily share a similar organization, with each polypeptide containing six putative transmembrane segments (S1–S6), a pore region between segments S5 and S6, and cytoplasmic N- and C-terminal domains. More than 20 functional Shaker superfamily voltage-gated K^+ channel α subunits have been experimentally investigated in heterologous expression systems.

Shab family K^+ channels (Kv2) are delayed rectifier channels and members of the Shaker superfamily [2]. Different from some of the other Kv channels, such as

Abbreviations

α subunit, pore-forming subunit; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; DOC, deoxycholate; GluR2/3, glutamate receptor 2/3; Kv2, Shab family K^+ channels; NR1, NMDA receptor R1 subunit; OG, octyl glucoside; PSD95, postsynaptic density 95; TAP, transcytosis-associate protein; VCP, valosin containing protein.

Kv1 with nine subunit members, the Kv2 subfamily has only two known mammalian members (Kv2.1 and Kv2.2), which are indistinguishable in their biophysical properties [4,5]. These two subunits are capable of forming heteromultimeric complexes in a heterologous expression [6]. However, immunohistochemical data suggest a very limited overlap in tissue distribution in brain [7,8]. Intriguingly, dominant negative mutants of either Kv2.1 or Kv2.2 selectively attenuate the formation of a functional Kv2.1 or Kv2.2 channel, respectively [9]. It is unclear how the specificity is established. This observation invites consideration of a more complex mechanism by which native channel complexes are formed during biogenesis.

Recently, several novel classes of α subunits have been cloned (Kv5, 6 and 8–11), which are electrically silent Kv channels, reflecting their inability to generate K^+ channel activity when heterologously expressed in either *Xenopus* oocytes or mammalian systems [10–12]. Interestingly, several studies have shown that coexpression of electrically silent Kv α subunits with Kv2.1 allows them to be transported to the plasma membrane from the ER, suggesting interaction between Kv2.1 and electrically silent channel subunits. The channel activities of Kv2.1 have been shown to be changed by coexpression of electrically silent Kvs [13,14], thereby suggesting a role in modulation.

To investigate the native composition of the Kv2.1 potassium channel and to develop a general strategy to isolate potassium channel complexes, we pursued and compared both conventional and affinity purification from native central nervous system tissues and from recombinant cell lines. Here we report the biochemical characterization of Kv2.1 protein complexes. The biochemical profile of the Kv2.1 potassium channel forms a foundation for subsequent large-scale purification and could serve as a useful guide for biochemical purification of other potassium channels.

Results

Expression and biochemical characterization of native Kv2.1

Regional distribution in various rat tissues of Kv2.1 channel protein was assessed using western blot analysis to identify a native source possessing significant amounts of Kv2.1 protein for purification. To this end, antibodies directed against the C-terminus of Kv2.1 (antibodies 2078 and 7088, see below) were developed, affinity-purified, and used in the following experiments. The antibodies detected the recombinant polypeptide around 100 kDa specifically from trans-

ected COS7 cells, which are consistent with the predicted molecular mass of Kv2.1 (Fig. 1A, lanes 2). Furthermore, Kv2.1 polypeptides from rat brain were recognized by the antibodies (2078 and 7088), and the

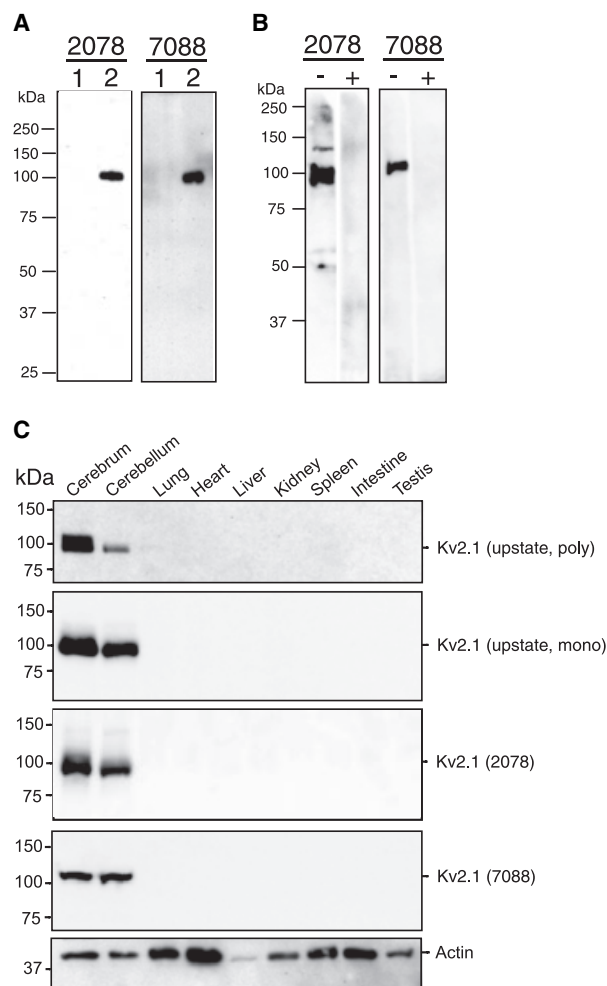


Fig. 1. Specificity of Kv2.1 antibodies used in this study and expression of Kv2.1 in various rat tissues. (A) Immunoblot analysis of recombinant Kv2.1 with anti-Kv2.1 (2078 and 7088) IgGs. Protein samples from untransfected (lane 1) and pCIS-Drk1 transfected (lane 2) COS7 cells were size-fractionated by SDS/PAGE and visualized either with 2078 or 7088 antibody as indicated. (B) Preincubation with synthetic antigen peptides blocks antibody binding to the native Kv2.1 polypeptide. Rat brain membranes were separated on SDS/PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. Membrane strips were treated with 1 : 500 dilutions of antibodies with no peptide addition (-) or addition of synthetic Kv2.1 peptide (+). (C) Western blot analysis of Kv2.1 expression in various rat tissues. Fifteen micrograms of whole cell extracts were loaded in each lane and immunoblotted by different anti-Kv2.1 IgG (upper four panels) and anti-actin IgG (bottom panel). Kv2.1 specific bands of 95–110 kDa proteins are detected in cerebrum and cerebellum.

signals were abolished by synthetic antigen peptides (Fig. 1B).

The expression of Kv2.1 mRNA was previously reported to be ubiquitous by RT-PCR, but found mainly in heart, skeletal muscle and brain by Northern blot analysis [10,15]. Using both commercial and our specific peptide antibodies (2078 and 7088), we found that Kv2.1, at the protein level, showed the most prominent expression in brain regions in a panel of examined tissues (Fig. 1C). In particular, Kv2.1 is considerably more abundant in cerebrum than cerebellum. Therefore, rat forebrain excluding cerebellum was chosen as a native source for biochemical characterization. In cerebrum, an additional band with slower mobility was visible, suggestive of heterogeneity at the levels of mRNA processing, post-translational modification and/or possible proteolytic degradation (see below).

Effective membrane solubilization is a prerequisite for purification of membrane-bound proteins. However, the relative solubility of the Kv2.1 protein under different detergent treatments has not been extensively studied. Comparative analyses were carried out to determine and optimize conditions suitable for solubilizing Kv2.1 from the chosen source, rat forebrain. The tested conditions include detergents at different concentrations. Solubility was judged by 105 000 *g* centrifugation. The partitioning of Kv2.1 proteins in either soluble or insoluble fractions was followed by immunoblotting using antibodies specific to the C-terminus of Kv2.1 polypeptide, and the signal intensity was quantified by densitometry within a linear range. The protein amounts were estimated using a standard obtained with a purified recombinant Kv2.1 fusion protein (see below). Solubility of Kv2.1 is shown in Fig. 2A,B when treated with different detergents, including SDS, deoxycholate (DOC), 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS), octyl glucoside (OG), Triton X-100, and Digitonin. More than 50% of Kv2.1 may be solubilized in the presence of 1% SDS. In addition, a significant amount of Kv2.1 could be recovered in soluble fractions with 1% DOC and Triton X-100 extraction. The effective concentrations of DOC and Triton X-100 to extract Kv2.1 were further examined by titrations of different concentrations (data not shown). We chose 2.5% Triton X-100 and a combination of 0.5% DOC and 0.1% Triton X-100 as primary solubilization conditions prior to chromatographic steps and immunoaffinity purification, respectively. The two Kv2.1 species in Fig. 2 showed differential behavior upon treatment by different detergents. In general, the lower band of 95 kDa was more soluble than the upper band (Fig. 2A,B). DOC extracts more of the lower band regardless of

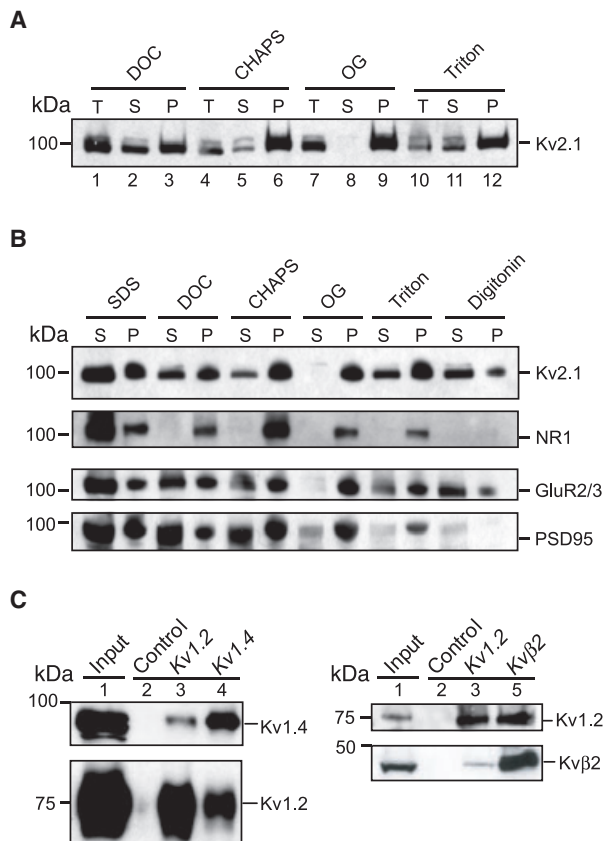


Fig. 2. Solubilizing Kv2.1 protein from rat forebrain. (A) Distribution of two Kv2.1 species upon treatment of indicated detergents by 105 000 *g* centrifugation is shown. Native Kv2.1 from equal amount of rat forebrain (100 mg) was extracted in the buffer containing 0.5% of detergents by Dounce homogenizer and centrifuged at 700 *g* to remove cell debris and nuclei. The supernatant (T) was further separated to soluble (S) and insoluble pellet (P) by ultracentrifugation at 105 000 *g*. (B) The relative solubility of Kv2.1 was compared to several post synaptic density (PSD)-enriched proteins, including GluR2/3, NR1 and PSD95 upon treatment with various detergents. Proteins corresponding to an equal volume of supernatant (S) and pellet (P) were loaded after homogenizing rat forebrain with 1% of various detergents as indicated. (C) Solubilized membrane proteins were immunoprecipitated by Kv1.2, Kv1.4 and Kvβ2 antibodies. Kv1.2, Kv1.4, and Kvβ2 input were visualized (lane 1). The immunoprecipitated materials by antibodies against Kv1.2 (lane 3), Kv1.4 (lane 4, left panel), and Kvβ2 (lane 5, right panel) were probed by antibodies as indicated on the right of each panel.

concentration while CHAPS and Triton X-100 solubilized two species equally well when lower concentrations of detergents were applied (Fig. 2A, lanes 2, 5 & 11). This suggests a different biochemical feature of the two protein species. We also tested membrane preparation of rat brain as a starting material and found a very similar result to what was obtained using whole brain extracts (data not shown). The behavior of Kv2.1 under different detergent treatments was

compared to that of three other brain-specific proteins, including NMDA receptor R1 subunit (NR1), glutamate receptor 2/3 (GluR2/3), and postsynaptic density 95 (PSD95). The level of solubility of Kv2.1 is more similar to GluR2/3, consistent with reports that NR1 and PSD95 are highly insoluble (Fig. 2B). We also observed similar level of solubility for Kv2.1 in stably transfected cells.

The quality of solubilization was evaluated by coimmunoprecipitation and size exclusion studies. To assess whether the applied conditions would disrupt the potassium channel complexes, we performed coimmunoprecipitation studies of Kv1.2 and Kv1.4, which were previously shown to interact and form heteromultimeric channels *in vivo* [16]. The results indicated that anti-Kv1.2 IgG was able to precipitate Kv1.4 subunits. Conversely, anti-Kv1.4 IgG was able to precipitate the Kv1.2 polypeptide (Fig. 2C). These results support the idea that the referenced condition for solubilization is compatible with the isolation of intact channel complexes.

To examine the hydrodynamic properties of the solubilized Kv2.1 complex, solubilized crude extracts from either whole cell or membrane fractions were evaluated by size-exclusion chromatography. The Kv2.1 polypeptides were detected by immunoblot. This analysis provides information on Stoke's radius and allows for estimation of their molecular masses, which permit evaluation of apparent oligomeric size. The solubilized material in 2.5% Triton X-100 behaved as a macromolecular complex(es) that was quantitatively recovered. The peak for the immunoblot signal migrates past void volume and overlaps with the standard, thyroglobulin, which has a Stoke's radius of about 85 Å and a molecular mass of 670 kDa (shaded area, Fig. 3). This is similar to that of Kv1.2 complexes including both Kv1.2 and Kvβ2 [17]. With the same solubilized extracts, other known potassium channel complexes such as Kv4.2 with dipeptidyl aminopeptidase X and Kv1.2 with Kv1.4 could be found by coimmunoprecipitation experiments (data not shown, and Fig. 2C), providing further support that the conditions used were compatible for the isolation of channel complexes [16,18]. Additional experiments using recombinant Kv2.1 expressed in HEK293 cells revealed a similar chromatographic profile (data not shown).

Heterogeneity of Kv2.1 complexes

In order to biochemically characterize the Kv2.1 protein complexes, we further evaluated the solubilized materials. Total solubilized membrane protein was quantified by Bradford assay, for which independent

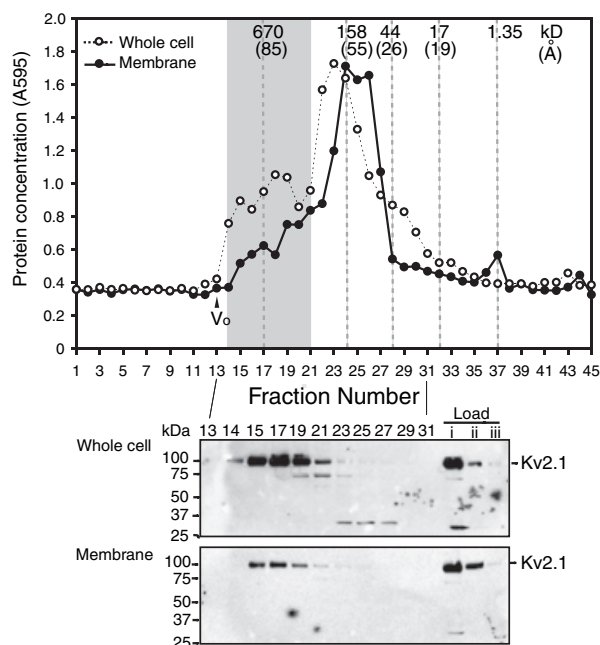
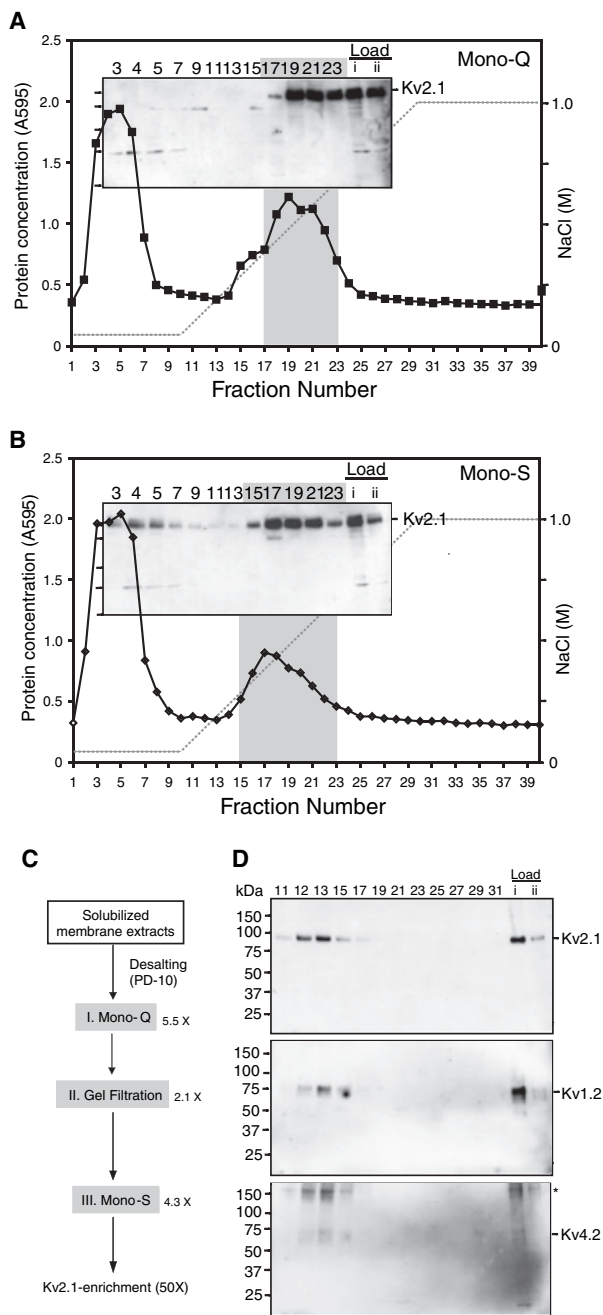


Fig. 3. Size-exclusion chromatography analysis of soluble Kv2.1 complexes. Native Kv2.1 complexes in either whole cell lysate (○) or crude membrane extracts from rat forebrain (●) with 2.5% Triton X-100 were fractionated by size-exclusion chromatography. The dotted lines on the chromatogram depict the peak fractions of standards, and the shaded area represents the locations of fractions showing Kv2.1 immunoreactivity. Two percent of each fraction was taken from the elution volume (V_0) and analysed by western blotting against Kv2.1. The amount of Kv2.1 immunoreactivity in each fraction was analyzed in comparison to Kv2.1 immunoreactivity in the load [1 (i), 0.1 (ii), and 0.02 (iii)% of the load].

preparations at concentration of $2\text{--}3\text{ mg}\cdot\text{mL}^{-1}$ gave consistent results with subsequent analyses. Quantitative immunoblots using Kv2.1 antibody were used to estimate the relative yield of native Kv2.1 compared to the purified recombinant C-terminal Kv2.1 protein of known concentration. Quantitative analyses estimated that the Kv2.1 protein was at a concentration of $50\text{ ng}\cdot\text{mg}^{-1}$ (less than 0.05%), a rare protein component in the detergent extract of rat forebrain, indicating that both substantial purification and high recovery yield would be necessary to reach homogeneity. The solubilized Kv2.1 protein was applied either to an anionic exchange Mono-Q column, or to a cationic exchange Mono-S column. Both Mono-Q and Mono-S columns were able to capture Kv2.1 protein at 50 mM NaCl when the same amount (6 mg) of solubilized protein was applied. The fractions with Kv2.1 proteins were identified by western blot analysis, highlighted in the shaded areas superimposed onto the chromatograms (Fig. 4). There are two additional antibody-reacted species with molecular masses of 70 and



30 kDa (Figs 3 and 4A,B). These are probably degraded fragments because the reactivity was detectable by different Kv2.1 antibodies. Interestingly, Kv2.1 protein was quantitatively retained to Mono-Q column (Fig. 4A). In contrast, 10–15% of the Kv2.1 material did not bind to Mono-S. The finding of Kv2.1 in the Mono-S flow-through fractions was independent of the quantities of loading materials, suggesting there are at least two biochemically distinct populations or channel complexes with different protein composition

Fig. 4. Chromatographic fractionation of native Kv2.1 complexes. (A and B) Elution profile of native Kv2.1 complex by Mono-Q (A) and Mono-S (B) ion exchange chromatographies. Native Kv2.1 complexes were solubilized from brain membrane and subjected to either Mono-Q or Mono-S. After binding of solubilized membrane proteins, the columns were washed with 50 mM NaCl; retained proteins were eluted by the application of increasing NaCl in a linear gradient as indicated by the dotted lines on the chromatogram. Shaded area represents the locations of fractions showing Kv2.1 immunoreactivity by the immunoblotting of every other fraction (inset). Molecular mass markers for 100, 75, 50, and 37 kDa are indicated on the left side of the gel from the top. The amount of Kv2.1 immunoreactivity in each fraction (2% on the gel) were analyzed in comparison to Kv2.1 immunoreactivity in the load [0.125 (i) and 0.05 (ii)% of the load]. (C) Schematic diagram of three-step conventional purification designed and its fold purification per step. Native Kv2.1 complexes were solubilized from brain membrane and subjected to sequential chromatography by Mono-Q, size-exclusion chromatography, and Mono-S. The eluate positive for Kv2.1 immunoreactivity from the Mono-Q was further fractionated by size-exclusion chromatography. Kv2.1 positive fractions from the size-exclusion chromatography column were then loaded onto the Mono-S column, washed, and the fraction with Kv2.1 immunoreactivity was eluted with NaCl as described. (D) Chromatographic cofractionation of voltage-gated potassium channels. The fractionations of different K⁺ channel subunits were followed subsequent to each step of chromatography by immunoblotting with Kv2.1-, Kv4.2-, and Kv1.2-specific antibodies. The peak fractions of the second size-exclusion chromatography step from the sequential chromatography described in (C) are shown.

(Fig. 4B). Fractionated proteins on Mono-Q and Mono-S were further analyzed by comparing the western signals of Kv2.1 from different antibodies. Kv2.1 in the flow-through and bound fractions of Mono-S was differentially detected in their mobility and intensity by 2078 and 7088 antibodies raised against different regions of Kv2.1 (Fig. 5). As this was one membrane probed sequentially with three indicated

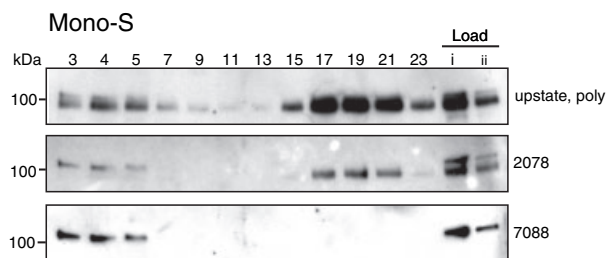


Fig. 5. Differential reactivity of Mono-S fractions of Kv2.1 to different Kv2.1 antibodies. Equal amount of fractionated samples from Mono-S was analyzed by SDS/PAGE. Numbers indicate the location of fractions in the Mono-S chromatography as shown in Fig. 4B. The immunoblotting analyses were performed sequentially using one membrane by three different Kv2.1 antibodies as indicated and as in Experimental procedures.

antibodies after removing bound immunoglobulins, the differential detection, e.g. lack of signal in fractions 17–23 for 7088, could not be attributed to the difference in affinity of antibodies. Hence it supports the notion of biochemical heterogeneity. In contrast, the Mono Q did not separate the different subpopulations since bound Kv2.1 populations were detected by all antibodies tested (data not shown). Sequential purification by ion exchange chromatographic steps and gel filtration steps yielded an ≈ 50 -fold purification (Fig. 4C). Heterogeneity in both chromatographic profiles and reduced recovery contributed to the poor overall purification. An examination of the elution fractions of the third chromatographic steps of Mono-S on SDS/PAGE showed the major peak of proteins and the peak fractions of Kv2.1 were identical. In addition, immunoblot analysis showed that GluR2/3 and other family members of Kv channels including Kv1.2 and Kv4.2 proteins were also found overlapping with the fractions containing Kv2.1 in all three chromatographic steps (Fig. 4D and data not shown). This is in agreement with information in earlier reports. For example, Kv1.2, Kv1.4 and Kv4.2 were comigrated in anion exchange chromatography [16].

Immunoaffinity purification of Kv2.1 complex and proteomic characterization

From the above analyses we next chose to make use of immunoaffinity purification. We therefore optimized immunoprecipitation methods for the enrichment of Kv2.1 channels by using different antibodies and titration. To keep the conditions for antibody binding consistent, the extracts were either diluted or dialyzed against buffer containing 0.1% Triton X-100 before immunoprecipitation regardless of the detergent used for the initial extraction. The optimal ratio of extract to antibody in small-scale immunoprecipitation was determined by titrating amounts of the affinity purified Kv2.1-specific antibodies with fixed amounts of extract (data not shown). These antibodies were used to immunoprecipitate Kv2.1 proteins from native or recombinant source.

To test the antibody specificity for immunoaffinity purification, a stable HEK293 clone expressing C-terminal Myc-tagged full-length rat Kv2.1 was established. The functional expression of Kv2.1 on cell surface was demonstrated by both immunocytochemistry and whole cell voltage clamp recording (data not shown). Purification of Kv2.1 channel complex was carried out by immunoprecipitation with either α -Myc antibody or Kv2.1 antibody (2078) in parallel using whole cell extracts of HEK293 cells stably expressing

rat Kv2.1 cDNA. The immunoprecipitated materials were visualized by Coomassie Blue stained upon SDS/PAGE fractionation with identified bands (Fig. 6A). Comparison of the precipitated materials from the positive cell line stably expressing recombinant

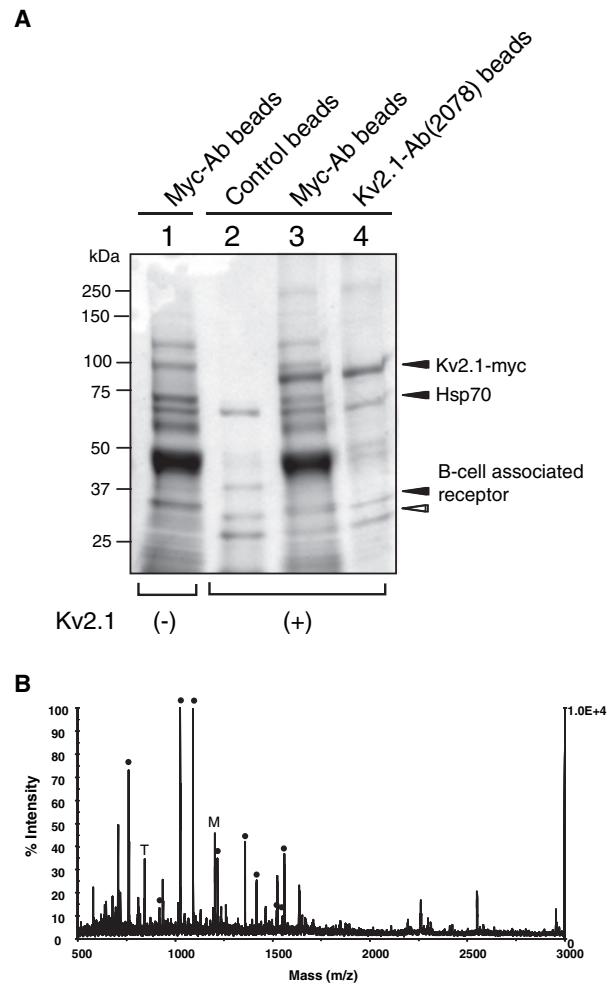


Fig. 6. Proteomic characterization of Kv2.1 complexes from HEK293 cells stably expressing Kv2.1. (A) Coomassie Blue staining of SDS/PAGE whole cell extracts by 1% Triton X-100 from negative (-) and positive (+) HEK293 clones for Kv2.1 expression. The first two lanes are negative controls showing proteins bound to α -Myc protein A-Sepharose with Kv2.1(-) lysate (lane 1) and to normal rabbit IgG protein A-Sepharose with Kv2.1(+) lysates (lane 2). Polypeptides immunoprecipitated with α -Myc from Kv2.1(+) cell lysates were compared to those with affinity-purified Kv2.1 antibody (lanes 3 and 4). Proteins in the last lane marked with arrowhead were analyzed by mass spectrometry, and unambiguously identified proteins are indicated by filled arrowheads. (B) MALDI-TOF peptide mass map obtained from the immunopurified Kv2.1 protein. Ion signals with measured masses that match calculated masses of protonated tryptic peptides of the identified protein within 50 p.p.m. are indicated with closed circles. T, Signals from autolysis products of trypsin; M, signals from matrix-related ions.

Kv2.1 to those from the control cell line revealed a specific polypeptide with a molecular mass just below 100 kDa. The size of this polypeptide is consistent with the calculated molecular mass of Kv2.1 from its deduced sequence (NP_037318). This polypeptide was visible by anti-Myc and anti-Kv2.1 IgGs from the stable cell line but not by control beads from the same source or anti-Myc IgG from control cell line (Fig. 6A, lanes 1 and 2). Bands indicated by arrowheads in Fig. 6A were excised from lane 4, subjected to digestion with trypsin, and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Positively identified bands are shown as filled arrowheads. The bands that were also found in control IgG-beads were not pursued further (Fig. 6A). The peptide mass map of Kv2.1 protein from this gel is illustrated in Fig. 6B. Sixteen of the measured peptide masses match theoretical tryptic peptide masses calculated for rat Kv2.1 (DRK1, accession number NCBI NP_037318), a protein with a predicted mass of 95.3 kDa. The matching peptides cover 19% of the Kv2.1 sequence. The distinct bands of ≈ 100 kDa were unambiguously identified as rat Kv2.1, showing that Kv2.1 can be successfully purified under the conditions used and further demonstrating the specificity of peptide-specific antibody for application of immunoprecipitation.

The Kv2.1 channel complex from rat forebrain membrane was also isolated by immunoprecipitation. The necessary amount of solubilized membrane extracts from rat forebrain for native Kv2.1 purification at the level of Coomassie Blue detection was calculated based on the comparison of the expression level of Kv2.1 from rat forebrain to that from the stable clone extracts (data not shown). Three independent Kv2.1 antibodies were used for the purification in parallel and the result from a commercial monoclonal antibody is shown (Fig. 7A). The commercial monoclonal antibody brought down a band with a molecular mass of ≈ 100 kDa that was specific to antibody but not the control (#4) (Tables 1 and 2). For affinity-purified peptide antibodies, 2078 and 7088, several bands were precipitated (see below). Among them is the 100 kDa polypeptide. Polypeptides of ≈ 100 kDa from all three antibody immunoprecipitations were unambiguously identified as Kv2.1 proteins. A representative mass spectrum and the list of peptides from band 4 are shown in Fig. 7B and Table 1. The specificity of affinity purified antibody binding was further confirmed by immunoblot (data not shown). Hence, native Kv2.1 may be specifically precipitated by one monoclonal antibody and two peptide antibodies against different regions of the same polypeptide.

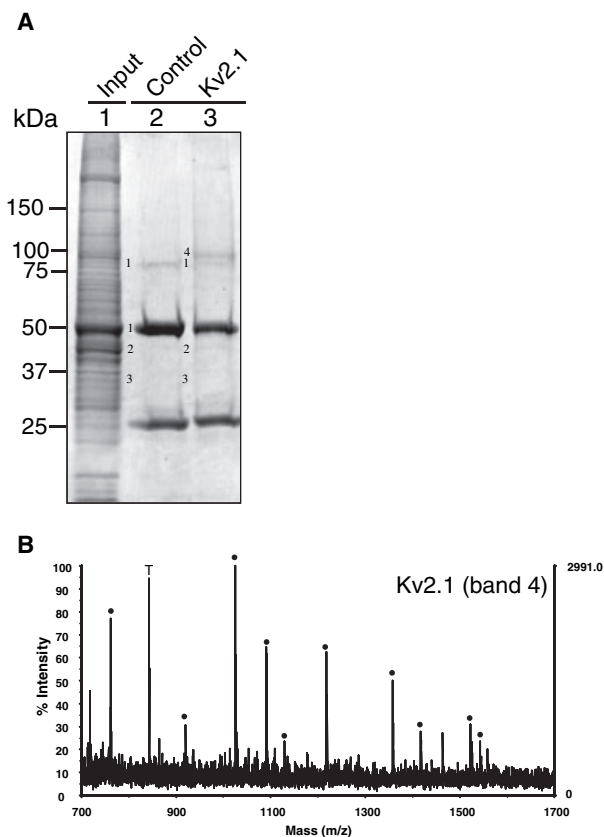


Fig. 7. Proteomic characterization of native Kv2.1 channel complexes from rat forebrain. (A) A whole image of Coomassie Blue stained SDS/PAGE gel of polypeptides immunoprecipitated from rat forebrain membrane extracts. Lane 1 is solubilized membrane extract used for immunoprecipitation. Immunoprecipitated proteins from monoclonal Kv2.1 antibody (lane 3) and control (lane 2, protein G-Sepharose beads) were visualized. Proteins positioned by numbers in lanes 2 and 3 were excised for MALDI-TOF MS. Unambiguously identified bands are as follows; IgG γ 2A (band 1), β and γ -actin (band 2), GAPDH (band 3), and Kv2.1 (band 4). (B) MALDI-TOF peptide mass map of Kv2.1 obtained from immunopurified Kv2.1 complexes. Peptide mass spectrum is shown with selected ion signals with measured masses that match calculated masses of protonated tryptic peptides of the Kv2.1 protein within 50 p.p.m. (●). T, Signals from autolysis products of trypsin.

Discussion

Native potassium channels are scarce proteins. Despite their biological significance and the critical need to understand their native composition, purification of native potassium channels has met only limited success and remains a considerable challenge. The successes in purifying Kv1.2 and large conductance Ca²⁺-gated potassium channels from native tissues highlight the needs for affinity reagents, such as toxins [19,20]. The Kv2.1 in rat forebrain represents less than 0.05% of

Table 1. Kv2.1 peptides identified from the MALDI-TOF peptide mass map shown in Fig. 7.

Measured mass	Matching mass	Δ Mass (p.p.m.)	Missed cleavage	Position	Peptide
761.4375	761.4674	-39	0	295–300	(R) VVQIFR (I)
920.4086	920.4511	-46	0	576–583	(R) TEGVIDMR (S)
1025.4747	1025.5056	-30	0	649–657	(R) SGFFVESPR (S)
1090.5750	1090.6009	-24	0	285–293	(K) SVLQFQNVK (R)
1129.5755	1129.6217	-41	0	539–548	(K) TQSQPILNTE (E)
1217.5664	1217.5915	-21	0	616–627	(K) AGSSTAPEVGVK (G)
1260.6581	1260.6588	-0.58	0	604–615	(R) FSHSPLASLSSK (A)
1357.6424	1357.6963	-40	0	637–648	(R) LTETNPIPETSR (S)
1416.7214	1416.7599	-27	0	313–325	(R) HSTGLQSLGFTLR (R)
1463.7598	1463.8123	-36	0	35–47	(R) LNVGGLAHEVLWR (T)
1522.7276	1522.7807	-35	0	88–100	(R) HPGAFTSILNFYR (T)
1542.7425	1542.7838	-27	0	12–25	(R) STSSLPPEPMEIVR (S)
2549.2225	2549.3404	-46	0	673–695	(K) VNFVEGDPTPLLPGLYHDPLR (N)

Table 2. Proteomic analysis of native Kv2.1 channel. SWISS-PROT and TrEMBL accession numbers are listed.

Specific protein identified	Accession number	Protein parameter		MALDI-TOF MS	
		Molecular mass (kDa)	pI value	Matching peptides	Protein coverage (%)
Kv2.1	P15387	95.638	8.4	11	17

total protein, suggesting a need for more than 2000-fold purification assuming quantitative recovery at each purification step. Our experiments indicated that Kv2.1 protein is more abundant in brain and is in a highly insoluble form (Figs 1 and 2). In addition, Kv2.1 protein is heterogeneous in size and biochemical behavior, which was demonstrated in differential detection of two species of Kv2.1 in their mobility and intensity when either whole cell lysates or fractionated samples from ion-exchange chromatography were analyzed by different Kv2.1 specific antibodies against the C-terminus of Kv2.1 (Figs 1 and 5). The Kv2.1 channels have been reported in other tissues such as pancreas [10]. But the biochemical properties and abundance compared to Kv2.1 in brain remain to be determined. Our experiments also highlight some of the key parameters and strategies that are specifically useful for Kv2.1 and potentially applicable to the pursuit of purification of other potassium channels.

Quality assessment of channel purification often relies on binding natural ligands. While hanatoxin has been shown in electrophysiological studies to block the Kv2.1 channel [21], biochemical studies of its binding preference concerning channel oligomeric structures have not been reported. The toxin interaction with Kv2.1 modulates the voltage-sensor and the modulation may require lipid–protein interaction [22,23]. It is unclear how detergent might affect the interaction between hanatoxin and Kv2.1 channels. To gain infor-

mation concerning the quality of complexes after solubilization, both coimmunoprecipitation and hydrodynamic studies have been performed (Figs 2B and 3). The applied condition preserved the Kv1.2–Kv1.4 channel complex as well as the association of Kv1.2 with its known auxiliary Kv β 2 subunit (Fig. 2C and [16]). The resultant protein complex has a Stoke's radius of 85 Å similar to that of Kv1.2 complex (86 Å) [17]. Using similar biochemical criteria, glutamate receptor complexes have also been purified and characterized by proteomic approaches [24,25], a study that has yielded useful information.

While affinity purification is advantageous over the yeast two-hybrid approach in isolation of multiprotein complexes, the biochemical heterogeneity of the Kv2.1 polypeptides from rat brain poses a major difficulty. This is further underscored by the fact that anti-Kv2.1 IgG identify brain as an abundant source (Fig. 1) while mRNA messages were detected in almost all tissues [10]. Operationally, the heterogeneity in our experiments is reflected at two levels – multiple and broadness of peaks in chromatographic separations. After three-step sequential conventional chromatography, the resultant material has only modest 50-fold purification. There is also a significant loss contributing to a low recovery. Concentrating steps were necessary for each step, which resulted in further loss of Kv2.1 protein (data not shown). Because 2078 and 7088 antibodies have differential affinity to subpopulations

of brain Kv2.1, sequential coimmunoprecipitation experiments may provide further insights into the biochemical nature of the Kv2.1 heterogeneity.

Mechanistically, the biochemical heterogeneity is the basis of functional diversity and may originate from several factors. First, at the genetic level, the molecular heterogeneity of Kv2.1 was previously reported, which may reflect tissue-dependent variations in Kv2.1 transcript size and/or post-translational modification [15,26,27]. For example, multiple transcripts of Kv2.1 from brain were reported while a major transcript was found in other tissues [15,26]. Second, native Kv2.1 may be in complex with a variety of different protein factors which may associate with the pore-forming subunits statically or dynamically in response to certain stimuli. Earlier studies reported phosphorylated Kv2.1 species in COS-1 cells and from brain [27,28]. Also, the tyrosine 124 within the T1 domain of Kv2.1 was identified as a target site for Src (or Fyn) and protein tyrosine phosphatase epsilon (PTP ϵ) in Schwann cells [29–31]. In rat brain, a currently unknown polypeptide of 38 kDa was also implicated in association with Kv2.1 [32]. In addition, the electrically silent Kv subunits show a different pattern of tissue distribution among their subfamilies [10]. Their association with Kv2.1 might have caused biochemical heterogeneity and consequently functional diversity. More recently, MinK-related peptide 2 was shown to be in the complexes of two structurally and functionally different Kv α subunits including Kv3.1b and Kv2.1 from rat brain, suggesting the existence of a β subunit influence over multiple delayed rectifier potassium channels [33]. Many of these proteins have molecular masses equal to or less than 50 kDa, the size of immunoglobulin heavy chain. The abundant immunoglobulin noise in the gel hampers positive identification of proteins with molecular masses less than 50 kDa. Third, the biochemical heterogeneity may be related to the complex cell biology. The heteromultimer formation of Kv2.2 and Kv2.1 has been reported when expressing them in *Xenopus* oocytes [6]. But the dominant negative constructs of these two subunits specifically affect only the corresponding homomultimeric channels in both HEK293 cells and cultured neurons. Furthermore, the Kv2.1 channels display a distinctive, vesicle-like clustering distribution with correlation to phosphorylation of Kv2.1 [34,35]. The protein complexes in different trafficking stages may be in different states of lipid and/or protein environments and it is possible that the cytoplasmic population of the brain Kv2.1 protein is more soluble under our detergent condition. Hence, for a given channel protein, these factors may contribute singularly or combinatorially to the biochemical

heterogeneity. This highlights the importance to profile a variety of detergent solubilization conditions in order to achieve a better homogeneity of biochemical behavior as a starting point.

Analyses of the associated proteins by mass spectrometry revealed several proteins that were precipitated by specific anti-Kv2.1 IgG (2078 and 7088). These proteins include rho/rac effector protein Citron-N, transcytosis-associate protein (TAP)/p115 and valosin containing protein (VCP) (data not shown). The specificity of their association was evaluated preliminarily by antibody-specific precipitation and restricted detection from brain lysates but not from stable HEK293 cells (data not shown). Because of these proteins roles in vesicular trafficking steps and coupling with signaling events, the tentative association of Kv2.1 with these factors may represent a collection of Kv2.1 channel complexes in transit to the cell surface. The potential roles of these proteins in Kv2.1 trafficking require additional follow-up studies.

Kv2.1 and Kv2.2 are homologous subunits. Our purification failed to detect Kv2.2. Within the range of molecular mass of 100 kDa, several proteins have been positively identified. Table 1 shows a list of identified Kv2.1 peptides; of these, the majority are known to be Kv2.1 sequence-specific, providing statistical support for the hypothesis that Kv2.2 was not at the detectable level when Kv2.1 was targeted for immunoprecipitation. These results are consistent with the evidence obtained from sympathetic neurons [9], *in situ* hybridization and immunohistochemistry in rat brain [7,8]. It would be interesting to test whether the Kv2.2 associates with Citron-N, TAP/p115 or VCP.

Experimental procedures

Antibodies

Antibodies specific for the Kv2.1 α subunit were generated by injection of the synthesized peptides corresponding to amino acids 743–761 (EAGVHHYIDTDTDEGQ, antibody 2078) (Invitrogen, Carlsbad, CA) and 837–853 (HMLPGGGAHGSTRDQSI, antibody 7088) (Antibody Designs, Huntsville, AL) into rabbits and were used for immunoaffinity purification of the Kv2.1 complex. A cysteine residue was added to the N-terminus of the peptides to facilitate coupling to keyhole limpet hemocyanin (KLH) for immunization, and to the resin for affinity purification. Affigel-10 resin (Bio-Rad, Hemel Hempstead, UK) and/or SulfoLink (Pierce, Milwaukee, WI) were used for affinity-purification. Polyclonal and monoclonal antibodies against Kv2.1 from Upstate Biotechnologies (Lake Placid, NY) were also used in some immunoblotting and immunopreci-

pitiation experiments in this study. Myc antibody (Sigma, St. Louis, MO) were used to immunoprecipitate recombinant Kv2.1 from stable cells. Antibodies against Kv1.4 and Kv1.2 were purchased from Upstate Biotechnologies and Chemicon (Temecula, CA), respectively.

Cell culture

Human embryonic kidney 293 (HEK293) cells were cultured as described previously [36] and transfected with linearized plasmid expressing rat Kv2.1(NP-037318) with Myc epitopes constructed using pCMV-Tag 5 A (Stratagene, La Jolla, CA). Stable cell lines were generated by single cell subcloning by selection made in a 96-well format on the basis of survival in the presence of G418 ($500 \mu\text{g}\cdot\text{mL}^{-1}$; Sigma, St. Louis, MO). The expression and subcellular localization of rat Kv2.1 of the positive clones were further confirmed by western blotting analysis and immunocytochemistry with Kv2.1 and Myc-specific antibodies, and whole cell recording. The established stable clones were kept in $250 \mu\text{g}\cdot\text{mL}^{-1}$ of G418.

Protein extraction from HEK cells

To prepare whole-cell lysate, HEK293 cells stably expressing rat Kv2.1 channels were washed with ice-cold NaCl/P_i three times, and harvested. After brief centrifugation (700 g), the cells were resuspended and lysed in buffer containing 10 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% of Triton X-100 and a cocktail of protease inhibitors: 10 μM benzamidine HCl, 1 $\mu\text{g}\cdot\text{mL}^{-1}$ phenanthroline, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ aprotinin, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ pepstatin, and 1 mM phenylmethanesulfonyl fluoride. After incubation on ice for 30 min, the cell suspension was homogenized by a Dounce homogenizer, and the homogenate was clarified by centrifugation. The supernatants from 105 000 g and 15 000 g were used for chromatography and immunoprecipitation, respectively.

Protein extraction from native tissues and solubilization studies

Separated forebrain from Sprague–Dawley rats (Pel Freez Biologicals, Roger, AR) was homogenized in 10 volumes of ice-cold sucrose buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.5, and a cocktail of protease inhibitors). The homogenate was centrifuged at 700 g for 10 min; the pellet was washed once with 7 volumes of sucrose buffer, and the combined supernatants were centrifuged further at 27 000 g for 40 min to yield a crude membrane pellet (P2). For screening the relative solubility of Kv2.1 proteins, samples of whole cell lysates or crude membranes (P2) were mixed with equal volumes of different detergents prepared in buffer containing 10 mM Hepes, pH 7.5, 150 mM NaCl,

1 mM EDTA and protease inhibitor cocktails. The detergents and final concentrations tested were 0.5, 1.0, and 2.5% (w/v) Triton X-100, sodium deoxycholate, CHAPS, digitonin and 1% (w/v) SDS and octyl-glucopyranoside. After stirring at 4 °C for 30–60 min, the samples were centrifuged at 105 000 g for 1 h. The resulting pellets and supernatants were collected, and equal volume amounts of the protein from pellet and supernatant were compared as insoluble and soluble proteins, respectively. The solubilized membrane extracts with 2.5% Triton X-100 were used for chromatographic studies. For immunopurification, the crude membrane was solubilized in 50 mM Tris/HCl, pH 9.0, 0.1% Triton X-100, 0.5% DOC for 1 h and then was dialyzed against 50 mM Tris/HCl (pH 7.4), 0.1% Triton X-100 overnight at 4 °C. The insoluble pellet was removed by centrifugation at 20 000 g for 30 min.

Chromatography

All procedures were carried out at 4 °C, unless stated otherwise. All buffers and solutions used during the FPLC chromatographic steps were filtered and degassed. The whole-cell extracts of the stable cells and the rat forebrain membrane extracts were subject to size-exclusion chromatography and ion-exchange (Mono-Q and Mono-S) independently and the behavior of the solubilized Kv2.1 channel complexes on each chromatography were analyzed. The buffers used in ion exchange chromatography were buffer A [10 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1% Triton X-100] and buffer B (buffer A with 1 M NaCl). Then, the combination of three consecutive columns was employed to enrich native Kv2.1 channel complex. Ten microliters of each fraction from all columns was analyzed for Kv2.1 immunoreactivity by SDS/PAGE followed by immunoblotting.

Size-exclusion chromatography

Protein sample (0.5 mL) from either the stable cells or native tissue was applied to a Superdex 200 10/30 column connected to the FPLC system equilibrated with buffer A, with 150 mM NaCl at 4 °C and calibrated with the following molecular mass (kDa) markers (Bio-Rad): bovine thyroglobulin (670), bovine γ -globulin (158), chicken ovalbumin (44), myoglobin (17), vitamin B12 (1.35). The column was eluted with 30 mL of the same buffer at a flow rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$, and 0.5 mL fractions were collected on ice for further analysis.

Mono-Q and Mono-S

The solubilized membrane extracts adjusted to a final salt concentration of 50 mM NaCl (2 mL) were applied onto a 1 mL Mono-Q column connected to an FPLC system

(AKTA FPLC, Amersham Bioscience, Piscataway, NJ), equilibrated previously with buffer A with 50 mM NaCl, at a flow rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$ at 4°C . After washing the column with buffer A with 50 mM NaCl, proteins were eluted with 10 mL of a linear gradient (0.05–1.0 M NaCl) in buffer A. Fractions (0.5 mL) were collected on ice for analyzing Kv2.1 immunoreactivity.

Immunopurification of Kv2.1 channel complexes

The whole-cell lysates from the stable cells (6 mg of protein at $2 \text{ mg}\cdot\text{mL}^{-1}$) and the solubilized rat forebrain membrane extract (usually 60 mg of protein at $2\text{--}3 \text{ mg}\cdot\text{mL}^{-1}$) were precleared with protein A–Sepharose 4B beads (Sigma) or protein A–Sepharose 4B beads crosslinked to Kv2.1 antibodies. The complexed beads were collected and washed three times for 10 min by resuspension/centrifugation (2000 g) with extraction buffer for the whole-cell lysate of the stable cells and dialysis buffer for the membrane extracts of rat forebrain. The bound Kv2.1 complexes were eluted from the beads by adding a sample buffer containing 2.5% 2-mercaptoethanol, 1 mM EDTA, 1.5% SDS, and 10% glycerol in 50 mM Tris buffer, pH 6.7, and the eluted proteins were separated by SDS/PAGE.

Immunoblot analysis

Immunoblots prepared as previously described [36] were incubated with the appropriate polyclonal antibodies at a 1 : 1000 dilution at 4°C for 14 h, and bound antibodies were detected using chemiluminescence with an ECL detection kit (Amersham Pharmacia, Piscataway, NJ). The following primary antibodies were used: Kv2.1, 7088 ($260 \mu\text{g}\cdot\text{mL}^{-1}$), 2078 ($380 \mu\text{g}\cdot\text{mL}^{-1}$), and Myc. Recombinant C-terminal domain of Kv2.1 (amino acids 421–853) was cloned into pRSET (Invitrogen, Carlsbad, CA) and purified by Nickel resin, and used to quantify Kv2.1 signal. For peptide competition experiments, affinity-purified antibodies were preincubated overnight at 4°C in 1 mL of 5% BSA in Tris-buffered saline containing 10 mM Tris, pH 7.5, 150 mM NaCl with 0.1% Tween-20 with synthetic antigenic peptides at $10 \mu\text{g}$ per μg of antibody. The strips of protein membranes then were treated with a final concentration of 1 : 500 dilution of antibody before use.

Mass spectrometry and protein identification

Kv2.1 channel complex were isolated from rat forebrain extracts by immunoprecipitating with the affinity purified Kv2.1 antibodies (7088 and 2078) and monoclonal Kv2.1 antibody. The proteins in the complex were then identified by mass spectrometry combined with sequence database search [37]. Protein samples were separated by SDS/PAGE and stained by colloidal Coomassie Blue. In-gel digestion was

performed on prominent protein bands identified in colloidal Coomassie Blue stained SDS-polyacrylamide gels using sequencing grade modified trypsin (Promega, Madison, WI) as described previously [38]. Extracted peptides were cocrystallized in 2,5-dihydroxybenzoic acid ($10 \text{ mg}\cdot\text{mL}^{-1}$ in 50% acetonitrile/0.3% trifluoroacetic acid) and analyzed by MALDI-TOF on a Voyager DE STR (Applied Biosystems, Foster City, CA) using the VOYAGER INSTRUMENT CONTROL PANEL (v5.1) and DATA EXPLORER (v4.0). Data was acquired in reflector mode, and masses were externally calibrated using a standard peptide mixture to better than 50 p.p.m. error. Proteins were identified by searching the acquired monoisotopic masses against the NCBI nonredundant or SwissProt databases using the MS-Fit search engine of ProteinProspector (<http://www.prospector.ucsf.edu/>) or PeptIdent (<http://www.us.expasy.org/tools/peptident.html>). Mass spectrometry and analyses were performed at the John Hopkins University Mass Spectrometry Facility.

Acknowledgements

We thank Dr Jason Pang for electrophysiological analyses. We also wish to thank members of the Li laboratory for valuable comments on this manuscript. The work is supported by grants from the National Institutes of Health (to M.L.), an Established Investigator Award (M.L.), and predoctoral training awards (J.-J. C.) from the American Heart Association and National Institute of Health.

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