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Subunit Combinations Defined for K^+ Channel Kv1 Subtypes in Synaptic Membranes from Bovine Brain†

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ABSTRACT: Voltage-dependent Shaker-related (Kv1) K^+ channels are composed of transmembrane α subunits and peripheral Kv β proteins that exist as octomers with $(\alpha)_4(\beta)_4$ stoichiometry. Although several α (designated Kv1.X) and three Kv β subunits are known to be expressed in brain, their oligomeric combinations in neurons have yet to be deciphered. Herein, the subunits comprising a number of neuronal K^+ channels from bovine brain cortex were deduced by immunoprecipitation and Western blotting, using antibodies specific for Kv1.X and Kv β subtypes. Only a subset of the theoretically possible oligomers was detected, showing that the synthesis and/or assembly of these multisubunit K^+ proteins is controlled to yield a limited variety of K^+ channels. Except for a small population of Kv1.4 containing K^+ channels, all the recognizable species contained Kv1.2 and β2 subunits. Furthermore, several subpopulations were identified including a fully defined complex of Kv1.2/1.3/1.4/1.6 and Kv β2, plus oligomers containing three or two assigned α subunits. Kv1.2 was also shown to occur in the absence of these other subunits as a putative homo-oligomer. Thus, for the first time, the complete subunit combination of an authentic K^+ channel has been elucidated; also, the strategy employed to establish this can now be applied to closely related members of other K^+ channel families.

In neurons, K^+ channels form the largest group of voltage-sensitive cation channels, where they perform many tasks including regulation of spike frequency and modulation of neurotransmitter release. α-Dendrotoxin (α-DTX) and related toxins, selective inhibitors of A-type K^+ currents (Stansfeld *et al*., 1987), have allowed the identification and isolation of a subset of these channels from mammalian brain (Rehm & Lazdunski, 1988; Parcej & Dolly, 1989). The oligomeric size of the K^+ channels was analyzed by sucrose gradient centrifugation and gel filtration. In the case of both the detergent crude extract of synaptic membranes (Black *et al*., 1988) and the resultant K^+ channels purified by affinity chromatography (Rehm & Lazdunski, 1988; Parcej *et al*., 1992), a similar M_r value (~400 kDa) was observed. Accordingly, the latter authors reported that hetero-oligomeric species, comprised of four α and four β subunits, accounted for this experimentally determined size. Although K^+ channels can be clustered by associating with proteins from the PSD-95 family when expressed heterologously in COS cells (Kim *et al*., 1996), no evidence could be obtained for the existence of K^+ channel aggregates in detergent extracts of brain membranes (see above) or GH_3 cell (Takimoto *et al*., 1996) preparation. The α subunits ($M_r \approx$ 56−65 kDa excluding post-translational modifications) are variants of the Shaker (Kv1 subfamily) proteins (Scott *et al*., 1990, 1994a; Pongs, 1992) and form the transmembrane, voltage-sensitive K^+ translocating pathway [reviewed by Dolly and Parcej (1996), Pongs (1992), and Jan and Jan (1992)]. In contrast, the smaller ($M_r \approx$ 39 kDa) β subunits, first identified in α-DTX sensitive K^+ channels purified from rat (Rehm & Lazdunski, 1988) and bovine (Parcej & Dolly, 1989) brain, appear to be peripherally associated auxiliary proteins (Scott *et al*., 1994b). To date, the Kv1 subfamily has seven characterized members, designated Kv1.1−1.7 (Gutman & Chandy, 1993). When expressed heterologously,

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each is individually capable of forming a homotetrameric K^+ channel with distinct biophysical and pharmacological properties [Stuhmer *et al*., 1989; reviewed in Dolly and Parcej (1996)], with Kv1.2 being most sensitive to α-DTX, consistent with its predominance in the toxin-affinity purified brain K^+ channels (Scott *et al*., 1990, 1994a). Furthermore, three β subunit isoforms have so far been identified (Kvβ1a, Kvβ1b, and Kvβ2; Scott *et al*., 1994b; Rettig *et al*., 1994; Morales *et al*., 1995; Majumder *et al*., 1995; McCormack *et al*., 1995; Rhodes *et al*., 1996), and each has been shown to modulate the properties of some of the Kv1.X channels. The ability of Kv1.X α subunits to assemble into hetero-oligomers when coexpressed *in vitro* (Isacoff *et al*., 1990; Ruppersberg *et al*., 1990), and the identification of such structures in brain (Wang *et al*., 1993; Sheng *et al*., 1993; Scott *et al*., 1994a), reaffirms the complex structures of the multimeric K^+ channels. A wide diversity of K^+ currents have been recorded in mammalian neurones, some of which are sensitive to α-DTX or its homologues [reviewed by Rudy *et al*. (1988) and Dolly and Parcej (1996)]. However, the molecular counterparts of each of these K^+ conductances are unknown because, as yet, no authentic K^+ channel oligomers have yet been fully defined, precluding their reconstruction and biophysical characterization of the K^+ currents *in vitro*.

In order to attack this daunting and important problem, α subunit-specific antibodies have been used previously to show that a purified preparation of α-DTX sensitive K^+ channels contains several oligomeric species and all of these possess a number of different α subunits (Scott *et al*., 1994a). A major advance on these studies is presented herein, where a rationally designed protocol was devised in which the various multimeric species of K^+ channels were immunoprecipitated by requisite α subunit-specific antibodies and the pelleted material analyzed by Western blotting with α and β subunit-selective IgGs. Subsequent rounds of immunoprecipitation/Western blotting were then performed on the unprecipitated material. By this approach, the subunit combinations present in a number of channel subtypes were identified, including the first fully defined oligomer.

EXPERIMENTAL PROCEDURES

Synthesis of Kv1.X and Kv β Peptides and Their Coupling to Carrier Proteins. Peptides CNSHMPYGYAAQARARERERLAHSR (Kv1.4 α subunit residues 13−37; Stuhmer *et al*., 1989), CGMNHSAFPQTPFKTGN (Kv1.3 α subunit residues 486−501, excluding the N-terminal cysteine; Stuhmer *et al*., 1989), CSILGNKPYSKKDYRS (residues 353−367, common to all β subunits cloned to date except for the N-terminal cysteine and adjacent residue; Scott *et al*., 1994b; Majumder *et al*., 1995; Morales *et al*., 1995; McCormack *et al*., 1995), and SPARLSLRQTGSPGMIYSTRC (Kv β2 N-terminus, residues 9−28 except C-terminal cysteine; Scott *et al*., 1994b) were synthesized by using standard Fmoc solid phase methods (Applied Biosystems Inc.) and coupled to keyhole limpet haemocyanin (KLH; α subunit peptides) or bovine serum albumin (BSA; Kv β subunit peptides) via their N- or C-terminal cysteine, as described previously (Foran *et al*., 1995).

Preparation of Fusion Proteins. The following Kv1.X-fusion proteins were expressed in *Escherichia coli* and purified by preparative SDS−PAGE, in accordance with published details (Scott *et al*., 1994a; Veh *et al*., 1995): Kv1.1-pGEX and -pHE fusion proteins containing amino acid residues 354−495 of Kv1.1 linked to the N-terminus of glutathione S-transferase and λN-protein, respectively, and Kv1.3-, 1.4-, 1.6-pUR, and 1.6-pGEX fusion proteins, containing amino acid residues 409−525, 578−655, and 438−530 of the respective Kv1.X subunits linked to the N-terminus of β-galactosidase (pUR).

Generation, Purification, and Biotinylation of Kv1.X- and Kv β-Specific Antibodies. Immunization of New Zealand White rabbits with Kv1.1- or Kv1.6-pGEX fusion proteins or the various α and β subunit peptide conjugates was carried out as described (Harlow & Lane, 1988). Peptides, coupled via an extra N- or C-terminal cysteine to Thiol-Sepharose (Pharmacia; according to the manufacturer's procedure) or iodoacetic acid-agarose (Hermanson *et al*., 1992), were used to purify antipeptide antibodies from serum (Harlow & Lane, 1988). Anti-Kv1.1 and -1.6 antisera were similarly purified using Kv1.1-pHE or Kv1.6-pUR fusion proteins, respectively, except these were immobilized on cyanogen bromide-activated Sepharose (Pharmacia). Titer of each antiserum and purified antibody preparation was determined by enzyme-linked immunosorbant assay (ELISA) using the respective peptide or the other forms of the fusion proteins (Kv1.1-pHE and 1.6-pUR), as detailed previously (Scott *et al*., 1994a). Monoclonal antibody 5 (mAb5), raised against purified K^+ channels and shown to be specific for Kv1.2 (Muniz *et al*., 1992), was purified using protein G-agarose. To facilitate Western blotting of immunoprecipitated material (see later), purified (anti Kv1.X and Kv β) antibodies were biotinylated exactly as described by Harlow and Lane (1988).

Immunoprecipitation and Western Blotting. Synaptic plasma membranes from bovine brain cortex were prepared and solubilized at 10 mg of membrane protein/mL in extraction buffer [0.0625 M imidazole-HCl, pH 7.5, 0.25 M KCl, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, 50 μg/mL bacitracin, and 50 μg/mL soybean trypsin inhibitor] containing 4% (w/v) Thesit, as previously described (Parcej & Dolly, 1989; Parcej *et al*., 1992). For immunoprecipitation, aliquots of the solubilized membrane (1−3 mL) were diluted 4-fold with extraction buffer and incubated overnight at 4 °C with 20 μg/mL of each α subunit-specific antibody. Immune complexes were then precipitated by incubation with 50−70 μL of protein A-agarose (rabbit polyclonal antibodies) or of anti-mouse IgG-agarose (mAb5) for 5−8 h at 4 °C. After centrifugation for 2 min at 10000*g*, the supernatants were removed for further rounds of immunoprecipitation and the pellets were washed four times by centrifugation/resuspension with extraction buffer/1% (w/v) Thesit. Material bound to the protein A- or anti-mouse IgG-agarose was recovered by boiling in 100 μL of SDS−PAGE sample buffer [50 mM Tris-HCl, pH 6.8, 5% (w/v) 2-mercaptoethanol, 20% (w/v) glycerol, 1.5% (w/v) SDS] for 5 min. Toxin I-affinity chromatography was used, as described previously (Parcej & Dolly, 1989; Parcej *et al*., 1992), to purify K^+ channels from a detergent solubilized extract of bovine cerebral cortex membranes. Synaptic membranes, purified channels (approximately 50 μg of protein and 1 pmol of K^+ channel/lane, respectively), fusion proteins (0.1−0.3 μg/lane), and immunoprecipitated samples were subjected to SDS−PAGE on 10% gels and transferred to poly(vinylidene difluoride) (PVDF) membrane, as detailed

previously (Scott *et al*., 1994a). After blocking with 5% (w/v) dried milk, 1% (w/v) BSA, and 0.5% (w/v) Tween 20 in 50 mM Tris-HCl pH 8.0/150 mM NaCl, the PVDF filters were probed overnight at 4 °C with 1−3 μg/mL of mouse or rabbit purified anti-Kv1.X, or anti-Kv β subunit IgGs that had been biotinylated. Immunoreactivity of the resultant filters was detected using streptavidin-biotinylated horseradish peroxidase, in combination with the ECL system (Amersham) or using alkaline phosphatase conjugated goat anti-rabbit IgG (Muniz *et al*., 1992).

Cell Lines Used for Screening the Antibodies. Human embryonic kidney cell lines HEK 293 permanently transfected with Kv1.1, 1.2, or 1.4 (a kind gift from Professor Olaf Pongs) were grown in DMEM containing 10% fetal calf serum and 0.8 mg/mL geneticin G-418 (Gibco BRL). After reaching confluence, cells were scraped off the culture flasks and collected by centrifugation at 2500*g* for 5 min. Cell pellets were solubilized, and detergent extracts were subjected to immunoprecipitation and Western blotting, using the conditions specified above for synaptic membranes.

Determination of Antibody Specificity. Newly prepared anti-Kv1.3 and -1.6 antibodies were checked for cross-reactivity in Western blots (see above) using purified C-terminal fusion proteins, as described previously (Scott *et al*., 1994a). For determination of the specificity in blots and immunoprecipitation reactions of anti-Kv1.4 N-terminal peptide antibodies, as well as anti-Kv1.1 and -1.2 IgG, the requisite individual Kv1 subunits expressed in transfected HEK 293 cells were used. Briefly, cells harvested from two 175 cm^2 culture flasks (~10 mg of membrane protein) were solubilized with 1 mL of extraction buffer, diluted as described above, and equal portions immunoprecipitated with 20 μg of anti-Kv1.1, -1.2, or -1.4 antibodies, followed by dissolution in 100 μL SDS−PAGE sample buffer. Aliquots (15 μL) were then subjected to electrophoresis, blotted and probed with biotinylated IgG (see above). Specificity of anti-Kv β antibodies was tested using ELISA, as described previously (Scott *et al*., 1994a).

RESULTS

Specificity and Reactivity of Anti-Kv1.X and Kv β Antibodies. The specificity of anti-Kv1 antibodies was tested in Western blots using human embryonic kidney cells (HEK 293) permanently transfected with individual Kv1 subunits in the case of anti-Kv1.1, -1.2, -1.4, or employing C-terminal fusion proteins for anti-Kv1.3 and -1.6. Each of these antibodies recognized on blots its own requisite antigen and only anti-Kv1.1 IgG showed detectable cross-reactivity, weakly labeling Kv1.4 subunit (data not shown). In addition, anti-Kv1.1, -1.2, and -1.4 IgG were tested for their ability to recognize their respective antigens in the *native* state, using detergent extracts of the transfected HEK 293 cells (detailed in Experimental Procedures). In this way, no cross-immunoreactivity was observed except with Kv1.1 antibodies which also precipitated a small amount of native channels from the HEK 293 cells transfected with Kv1.4. This small level of cross-reactivity was taken into account in subsequent experiments. Since no source of expressed Kv1.3 subunit was available, and because the anti-Kv1.6 antibodies used herein were unable to immunoprecipitate K^+ channels, antibodies against these subunits could not be analyzed in this way. In brain membranes and toxin-affinity purified

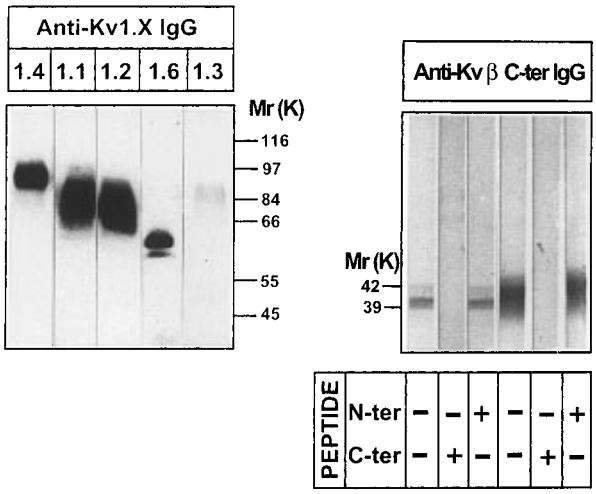

FIGURE 1: Identification of Kv1.X and Kv β subunits present in synaptic membranes and affinity-purified K^+ channels from bovine brain cortex. Crude membranes (50 μg of protein, first three lanes of each panel) and purified K^+ channels (1 pmol of ^{125}I-α-DTX-binding activity, last two and three lanes of the left and right panels, respectively) were subjected to SDS−PAGE and, after transfer to PVDF membrane, probed with 1−3 μg/mL of biotinylated anti-Kv1.X or β antibodies, as indicated. Anti-Kv β C-terminal antibodies (2 μg/mL) were added alone or in the presence of 5 μg/mL Kv β C-terminal or N-terminal peptide, as shown. Labeled blots were visualized using biotinylated-streptavidin horseradish peroxidase in conjunction with the ECL system (first four lanes in left panel) or secondary antibody conjugated to alkaline phosphatase. Numbers indicate the position of molecular weight markers (left panel) or the deduced M_r values.

K^+ channels, the antibodies used here reacted (Figure 1) similarly to those previously reported (Scott *et al*., 1994a; Muniz *et al*., 1992). In the case of anti-Kv1.1, minimal reactivity was sometimes seen with a protein whose molecular mass corresponds to that seen with the anti-Kv1.4 antibodies (e.g., Figure 1 and see later), as observed with the transfected cells.

The two anti-Kv β antibodies prepared were assayed by ELISA for reactivity against each immunogen and for any cross-reaction with other Kv β subunit peptides (see Experimental Procedures) and found to react only with the requisite peptide (data not shown). Thus, these antibodies would be expected to be Kv β subunit subtype-specific in Western blots. Two or sometimes three bands with apparent M_r of 36, 39, and 42 kDa were detected by the purified anti-Kv β C-terminal antibody in Western blots of crude membranes and toxin-affinity purified K^+ channels (Figure 1). Labeling of all bands was blocked when the C-terminal peptide was included in the incubation, while the N-terminal peptide proved ineffective, indicating the interaction to be specific. The relative abundance of the two major labeled bands ($M_r \approx 39$ and 41 kDa) was similar to that observed in protein stained SDS−PAGE gels of purified α-DTX sensitive K^+ channels (Parcej & Dolly, 1989; Parcej *et al*., 1992). These bands were also specifically labeled, as judged by blockade only with N-terminal peptide (not shown), by anti-Kvβ N-terminal antibody, in agreement with recent observations using similar polyclonal as well as monoclonal antibodies (Rhodes *et al*., 1996). Previously, protein sequencing of peptide fragments from the smaller, more prominent polypeptide (Scott *et al*., 1994b) yielded sequences which were subsequently found to be in the cloned Kv β2 subunit only (Scott *et al*., 1994b) or common to all three

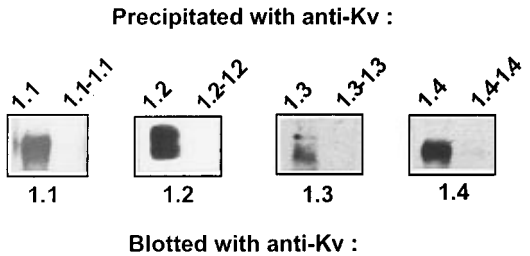

FIGURE 2: Complete precipitation of K^+ channel α subunits from a solubilized extract of synaptic membranes by anti-Kv1.X antibodies. Aliquots (1 mL) of a detergent extract of bovine cortex membranes were precipitated with 20 μg of each anti-Kv1.X antibodies, and the resultant supernatants were then reprecipitated with the same concentration of antibody, as indicated. The pellets were subjected to SDS−PAGE and probed with the respective antibodies to determine the amount of subunit precipitated.

Kv β subtypes. Thus, the 39 kDa band would appear to be Kvβ2, while the larger band(s) are probably post-translationally modified variants, or a new form of Kv β protein. Unfortunately, like anti-Kv1.6 antibodies, neither of the anti-Kv β antibodies was able to immunoprecipitate K^+ channel complexes from solution (data not shown), even though Kv β subunits were present in anti-Kv1.X immunopellets (see later); possibly, the epitopes chosen for antibody production are sterically hindered in the intact K^+ channel complexes. As a prelude to multistage immunoprecipitation aimed at deducing the oligomeric composition of K^+ channels in brain detergent extracts, the anti-Kv1.X antibodies were tested for their ability to quantitatively remove their requisite antigens from solution. When these were used at a concentration of 20 μg/mL each was able to sediment all of the appropriate Kv1.X protein, no further α subunit being precipitated by a second treatment (Figure 2).

Design of a Multistage Precipitation Protocol: Identification of Pairs of Subunits Present in K^+ Channel Populations. Using ^{125}I-labeled α-DTX as a marker, we have shown previously (Scott *et al*., 1994a) that Kv1.2 subunit is present in all α-DTX sensitive K^+ channels while Kv1.1 resides in about half; Kv1.6 and 1.4 subunits occur in a minor population only (∼20 and ∼10%, respectively). The latter values represent the proportion of K^+ channel oligomers that contain each of these subunits. Thus, as a first step toward designing a multistage precipitation protocol, pairs of subunit-specific antibodies (1.3 and 1.4, 1.4 and 1.1, 1.1 and 1.2) were chosen according to this relative abundance of the multimeric complexes (plus the data presented below for Kv1.3) and used sequentially in precipitation experiments, followed by Western blotting. Anti-Kv1.3 antibody removed from the crude extract all of the Kv1.3-containing channels (no further subunit being pelleted in a second round of this immunoprecipitation, see Figure 2) together with some Kv1.4 subunit (Figure 3A); this result suggests that both subunit types are present together in K^+ channel oligomeric complex-(es). However, the Kv1.4 subunit precipitated by anti-Kv1.3 is only a fraction of the total present because Kv1.4-containing channels remained in the supernatant from the precipitation with anti-Kv1.3 IgG, and these could be precipitated with anti-Kv1.4 antibody (Figure 3A). On the other hand, when crude extract that had been pretreated with anti-Kv1.4 (which removed all of Kv1.4; Figure 2) was incubated with anti-Kv1.3 IgG, no additional Kv1.3 subunit was precipitated. Thus, it appears that all Kv1.3 in bovine cortex membranes is complexed with Kv1.4, and the Kv1.3-

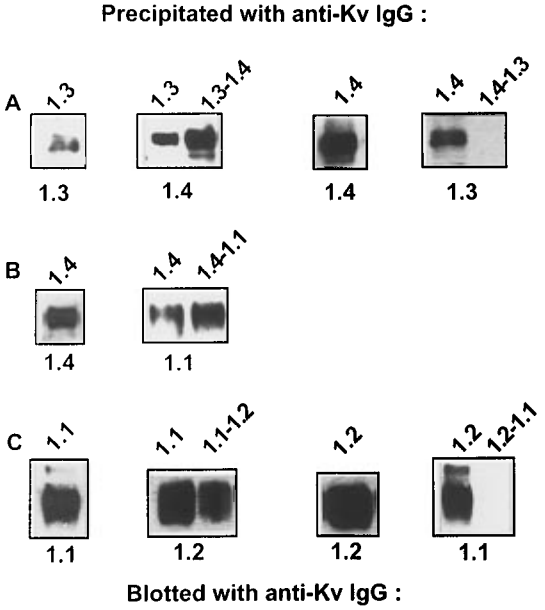

FIGURE 3: Precipitation of K^+ channels using pairs of anti-Kv1.X antibodies. A detergent extract of bovine synaptic membranes (1 mL) was precipitated initially with 20 μg/mL of the antibodies indicated above the panels and the resultant supernatants were then treated with the same concentration of the second member of each pair shown. The pellets were subjected to SDS−PAGE and Western blotted with the biotinylated antibodies specified beneath the panels.

containing channels are a subpopulation of those possessing Kv1.4. Based on the low relative abundance of K^+ channels in which Kv1.4 resides (<10% of α-DTX sensitive K^+ channels; Scott *et al*., 1992a), 1.3 must be a constituent of only a minor fraction of the oligomers. Kv1.1 subunits were coprecipitated with Kv1.4 from the crude extract by anti-Kv1.4 antibody (Figure 3B), showing that the Kv1.4 and 1.1 subunits must be complexed together. The total amount of Kv1.1 in the membrane extract exceeds that precipitated by anti-Kv1.4 because addition of anti-Kv1.1 antibody to the supernatant from the precipitation with anti-Kv1.4 led to complete precipitation of the remaining Kv1.1-containing channels (Figure 3B). On the basis of this, it can be concluded that there are subpopulations of the Kv1.1-containing channels that possess or lack Kv1.4. Treatment of the initial extract with anti-Kv1.1 completely removed from solution Kv1.1 and a trace of 1.4 subunit. However, since anti-Kv1.1 cross-reacts to some extent with Kv1.4 in both the native and denatured states (see later), it cannot be excluded that channels containing Kv1.4 but lacking Kv1.1 were precipitated by anti-Kv1.1. Anti-Kv1.1 precipitated a fraction of Kv1.2 subunits (Figure 3C), showing that both of these subunits can reside together in channel complexes. The substantial fraction of Kv1.2, which was not removed by anti-Kv1.1, could subsequently be precipitated using anti-Kv1.2 (Figure 3C). On the other hand, when anti-Kv1.2 was used as the first antibody with the initial extract, it precipitated completely both Kv1.2 (Figure 2) and Kv1.1 subunits (Figure 3C). Thus, all Kv1.1 is associated with Kv1.2, but there are Kv1.2-containing complexes which lack Kv1.1. This means that the Kv1.1-containing complexes are a subpopulation of those possessing Kv1.2. Since Kv1.2 is shown here to be in the great majority of channel oligomers (detailed later), anti-Kv1.2 reactivities observed with precipitates obtained using antibodies specific for Kv1.3, 1.4,

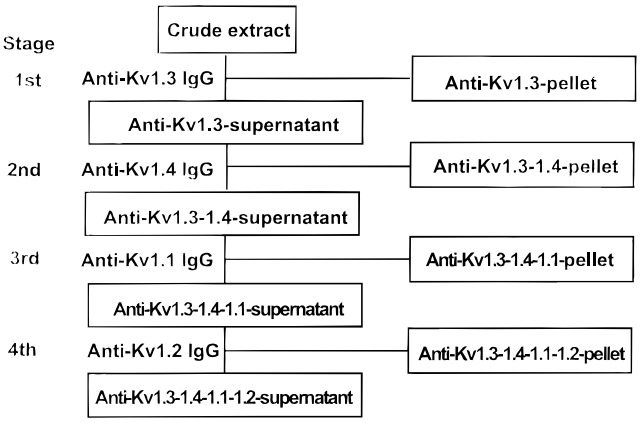

FIGURE 4: Scheme of multistage immunoprecipitation of the K^+ channel oligomers in a detergent crude extract of synaptic membranes.

1.1, and 1.2 in sequence can be normalized against the aggregate level of this staining for all of the pellets in this set. Such analysis revealed the rank order for the relative sizes of oligomeric populations, containing each of the subunits tested, to be Kv1.3 < 1.4 < 1.1 < 1.2; thus, for multistage precipitation, their respective antibodies were used in that order. Note that this ranking should not be confused with the absolute levels of each subunit present in the extract; such values could not be determined with accuracy due to the different efficiencies of the various antibodies used.

Elucidation of Subunit Combinations Comprising Kv1.X-Containing Channel Complexes. The multistage immunoprecipitation is shown schematically in Figure 4. At each stage, the precipitated complexes were tested for the presence of other subunits by Western blotting and where necessary, supplementary experiments were performed in order to address any ambiguities which arose. Anti-Kv1.3 antibody used at the first stage precipitated, along with Kv1.3, 1.4, 1.2, and 1.6 but not 1.1 (Figure 5A); note that a doublet was seen with anti-Kv1.6, possibly due to proteolysis or different states of post-translational modification (also seen in Figure 1). Having established previously (Figure 3A) that all Kv1.3 is complexed with Kv1.4, additional experiments were performed to determine whether the Kv1.3/Kv1.4 combination can ever occur without Kv1.2. When anti-Kv1.2 antibodies were used to immunoprecipitate K^+ channels from crude membrane detergent extract, they precipitated, along with Kv1.2, all of the Kv1.3 subunit because subsequent treatment of the resultant supernatant with anti-Kv1.3 antibody did not precipitate any additional Kv1.3 (Figure 5B). Thus, all Kv1.3 must be complexed with Kv1.2 subunit. Since Kv1.3 has been shown to always be complexed with Kv1.4, it is clear that Kv1.3 has to be associated simultaneously with Kv1.4 and 1.2 in a single type of channel complex. The presence of Kv1.6 subunits in the anti-Kv1.3 pellet means that at least a fraction of Kv1.3/1.4/1.2 oligomers contain Kv1.6 as the fourth subunit in the tetramer (Figure 6). However, the ineffectiveness of anti-Kv1.6 antibodies in the immunoprecipitation experiments means that we can neither exclude nor confirm the existence of a complex composed of Kv1.3/1.4/1.2 plus a second copy of Kv1.3, 1.4, 1.2, or another Kv1 subunit not tested. In the second stage, the supernatant obtained after treatment with anti-Kv1.3 antibody was precipitated using anti-Kv1.4 IgG. Immunoblotting of this precipitate revealed the presence of

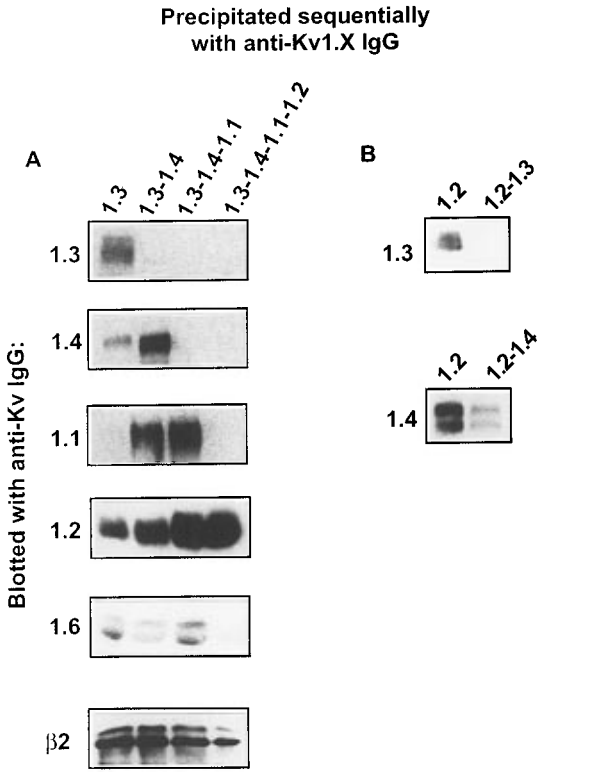

FIGURE 5: Sequential precipitation of K^+ channels solubilized from synaptic membranes. (A) An aliquot (3 mL) of detergent solubilized bovine synaptic membranes was precipitated with different anti-Kv1.X antibodies (20 μg/mL) in the sequence illustrated. The resultant pellets from each step were subjected to SDS−PAGE and blotted with the biotinylated anti-Kv1.X and -Kv β2 N-terminal IgGs shown, as described in Figure 1. (B) Aliquots (1 mL) of extract were precipitated with anti-Kv1.2; the resultant supernatant was subsequently precipitated with anti-Kv1.3 or 1.4. Western blotting of the pellets was performed with biotinylated anti-Kv1.X specified on the left of the blots.

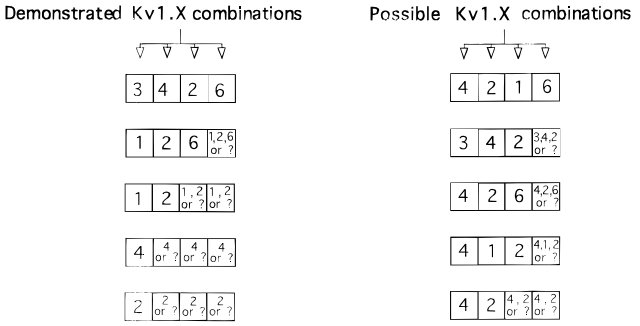

FIGURE 6: Kv1.X subunit combinations established for K^+ channel subtypes occurring in synaptic membranes. K^+ channels of the Kv1 subfamily have been shown to be octomeric proteins containing four α and four β subunits (Parcej *et al*., 1992). For clarity, only the α subunits are shown in this illustration which indicates the members found to be present in the oligomers separated by sequential precipitation with antibodies specific for each of four α subunits. Direct evidence was obtained for the combinations shown on the left panel whereas those depicted in the second column are tentative. In cases where one or more of the four α subunit partners were not identifiable, the only candidates are an additional copy (or copies) of the subunit(s) shown to be present or others (represented by ?) for which antibodies were unavailable.

Kv1.4, 1.1, 1.2, and 1.6 α subunits (Figure 5A). All four types of subunits could be bound in a single complex and/or could form multiple Kv1.4-containing oligomers. Additional experiments were, therefore, performed to distinguish these possibilities. From crude extract, anti-Kv1.2 antibody

precipitated all Kv1.2 along with the majority of Kv1.4 (Figure 5B) subunit. When the supernatant, completely devoid of the Kv1.2-containing complexes, was treated with anti-Kv1.4, the remaining Kv1.4 subunit was found in the pellet (Figure 5B). The latter weakly labeled pellet did not yield any reactivity when blotted with antibodies against Kv1.1, 1.2, 1.3, or 1.6 (data not shown); this highlights the possible existence in brain cortex membranes of a sparse, Kv1.4-containing complex lacking the other subunits monitored. In view of these findings, and because Kv1.4 resides in only 10% of Kv1.2-containing oligomers, such K^+ channels must comprise a minor population (Scott *et al*., 1994a). Further elucidation of the combinations of the subunits detected at this second precipitation stage (Figure 5A) could have been achieved by precipitation with anti-Kv1.1 IgG to deplete Kv1.1-containing complexes without removing the subpopulation of Kv1.4-containing channels, which are devoid of Kv1.1; unfortunately, this was precluded by the cross-reactivity of this antibody with Kv1.4. Thus, it is not possible to confirm or exclude the presence of complexes containing Kv1.4/1.2 or 1.4/1.2/1.6 that lack Kv1.1. However, since we have shown that Kv1.1 is always complexed with Kv1.2 (Figure 3C), the existence of channels possessing Kv1.1/1.4 or 1.1/1.4/1.6 but devoid of Kv1.2 is unlikely. Again, due to the aforementioned inability of anti-Kv1.6 antibodies to immunoprecipitate, combinations with or without Kv1.6 could not be distinguished. Thus, possible subunit combinations deduced from these sets of experiments include Kv1.4/1.1/1.2/1.6, 1.4/1.2/1.6, 1.4/1.1/1.2, and 1.4/1.2, along with oligomers containing Kv1.4 but not the others (Figure 6). The third stage of immunoprecipitation was performed with anti-Kv1.1 antibody. Since all the Kv1.4-containing complexes were removed at the previous stage, the cross-reactivity of anti-Kv1.1 with Kv1.4 could not introduce the ambiguity in the interpretation of the results seen above. The precipitate obtained at this stage contained three types of subunits: Kv1.1, 1.2, and 1.6 (Figure 5A). It is already known that all Kv1.1 is precipitated with anti-Kv1.2 antibody (Figure 3C), demonstrating the presence of Kv1.1/1.2/1.6-containing oligomers. However, as mentioned previously, Kv1.6 has been identified as being in only about 20% of α-DTX sensitive (Kv1.2 containing) K^+ channels (Scott *et al*., 1994a), while Kv1.1 is present in 50−60%. Thus, K^+ channels containing Kv1.1 and 1.2 but not Kv1.6 are almost certainly present. The fourth and last stage of the multistage precipitation was performed with anti-Kv1.2 antibody; only Kv1.2 was found in the pellet (Figure 5A). Judging by the intensity of Kv1.2 band on the immunoblots, this is a major species of K^+ channel in cortex membranes, perhaps as much as 30% of all oligomers. Auxiliary Kv β subunits were found in at least a fraction of each of the K^+ channel subtypes unveiled (Figure 5A), as expected from the previous demonstration (Parcej & Dolly, 1989) that the majority of α-DTX-sensitive K^+ channels in bovine brain are $(\alpha)_4(\beta)_4$ octomers. An identical labeling pattern was observed in Western blots of the Kv1.X subtypes when either a Kv β2-specific (anti-N-terminal peptide) IgG (Figure 5A) or broad specificity (C-terminal directed) anti-Kv β (not shown) antibodies were used. Thus, Kv β2 is the major auxiliary subunit in K^+ channels from bovine synaptic membranes, as noted recently for rat brain by others (Rhodes *et al*., 1996).

DISCUSSION

The expression in mammalian tissues of a large number of genes of the Shaker-related K^+ channel subfamily (Jan & Jan, 1992; Pongs, 1992) and their proven ability to assemble *in vitro* to form hetero-oligomeric structures (Ruppersberg *et al*., 1990; Isacoff *et al*., 1990) provide two mechanisms, along with the association of auxiliary β subunits (Parcej & Dolly, 1989; Parcej *et al*., 1992; Rettig *et al*., 1994) for potentially generating an enormous number of K^+ channel subtypes. Recently, a stretch of amino acids within the N-terminus of Shaker α subunits has been shown (Li *et al*., 1992; Shen *et al*., 1993) to be chiefly responsible for the subunit associations. Since all mammalian Shaker-related α subunits possess this sequence, the potential for hetero-oligomeric association within the subfamily would appear to be unlimited. Similarly, a sequence found in the N-terminus of all the Kv1 α-subunits, but absent from other subfamilies, has been shown to be necessary for the association with Kv β1 subunit (Sewing *et al*., 1996). This suggests that the Kv β subunti can associate with all members of the Kv1 subfamily. Latterly, several Kv1.X subunits have been found to be colocalized (Wang *et al*., 1993; Sheng *et al*., 1993; Veh *et al*., 1995) in specific brain regions. For example, Kv1.1 and 1.2 have overlapping distribution in the juxtaparanodal regions of mouse brain stem (Wang *et al*., 1993) while Kv1.2 and 1.4 are found together in several layers of rat cerebellum (Sheng *et al*., 1993; Veh *et al*., 1995). The physical association of several different Kv1.X subunits in single oligomeric complexes has also been demonstrated by immunoprecipitation using subunit-specific antibodies (Sheng *et al*., 1993; Wang *et al*., 1993; Scott *et al*., 1994a). However, to date, it had not been possible to fully define the subunit combination of any authentic K^+ channels. The physicochemical similarity of the various oligomers precluded the use of conventional separation methods for their isolation. Thus, the fractionation of channel populations was approached immunologically herein by using subunit-specific antibodies for the selective depletion of separate channel populations from the mixture in the membrane extract. In this study, four different subunit-specific antibodies directed against Kv1.1, 1.2, 1.3, and 1.4 α subunits were employed for precipitation and these plus Kv1.6 and β subunit antibodies utilized for subunit identification in blots. By using these antibodies for immunoprecipitation in various combinations and in a sequential manner, followed by Western blotting of the precipitates, several oligomeric K^+ channels were defined. It was clearly demonstrated that only a small fraction of the possible oligomeric subtypes is actually present in bovine brain synaptic membranes. Strikingly, all but one (Kv1.4 putative homo-oligomer) of the oligomers identified contained at least one copy of Kv1.2, the α subunit found to be the prominent constituent of K^+ channels purified from bovine brain using toxin I (Scott *et al*., 1990). Also, neither Kv1.1 (the second most abundant subunit), Kv1.3 (the least abundant), nor Kv1.6 occur as homo-oligomers, and Kv1.1 and 1.3 are not partners in any of the K^+ channel complexes identified. However, Kv1.4 appeared to be present with and without (albeit to a lesser extent) other Kv1.X subunits; Kv1.2, too, may also form homo-oligomeric K^+ channels. One fully defined tetramer (Kv 1.3/1.4/1.2/1.6) was identified (Figure 5). Moreover, several putative complexes containing three or two identified

subunits, plus another unidentified variant (or a copy of one of the tested subunits) were shown to definitely exist; the results also suggest a number of possible subunit combinations whose existence could not be established conclusively.

Previously, it has been shown that the great majority of α-DTX sensitive K^+ channels contain Kvβ subunits (Parcej *et al*., 1992); however, a small but significant fraction lack these auxiliary proteins. Herein, a portion of all of the oligomeric K^+ channel subtypes identified were demonstrated to contain Kv β2, but it has not been possible to determine whether any particular oligomeric subtypes represent the α-only containing channels. The importance of the prevelance of β2 has yet to be fully realized because its effects on the biophysical properties of K^+ channels have not been fully elucidated. Some subtle modification of K^+ channel kinetics (McCormack *et al*., 1995) and a possible role in folding/trafficking (Shi *et al*., 1996) have been reported. Together, these data suggest that a proportion of Shaker-related K^+ channels found in bovine synaptic membranes would inactivate relatively slowly, since some (Figure 6) do not contain the rapidly inactivating Kv1.4 subunit; an antibody was not available for Kv β1, which is known to accelerate K^+ channel inactivation when associated with Kv1.1 or 1.4 (Rettig *et al*., 1994). Nevertheless, it may be envisaged that each of the identified complexes has an unique kinetic and pharmacological profile. On the basis of the demonstrated sensitivity and kinetics of expressed homo-oligomeric channels, the combinations shown here may be postulated to range from slowly- or noninactivating channels with very low (Kv1.2 homo-oligomer) or high (Kv1.1/1.2) TEA sensitivity to fast-inactivating, TEA-insensitive variants (Kv1.4 homo-oligomer), through moderately fast-inactivating channels with medium (e.g., Kv1.3/1.4/1.2/1.6, a fully defined channel) or moderately high (e.g., Kv1.2/1.1/1.4/1.6) TEA sensitivity. In addition, the presence of the carbohydrate-deficient Kv1.6 subunit (Scott *et al*., 1990) may influence the activation threshold of the channels, since desialidation of expressed Kv1.1 shifts the voltage dependence of activation to more positive values (Thornhill *et al*., 1996). The precise physiological functions of each of these channel types are not yet clear, but they may play subtly distinct roles in different cell types or within the same cell. Indeed, it is possible that the cellular location of a particular subtype may be determined by the subunits present. Recently, electron microscopy in conjunction with high-resolution immunocytochemistry was used to pinpoint Kv1.2 containing K^+ channels at intriguing locations on nerve terminal and axonal membranes in rat cerebellum (McNamara *et al*., 1996). By genetically reconstructing the identified complexes and examining their biophysical attributes *in vitro*, it is hoped that clues to their functions may be gleaned. In addition, extension of the successful approaches used in this paper to include other Kv1 subtypes and, further, to elucidate the subunit stoichiometry and position within the K^+ channel complexes, should allow the properties of a whole range of authentic K^+ channels to be determined.

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