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Antibodies Specific for Distinct Kv Subunits Unveil a Heterooligomeric Basis for Subtypes of α-Dendrotoxin-Sensitive K^+ Channels in Bovine Brain†

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ABSTRACT: The authentic subunit compositions of neuronal K^+ channels purified from bovine brain were analyzed using a monoclonal antibody (mAb 5), reactive exclusively with the Kv1.2 subunit of the latter and polyclonal antibodies specific for fusion proteins containing C-terminal regions of four mammalian Kv proteins. Western blotting of the K^+ channels isolated from several brain regions, employing the selective blocker α-dendrotoxin (α-DTX), revealed the presence in each of four different Kvs. Variable amounts of Kv1.1 and 1.4 subunits were observed in the K^+ channels purified from cerebellum, corpus striatum, hippocampus, cerebral cortex, and brain stem; on the other hand, contents of Kv1.6 and 1.2 subunits appeared uniform throughout. Each Kv-specific antibody precipitated a different proportion (anti-Kv1.2 > 1.1 >> 1.6 > 1.4) of the channels detectable with radioiodinated α-DTX in every brain region, consistent with a widespread distribution of these oligomeric subtypes. Such heterooligomeric combinations were further documented by the lack of additivity upon their precipitation with a mixture of antibodies to Kv1.1 and Kv1.2; moreover, cross-blotting of the multimers precipitated by mAb 5 showed that they contain all four Kv proteins. Collectively, these findings demonstrate that subtypes of α-DTX-susceptible K^+ channels are prevalent throughout mammalian brain which are composed of different Kv proteins assembled in complexes, shown previously to also contain auxiliary β-subunits [Parcej, D. N., Scott, V. E. S., & Dolly, J. O. (1992) *Biochemistry 31*, 11084–11088].

Although multiple K^+ channels in the nervous system have been characterized according to their biophysical and pharmacological properties (Halliwell, 1990), little is known about the basis of their diversity because, until recently, biochemical investigation has been hampered by the lack of high-affinity probes. However, a snake venom polypeptide, α-DTX,[1] has been instrumental in identifying, characterizing, and purifying a family of fast-activating, slow-inactivating, voltage-dependent K^+ channel proteins from mammalian brain [reviewed in Dolly et al. (1994)]. The existence of subtypes of α-DTX acceptors was first postulated from localization studies on rat brain sections using ^{125}I-labeled α-DTX (^{125}I-α-DTX), together with β-bungarotoxin (β-BuTX) and δ-dendrotoxin (δ-DTX). While ^{125}I-α-DTX bound to acceptors in both gray

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[1] Abbreviations: TBS, Tris-buffered saline; α-DTX, α-dendrotoxin; ^{125}I-α-DTX, ^{125}I-labeled α-DTX; β-BuTX, β-bungarotoxin; δ-DTX, δ-dendrotoxin; mAb, monoclonal antibody; ^{125}I-mAb 5, ^{125}I-labeled mAb 5; BSA, bovine serum albumin; IgG, immunoglobulin class G; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; M_r, relative molecular weight; PVDF, poly(vinylidene difluoride); ELISA, enzyme-linked immunosorbant assay; K_D, dissociation constant; GST, glutathione-S-transferase; PCR, polymerase chain reaction.

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and white matter areas, β-BuTX and δ-DTX unveiled subtypes that reside at certain synaptic and gray matter regions, respectively (Pelchen-Matthews & Dolly, 1988, 1989; Awan & Dolly, 1991). In addition, binding studies with α-DTX, β-BuTX, and δ-DTX on rat (Rehm et al., 1988; Breeze & Dolly, 1989) and guinea pig (Muniz et al., 1990) synaptosomes revealed a complex pattern of apparent mutual noncompetitive inhibition (Bidard et al., 1987), suggesting that each of the toxins bind to distinct but interacting sites.

α-DTX-susceptible K^+ channels purified from rat (Rehm & Lazdunski, 1988) or bovine (Parcej & Dolly, 1989; Parcej et al., 1992) brain showed on SDS–PAGE a broad, diffusely staining α band ($M_r \approx 78K$) and a more distinct β subunit of $M_r = 39K$; these are associated in 4α4β octomers (Parcej et al., 1992). Interestingly, although removal of N-linked carbohydrate decreased the size of the α subunit (giving a core protein with M_r of 65K), it did not reduce its diffuse appearance (Scott et al., 1990) suggesting microheterogeneity. This inference was supported by N-terminal sequence analysis of the α subunit(s) (Scott et al., 1990) which yielded a major signal homologous to Kv 1.2, a K^+ channel α gene cloned from rat (McKinnon, 1989; Stuhmer et al.,1989) and bovine (Reid et al., 1992) brain, together with a minor sequence resembling another of the rat α genes, Kv1.1 (Baumann et al., 1988). Collectively, these findings provided convincing evidence that α-DTX acceptors are a family of K^+ channel proteins and validated the use of toxins for studying K^+ channel diversity and structure.

In mammals, K^+ channel diversity appears to be due to the presence of a large number of separate genes coding for proteins equivalent to the α subunits of purified α-DTX acceptors [reviewed by Pongs (1992), Jan and Jan (1992), Salkoff et al. (1992), and Christie et al. (1989)]. Despite numerous reports of expressed Kv genes forming homooligomeric K^+ channels, recent coexpression studies have shown that some are capable of yielding functional heterooligomeric K^+ channels in *Xenopus* oocytes (Christie et al., 1990; Ruppersberg et al., 1990). This could provide a means of generating K^+ channel subtypes (Jan & Jan, 1990), in addition to the inclusion of the β subunit found associated with the bulk of native K^+ channel complexes (Parcej et al., 1992). However, the relationship of these artificially created α subunit containing K^+ channels to the apparently more complex composition of the oligomers found in neuronal membranes has not been established.

In the present investigation, mono- and polyclonal Kv-specific antibodies were used to demonstrate directly the existence in brain of subtypes of voltage-dependent K^+ channels and to establish their heterooligomeric nature, thereby highlighting one possible mechanism employed *in vivo* to create the amazing spectrum of biophysical properties of neuronal K^+ currents.

EXPERIMENTAL PROCEDURES

Materials. Poly(vinylidene difluoride) (PVDF) membrane was from Millipore and $Na^{125}I$ from ICN. Peptide-*N*-glycosidase F and Thesit were both obtained from Boehringer Mannheim. α-DTX and toxin I were fractionated as detailed elsewhere (Dolly, 1992). Enzymobeads and goat anti-rabbit or -mouse alkaline phosphatase conjugates were from Bio-Rad. Anti-mouse (or anti-rabbit) immunoglobulin G (IgG) (whole molecule)-agarose and all other reagents were bought from Sigma Chemical Co.

Production of K^+ Channel Kv-Specific Polyclonal Antisera. cDNA fragments coding for the C-terminal sequences of Kv1.1, 1.2, 1.4, and 1.6 were cloned into bacterial expression vectors pHE6 (Milman, 1987; Kv1.1), pGEX (Smith & Johnson, 1988; Kv1.1, 1.2, 1.4, and 1.6), and pUR 288 (Ruether & Mueller-Hill, 1983; Kv1.2, 1.4, and 1.6) as follows. A Kv1.1 cDNA *Hinf*1 fragment [nucleotide (nt) 1058–1538; Baumann et al., 1988], including amino acids 354–495, was cloned into a pHE6 *Sma*I vector after filling in 5′ overhanging ends with Klenow polymerase I to produce Kv1.1-pHE. The Kv1.1 sequence was cut out of this clone with *Bam*HI and cloned into a *Bam*HI cut pGEX-3X vector after all *Bam*HI ends were blunt ended with Klenow polymerase I, to preserve the reading frame of glutathione-S-transferase (GST)-fusion protein (Kv1.1-pGEX). Kv1.2, 1.4, and 1.6 cDNA sequences coding for the C-terminus of the proteins were amplified in the polymerase chain reactions (PCR) using the following oligonucleotide primers:

Kv1.2: 5′ ATT GGA TCC GGA GAG GAG GAG GCC CAG TAC 3′

5′ ATT GAA TTC TCA GAC ATC AGT TAA CAT TTT 3′

Kv1.4: 5′ ATT GGA TCC CAG ACC CAG CTG ACC CAA AAC 3′

5′ ATT GAA TTC TCA CAC ATC AGT CTC CAC AGC 3′

Kv1.6: 5′ TTA GGA TCC ATT GTG GGC TCA CTG TGT GCC 3′

5′ TTA GAA TTC CAT CCA TCA AAC CTC GGT GAG 3′

The denaturing temperature was 94 °C; annealing temperatures were 54–58 °C, 30 cycles at 72 °C. For cloning purposes, a *Bam*HI recognition site was included in the sense and an *Eco*RI recognition site in the antisense primers. After amplification, DNA fragments [Kv1.6, nt 1742–2029, aa 438–530, EMBL Acc. No. X17621, Grupe et al. (1990); Kv1.4, nt 1732–2068, aa 578–655; Kv1.2, nt 1826–2059, aa 422–498, EMBL Acc. No. X16003, Stuhmer et al. (1989)] were digested with *Bam*HI and *Eco*RI and then ligated with *Bam*HI/*Eco*RI cut pGEX-2T DNA yielding Kv1.6-, 1.4-, and 1.2-pGEX expression clones, respectively. Kv-C-terminal sequences were similarly cloned into the expression vector pUR 288 (Kv1.2, 1.4-, and 1.6-pUR) using DNA fragments amplified by PCR with primer pairs as above except that the *Eco*RI recognition sites were substituted by an *Xba*I site. Clones were checked by sequencing (Sanger et al., 1977).

Expression of the fusion proteins was either induced by heat shock in the case of Kv1.1-pHE giving a fusion protein with 33 aa λ N-protein at the N-terminus or with 0.5 mM isopropyl β-D-thiogalactopyranoside for both pGEX (yielding a fusion protein with 27.5K GST at the N-terminus) and pUR constructs (N-terminal fusion with 116 K of β-galactosidase). The cells were lysed 4 h after induction by sonication and centrifuged at 10000*g*. The pellets were resuspended in SDS sample buffer and subjected to preparative SDS–PAGE; bands containing the induced fusion proteins were excised and electroeluted using a Biotrap apparatus (Schleicher & Schull, Germany). Purity of the recovered proteins was assayed by analytical SDS–PAGE. Protein concentrations were determined colorimetrically (Bradford, 1976). The Kv1.1-pHE, Kv1.2-, 1.4-, and 1.6-pGEX fusion proteins were used to immunize 4–5-month-old New Zealand rabbits following standard protocols (Harlow & Lane, 1988). Specificities of the antisera were determined by enzyme-linked immunosorbent-assay (ELISA; Harlow & Lane, 1988) using either the pUR fusion proteins (Kv1.2, 1.4, 1.6) or the pGEX construct (Kv1.1) as antigens. Antisera which showed cross-reactivities to fusion proteins containing the C-terminus of other Kv subunits were purified either by sequential adsorption

onto nitrocellulose strips containing the cross-reacting antigens and/or affinity-purified using nitrocellulose-immobilized fusion proteins, respectively, with elution by 0.2 M glycine-HCl, pH 2.5, 150 mM NaCl, and 0.1% BSA. Sera were stored in aliquots at −20 °C until used. An additional construct [pUR(I)] encompassing a C-terminal portion of Kv1.3 (Stuhmer et al., 1989; residues 443–525) was prepared similarly for use in purifying and characterizing antibodies to the other fusion proteins.

Purification and Characterization of a Monoclonal Antibody Raised against α-DTX Acceptors. Monoclonal antibody (mAb) 5, which recognizes the α subunit of neuronal α-DTX-sensitive K^+ channel (Muniz et al., 1992), was purified by hydroxyapatite and anion-exchange chromatography. Ascitic fluid (2 mL) was diluted 10-fold in 10 mM sodium phosphate, pH 6.8, applied to an hydroxyapatite column equilibrated in the above buffer and eluted with a linear salt gradient (75–250 mM sodium phosphate, pH 6.8). Further purification was achieved by chromatography on a DEAE Sepharose column equilibrated in 10 mM Tris-HCl, pH 8.5; elution was with a linear salt gradient (50–300 mM NaCl in the latter). The column fractions were analyzed on SDS–PAGE and pooled according to immunoreactivity on a dot-binding assay (Muniz et al., 1992). The concentration of antibodies was determined spectrophotometrically (assuming an absorbance at 280 nm of 1.33 for a 1 mg/mL solution). The reactivity of the mAb 5 with the Kv fusion proteins (50 ng) was determined using the dot-binding assay.

Radiolabeling of mAb 5: Measurement of Its Binding to K^+ Channels and Antagonism by Antibodies to Kv1.2. Purified mAb 5 was radioiodinated using Enzymobeads as detailed elsewhere (Parcej et. al., 1992) using 10 μg of the pure antibody and 1m Ci of $Na^{125}I$. Binding of ^{125}I-labeled mAb 5 IgG (^{125}I-mAb 5) to α-DTX-sensitive K^+ channels, purified from bovine brain, was determined using the dot-binding assay; 0.1 pmol of α-DTX binding activity/dot was used to coat the nitrocellulose membrane. The latter was blocked with 3% (w/v) BSA prepared in Tris-buffered saline (TBS), cut into equal squares, and transferred to a 96-well ELISA plate. Aliquots of polyclonal antibodies (100 μL of several dilutions) or pure mAb 5 were incubated with coated membrane sections for 1 h prior to adding the labeled ^{125}I-mAb 5 (~60 000cpm/assay) and leaving overnight at 4 °C. The dots were washed three times with TBS containing 0.05% (w/v) Tween 20, and their radioactive content was quantified by γ-counting.

Western Blot Analysis. Synaptic plasma membranes were prepared from bovine cortex by the method of Bennett et al. (1986) while P_2 fractions were isolated from the cerebellum, hippocampus, corpus striatum, and brain stem. Aliquots (0.2–2.0 pmol) of α-DTX acceptors, purified from each of these regions according to the protocol of Parcej et al. (1992), were subjected to SDS–PAGE under reducing conditions, followed by electrotransfer and immunoblotting, as previously described (Muniz et al., 1992).

Immunoprecipitation Assay. Membranes prepared as above from bovine cortex, cerebellum, hippocampus, and corpus striatum were solubilized as detailed elsewhere (Parcej & Dolly, 1989). Aliquots (0.1pmol/assay) of the detergent extract or purified acceptor preparation were preincubated with 3 nM ^{125}I-α-DTX, in the presence or absence of an excess of unlabeled α-DTX, and immunoprecipitated as in Muniz et al. (1992).

Detection of α Subunit Isoforms in K^+ Channels Precipitated by mAb 5. Purified K^+ channels (10–20 pmol) were precipitated with mAb5 ascitic fluid (2 μL) and subjected to SDS–PAGE and Western blotting with Kv-specific antibodies, as outlined earlier.

RESULTS

Anti-Kv Antibodies and mAb 5 Are Specific for Different α Subunits of Mammalian Neuronal K^+ Channels. The gene encoding Kv1.2 (Stuhmer et al., 1989), known to be virtually identical to a constituent of α-DTX-sensitive K^+ channels from bovine cortex (Reid et al., 1992), and those for other members of this *Shaker* related subfamily are widely expressed in rat brain (Beckh & Pongs, 1990; Grupe et al., 1990). The cloned genes for Kv1.1, 1.2, 1.4, and 1.6 have been used herein to construct fusion proteins for the production of antibodies against the various subunits and, also, to examine the specificities of available mAbs to authentic K^+ channels. For this purpose, the most variant C-terminal regions of each were employed, as detailed under Experimental Procedures. To characterize the resultant antisera, each Kv sequence was additionally expressed in a second *Escherichia coli* expression system to obtain fusion proteins with an alternative fusion part, thereby allowing detection of reactivity towards specific Kv-sequences.

Specificities of the antisera were tested in ELISA (Figure 1A) as well as in Western blots (Figure 1B). In the case of anti-Kv1.6 and anti-Kv1.2 sera, ELISA signals were only detected with the corresponding antigen. Therefore, these sera were used without further purification. Anti-Kv1.1 and anti-Kv1.4 antisera showed some reactivity with other Kv antigens in ELISA. Cross-reactivity of anti-Kv1.4 serum could be removed by affinity purification with nitrocellulose-bound Kv1.4-pUR fusion protein. Crude antiserum to Kv1.1 cross-reacted with Kv1.4 and Kv1.2 fusion proteins. Removal of these cross-reactivities was achieved by sequential adsorption of anti-Kv1.1 serum onto nitrocellulose coated with Kv1.4 or Kv1.2 fusion proteins, followed by affinity purification employing nitrocellulose bound Kv1.1 fusion protein (see Experimental Procedures). Finally, the specificity of each anti-Kv antibody preparation was investigated by Western blotting of all the fusion proteins. This revealed that the antisera to Kv1.6 and Kv1.2 recognized only their respective antigens, as was also shown to be the case for purified anti-Kv1.1 and 1.4 sera (Figure 1B).

Each of the mouse mAbs (1–7), raised against α-DTX-sensitive K^+ channels purified from synaptic plasma membranes of bovine cerebral cortex, reacted in Western blots with a band containing Kv1.2 and, possibly, other subunits (Muniz et al., 1992). In an attempt to define their specificities, reactivities with the various fusion proteins were evaluated. In a dot blot assay, mAbs 1, 2, 3, 4, 6, and 7 failed to recognize epitopes in any of these antigens; in contrast, mAb 5 reacted strongly with the Kv1.2 adduct but not significantly with any of the others (Figure 2A). Although some reactivity was recorded with the fusion proteins Kv1.3 and 1.6, this was due to high nonspecific binding; an unrelated monoclonal antibody gave similar signals (data not shown). This Kv1.2 specificity of mAb 5 was confirmed using native oligomeric α-DTX acceptors and the IgG purified from the ascitic fluid. After radioiodination, saturable and avid interaction of ^{125}I-mAb 5 with the K^+ channels was detected; an apparent $K_D \approx 0.5$ nM was determined from analysis of its displacement by unlabeled mAb 5 IgG (Figure 2B inset), using the LIGAND program (Munson & Rodbard, 1980). Most importantly, the anti-Kv1.2 antibody antagonized the binding of ^{125}I-mAb 5 to the neuronal K^+ channels whereas antibodies to Kv1.1 or 1.6

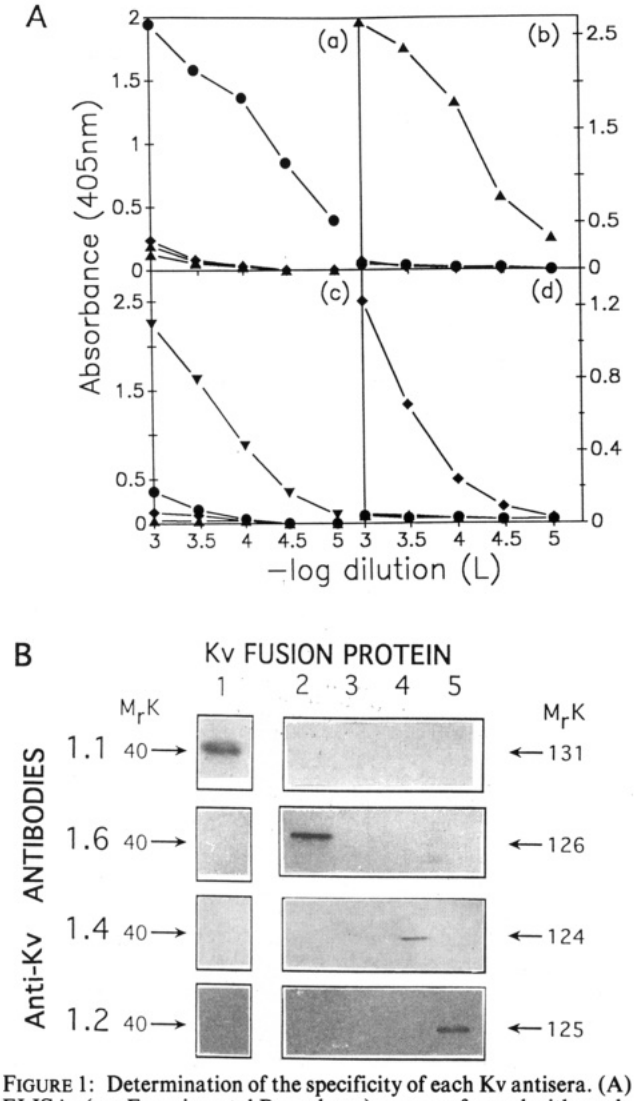

FIGURE 1: Determination of the specificity of each Kv antisera. (A) ELISAs (see Experimental Procedures) were performed with crude anti-Kv1.6 and anti-Kv1.2 or with affinity-purified anti-Kv1.1 and anti-Kv1.4 antisera as indicated. The following fusion proteins were used as antigens for testing the anti-Kv1.1 antibodies (top left): Kv1.1-pGEX (●), Kv1.6-pUR (▲), Kv1.4-pUR (▼), Kv1.2-pUR (◆). For testing anti-Kv1.6 (top right), anti-Kv1.4 (bottom left), and anti-Kv1.2 (bottom right) antibodies: Kv1.1-pHE (●), Kv.1.6-pUR (▲), Kv1.4-pUR (▼), and Kv1.2-pUR (◆). Absorbance was read at 405 nm and plotted against log dilution (L) of antiserum. (B) Aliquots (10 ng) of all of the fusion proteins (Kv1.1, -1.3, -1.4, -1.6 and -1.2) were subjected to SDS–PAGE on four 8% (w/v) gels, followed by electrophoretic transfer onto PVDF membrane. Each blot was then incubated with one of the Kv specific antisera (anti-Kv1.1 or 1.2 and anti-Kv1.6 or 1.4 were diluted 1:1000 and 1:250, respectively), prior to development with goat anti-rabbit second antibody conjugated to alkaline phosphatase. Strips from each of the blots encompassing the M_r range of the fusion proteins are shown: lane 1, Kv1.1 (M_r ~40K); lane 2, Kvl.6; lane 3, Kvl.3; lane 4, Kv1.4, and lane 5, Kv1.2 (all M_r ~ 125 K). Arrows indicate the mobilities of each of the conjugates.

(Figure 2B) and all the other mAbs proved ineffective, at the highest concentrations available. It was, thus, established conclusively that this very reactive monospecific IgG is selective for an epitope in the C-terminal portion of the Kv1.2 subunit, a finding that allowed mAb 5 to be employed in elucidating the α subunit combinations in K^+ channels.

Immunoidentification of Multiple Kvs in α-DTX Acceptors Purified from Various Brain Regions. By Western blotting of purified 4α4β octomeric (Parcej et al., 1992) K^+ channels from bovine cerebral cortex with each of the antibodies characterized above, four different subunits could be distinguished (Figure 3A). Anti-Kv1.1 IgG intensely labeled a

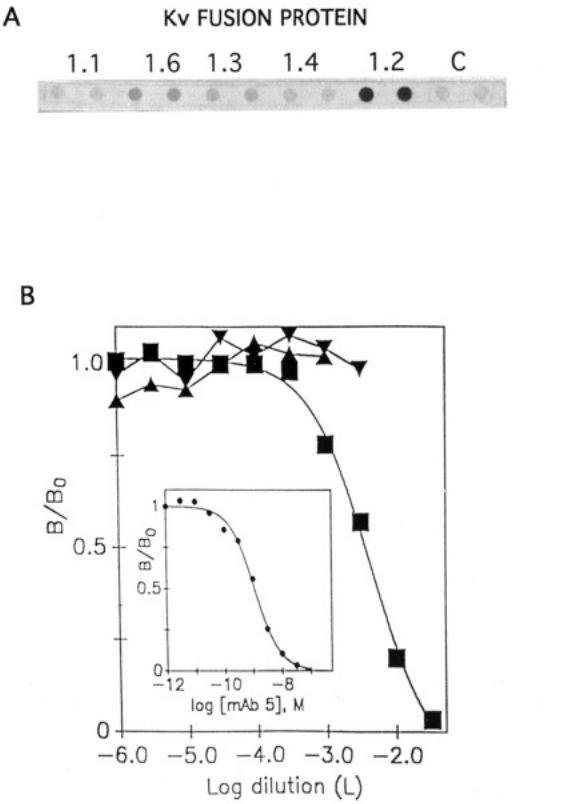

FIGURE 2: Selectivity of mAb5 for Kv1.2 fusion protein and displacement by anti-Kv1.2 antisera. (A) Duplicate samples (50 ng) of each fusion protein were applied to a strip of nitrocellulose membrane in a dot blot apparatus. After blocking with TBS containing 3% (w/v) BSA, the strip was incubated with mAb5 diluted 1:1000 in the above solution overnight at 4 °C. Antibody binding was detected using goat anti-mouse IgG conjugated to alkaline phosphatase. (B) Various concentrations of polyclonal Kv1.1 (▲), 1.6 (▼), and 1.2 (■) antisera were incubated with a fixed amount of ^{125}I-mAb 5 (~60 000cpm/assay) in a total volume of 100 μL of TBS containing 3% (w/v) BSA and an aliquot (0.1 pmol of α-DTX binding/dot) of α-DTX acceptor purified from bovine brain and immobilized on nitrocellulose. The dots were washed with ice-cold TBS, and the binding was quantified by γ-counting. (Inset) As detailed above, except that varying concentrations of purified mAb 5 were incubated with ^{125}I-mAb 5 and the immobilized purified acceptors, in order to determine the binding affinity of the radiolabeled mAb.

band with an apparent M_r = 86K; the latter value was decreased after N-deglycosylation (data not shown) consistent with the presence of consensus sites for glycosylation on this subunit cloned from rat (Baumann et al., 1988; Christie et al., 1989). The subunit recognized by Kv1.6 antiserum gave a lower M_r = 58K (Figure 3A), a value corresponding exactly to that predicted from its gene cloned from rat cortex (Grupe et al., 1990). On the other hand, anti-Kv1.4 purified IgG stained a band of apparent M_r = 95K, again in accord with the relatively large size predicted from the nucleotide sequence (Stuhmer et al., 1989). Its presence in the pure α-DTX acceptors is noteworthy since expression of the rat Kv1.4 gene in *Xenopus* oocytes produces a K^+ current that is resistant to α-DTX (Stuhmer et al., 1989). Hence, this observation indicates that heterooligomeric complexes composed of α-DTX binding α subunits, together with those such as Kv1.4 that lacks this activity, may exist; otherwise their copurification by toxin I affinity chromatography could not be explained. Lastly, both anti-Kv1.2 and mAb 5 strongly labeled a broad band with $M_r \approx$ 78K; notably, its diffuseness resembles the protein staining pattern of this subunit (Parcej & Dolly, 1989; Scott et al., 1990).

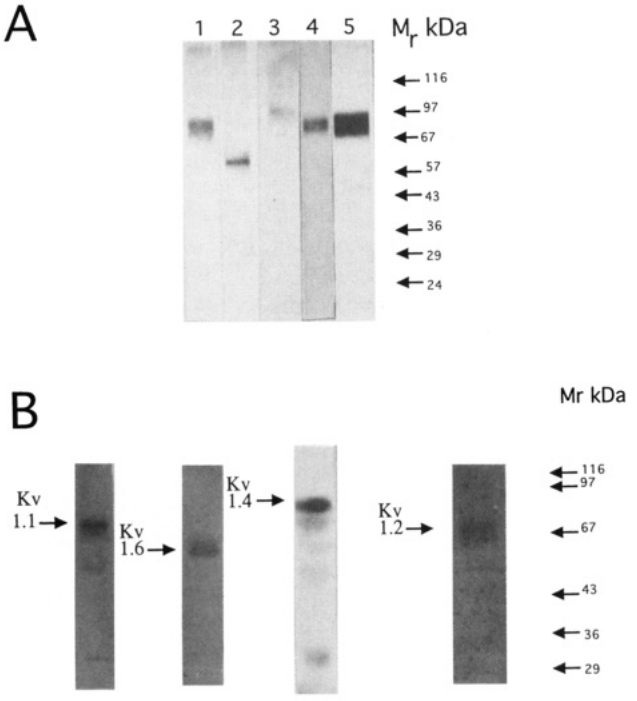

FIGURE 3: Analysis of the α subunit composition of purified α-DTX acceptors using subtype-specific antibodies. (A) Purified acceptors from bovine cortex were subjected to 10% (w/v) SDS–PAGE followed by electrophoretic transfer onto PVDF membranes, prior to probing with type-specific antisera and development with goat anti-rabbit (or anti-mouse) second antibody conjugated to alkaline phosphatase. Lane 1, anti-Kv1.1 (diluted 1:1000); lane 2, anti-Kv1.6 (diluted 1:250); lane 3, anti-Kv1.4 (diluted 1:250); lane 4, anti-Kv1.2 (diluted 1:1000), and lane 5, mAb 5 (diluted 1:1000) (0.2 and 2.0 pmol of ^{125}I-α-DTX binding activity was loaded onto lane 1 or 4 or 5 and lane 2 or 3, respectively). Molecular weight markers were as in Parcej et al. (1992). (B) An aliquot (10–20 pmol) of purified K^+ channels was selectively precipitated using mAb 5 (2 μL of ascitic fluid) prior to SDS–PAGE analysis of the sedimented immune complexes on a 10% (w/v) gel, with subsequent electrophoretic transfer onto PVDF. The blotted membrane was then probed with the four subtype-specific antibodies as detailed above. Arrows indicate the relative mobilities of each of the α subunits [tracks to be blotted with anti-Kv1.1 or 1.2 and Kv1.6 or 1.4 contained $\simeq$0.2 pmol and 2 pmol of α-DTX binding respectively; Kv1.4 was detected using the enhanced chemiluminesence method (Amersham International)].

It should be emphasized that α-DTX acceptors purified from bovine cerebellum, corpus striatum, hippocampus, and brain stem (Figure 4A) contain Kv1.1, 1.2, and 1.4 (except in cerebellum where levels were too low to be readily detected), as noted for cerebral cortex; the low levels of Kv1.6 and 1.4 (see later) necessitated the use of increased amounts of acceptor to facilitate their detection. A striking feature was the difference in the relative amounts of Kv1.1 and 1.4 subunits in the various brain regions; for example, Kv1.1 reactivity was highest in brain stem and lowest in cerebellum, whereas Kv1.4 was most prevalent in hippocampus. In contrast, a near equal distribution of Kv1.6 and 1.2 subunits was seen. Notwithstanding such quantitative variations, four Kvs are clearly demonstrable in brain K^+ channels, a finding consistent with studies that have shown mRNAs for these same subunits to be distributed throughout rat brain (Beckh & Pongs, 1990).

Dissimilar Levels of Precipitation of ^{125}I-α-DTX labeled K^+ Channels by Kv-Specific Antibodies Reaffirm the Occurrence of Oligomeric Subtypes. To establish what fraction of α-DTX-sensitive K^+ channels contain each of the Kvs, immunoprecipitations were performed initially with the proteins solubilized from bovine cerebral cortex. All four polyclonal antibodies precipitated the ^{125}I-α-DTX/K^+ channel

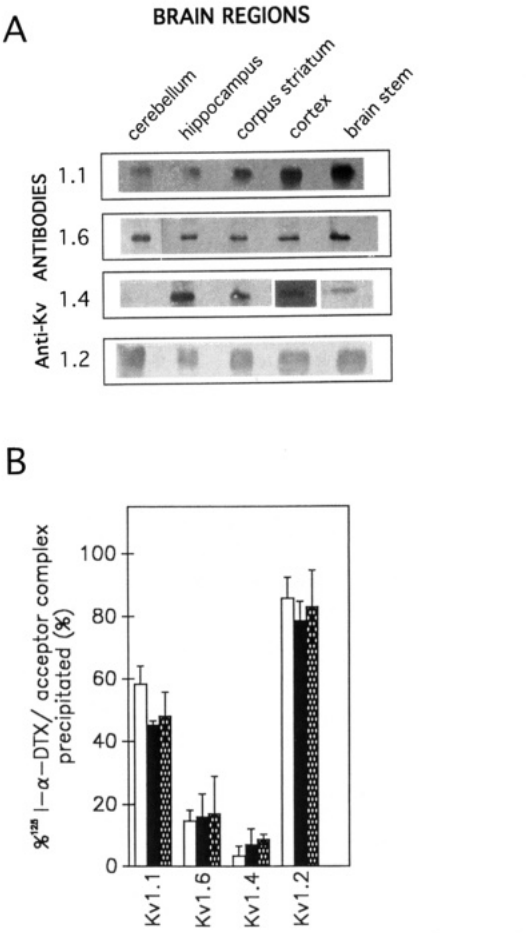

FIGURE 4: Regional occurrence of Kvs in bovine brain. (A) α-DTX acceptors were purified from different brain regions according to the method of Parcej *et al.* (1992). An aliquot (0.2 or 2.0 pmol) was subjected to SDS–PAGE on a 10% (w/v) gel followed by Western blot analysis. Aliquots (0.2 pmol) were probed with anti-Kv1.1 and 1.2 antibodies (diluted 1:1000); 2.0 pmol of α-DTX binding was probed with anti-Kv1.6 and 4 antibodies (diluted 1:250). Each blot was developed with goat anti-rabbit second antibody conjugated to alkaline phosphatase as detailed under Experimental Procedures. (B) Solubilized membranes from bovine brain cerebellum, hippocampus, and corpus striatum were incubated with 3 nM ^{125}I-α-DTX for 30 min and reacted with the optimal amount of first antibody (5 μL for anti- Kv1.1, 1.4, or 1.6 and 2 μL for Kv1.2), and the immune complexes were sedimented with goat anti-rabbit second antibody coupled to agarose beads. The quantities of radioactivity obtained in each pellet were plotted relative to the amounts of saturable ^{125}I-α-DTX binding sites measured in aliquots of the crude extract. Different regions are indicated by the open (cerebellum), filled (hippocampus), and hatched (corpus striatum) bars. Error bars are +SD and represent triplicate values from each of three independent experiments.

complexes but to varying extents (Figure 5A), highlighting the presence of multimeric subtypes. Under optimal conditions (Figure 5A), the anti-Kv1.2 antibody sedimented the majority (83 ± 6%, n = 9) of the complexes while lesser amounts (Figure 5B) were removed by those specific for Kv1.1 (47 ± 4%; n = 16), 1.6 (16 ± 5%; n = 7) and 1.4 (8 ± 2%; n = 7). These results suggest that Kv1.2 is the predominant subunit present, a deduction previously drawn from N-terminal sequence analysis of the purified α subunit and its immunoprecipitation from detergent extract of bovine cortex membranes by mAb 5 (Muniz et al., 1992). Thus, it can be concluded that almost all α-DTX-sensitive K^+ channels contain Kv1.2 while about half possess Kv1.1; 1.6 and 1.4-containing oligomers are less abundant. The results observed for the detergent extract of synaptic plasma membranes were very similar to those obtained for the purified proteins (Figure 5B), suggesting that no

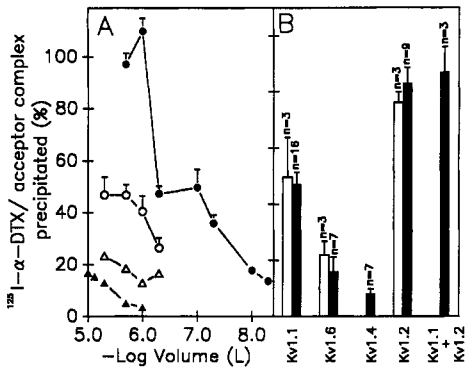

FIGURE 5: Differential immunoprecipitation of ^{125}I-α DTX/acceptor complexes by anti-Kv antibodies. (A) Detergent extracted bovine synaptic plasma membranes (0.1 pmol of α-DTX binding/assay) were incubated with 3 nM ^{125}I-α-DTX and varying concentrations of each subtype specific antibody in the presence and absence of 500 nM α-DTX (to adjust for nonsaturably bound ^{125}I-α-DTX). The total amount of IgG in each tube was kept constant by inclusion of preimmune serum or purified IgG with the crude and purified antisera, respectively. The immune complexes were sedimented with goat anti-rabbit second antibody coupled to agarose beads, and the quantities of radioactivity obtained in each pellet were plotted relative to the total amounts of saturable ^{125}I-α-DTX binding sites quantified in aliquots of the crude extract. Anti-Kv1.1 (○); Kv1.6 (△); Kv1.4 (▲) and Kv1.2 (●) antibodies. (B) Solubilized membranes (filled bars) and α-DTX acceptors purified from bovine cortex (open bars) were incubated with ^{125}I-α-DTX for 30 min and reacted with the optimal amounts of first antibody (5 μL for Kv1.1, 1.6, or 1.4, 2 μL for Kv1.2 and 5 μL + 2 μL Kv1.1 + Kv1.2), and the immune complexes were sedimented with the secondary antibody as detailed in for panel A. Values are the mean (+SD) of 3–16 experiments (as indicated in the plot) performed in triplicate.

enrichment or selective denaturation of oligomeric forms occurred during the K^+ channel isolation. It is noteworthy that a similar spectrum of subtypes was observed in immunoprecipitation assays with cerebellum, hippocampus, and corpus striatum (Figure 4B), although analysis of the latter data by student *t* test showed that Kv1.4 was present in significantly fewer acceptors in cerebellum than in cortex. Confirmatory evidence was gained for the predominance of heterooligomeric species in cortex synaptic membranes by the inability of additional saturating quantities of anti-Kv1.1 IgG to precipitate the 17% of oligomers not sedimented by anti-Kv1.2 antisera alone (Figure 5B). It can be deduced from these revealing observations that the Kv1.1 subunit must be present only in association with Kv1.2; interestingly, both of these are known to yield α-DTX-sensitive K^+ current when their cDNAs are expressed (Stuhmer et al., 1989).

Different Kv Combinations Demonstrated in α-DTX-Sensitive K^+ Channels by Cross-Blotting with Kv-Specific Antibodies. To investigate the various combinations of α subunits present in oligomers of the purified acceptors, a series of cross-blotting experiments with subtype-specific antibodies was performed. After precipitation with mAb 5 which, like anti-Kv1.2 polyclonal antibodies, is known to sediment most of the α-DTX-sensitive channels (Muniz et al., 1992), the sedimented immune complexes were then subjected to Western blotting with all of the available Kv-specific antibodies. This powerful technique (Figure 3B) showed that each subunit tested (Kv1.1, 1.4, and 1.6) can occur in oligomeric complexes with Kv1.2, thereby providing conclusive evidence that the bulk of α-DTX-sensitive K^+ channels are heterooligomers.

DISCUSSION

The multiplicity of K^+ channel Kv genes in mammalian neurons together with their ability to form heterooligomeric structures in expression studies are two potential mechanisms for generating the enormous diversity of K^+ currents observed electrophysiologically. However, the existence of K^+ channels containing multiple Kvs, together with the auxiliary β subunit, and the spectrum of such macromolecular complexes in the nervous system have yet to be demonstrated. *In situ* hybridization (Sheng et al., 1992; Kues & Wunder, 1992) and RNA blotting (Beckh & Pongs, 1990), although providing invaluable information as to the possibly overlapping regions of Kv expression, are of insufficient resolution to detect their coassembly into oligomeric structures. In order to overcome this problem, antibodies specific for Kvs were used to probe voltage-dependent K^+ channels purified from several bovine brain regions by toxin affinity chromatography, as well as channels identifiable by ^{125}I-α-DTX binding in detergent extracts therefrom. Each antibody precipitated α-DTX acceptors to varying extents, indicating oligomeric subtypes. As expected, Kv1.2 was the predominant constituent present in all the brain regions examined. Antibodies specific for another α-DTX sensitive protein, Kv1.6, recognized a band of appropriate M_r on Western blots of purified K^+ channels and precipitated the ^{125}I-α-DTX binding sites from detergent extracts of membranes from the various brain regions, to similar extents. However, although anti-Kv1.1 antibodies precipitated the same proportion (~50%) of the K^+ channels from all the regions, Western blot analysis indicated a relatively lower abundance of this subunit in K^+ channels purified from cerebellum and hippocampus, compared with those from corpus striatum, cerebral cortex, and brain stem. This apparent anomaly may be explained by the presence of dissimilar oligomeric species in the different regions, those in brain stem and cortex containing, on average, more copies of Kv1.1 per oligomer than their counterparts in cerebellum and hippocampus. The identification of Kv1.4 on Western blots of K^+ channels purified from cortex, hippocampus corpus striatum, and brain stem and the ability of anti-Kv1.4 antibodies to precipitate ^{125}I-α-DTX-labeled K^+ channels in detergent extracts of membranes from these regions are of particularly significance. When expressed in *Xenopus* oocytes, cRNA encoding Kv1.4 generated a K^+ current insensitive to α-DTX (Stuhmer et al., 1989); thus, this isoform must be associated with Kv variants susceptible to this toxin. This provides direct evidence for the existence of naturally occurring, neuronal K^+ channel α subunit heterooligomers. Conclusive evidence for the presence of K^+ channels composed of combinations of α subunit variants was provided by the lack of additivity observed when both anti-Kv1.1 and anti-Kv1.2 antibodies were used to precipitate ^{125}I-α-DTX labeled K^+ channels compared with the anti-Kv1.2 antisera alone. Hence, these data show that at least half of the α-DTX-sensitive K^+ channels from bovine cortex contain more than one Kv subtype. It must be emphasized that the data presented herein on K^+ channels do not exclude the occurrence of homooligomers of certain subunits in the brain regions investigated. For example, although all α-DTX sensitive K^+ channels containing Kv1.1 must also contain the Kv1.2 variant (see above), the 36% of the latter not complexed with Kv1.1 subunit may form homooligomers. Similarly, the Kv1.4 variant, while being associated with α-DTX binding α subunits in channels complexes which are susceptible to this toxin, could form additional toxin-insensitive homooligomers. Finally, the arrangement in K^+ channel complexes of α subunits not studied here is unknown.

These characterized antibodies will allow localization in the nervous system of K^+ channels containing various Kv

subunits and, thus, afford correlation with the established distribution of acceptors for K^+ channel toxins (see the introduction). This provides a more reliable method than *in situ* hybridization, whereby the mRNA content may not necessarily reflect the channel diversity in the membrane. They should also prove invaluable in determining whether individual cells or cell types contain single or multiple hetero- or homomultimeric channels and, thereby, provide a basis for the numerous roles they can perform in different cellular locations. Finally, the ability of these antibodies to discriminate between oligomers comprising different Kvs ought to permit their immunoaffinity separation from the mixture present in the purified α-DTX acceptor preparations and, eventually, determination of the exact number and subunit composition of oligomeric species.

In conclusion, immunological data have shown that heterooligomeric complexes composed of various Kvs together with the β subunit (Parcej et al., 1992; Muniz et al., 1992) are found *in vivo* which could account for some of the wide diversity of these proteins that control cellular excitability in the mammalian nervous system.

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