Articles

Characterization of Monoclonal Antibodies against Voltage-Dependent K^+ Channels Raised Using α-Dendrotoxin Acceptors Purified from Bovine Brain†

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ABSTRACT: Seven monoclonal antibodies raised against α-dendrotoxin-sensitive K^+ channels, purified from bovine cerebral cortex, recognize these proteins in their native or denatured states, via interaction with the α- but not the β-subunit. This finding, together with a similar observation made with polyclonal antibodies, shows that the latter is a distinct protein and not a proteolytic fragment of the larger subunit. Also, coimmunoprecipitation of α- and β-subunits provides further evidence that both are tightly associated constituents of the K^+ channel complexes. At least three isoforms of the K^+ channel α-subunit are distinguishable by immunoblotting of a detergent extract of synaptic membranes with mAb 5. Likewise, multiple forms are also detectable in the purified protein with mAb 5 although deglycosylation, which does not alter reactivity with any of the mAbs, was required to achieve adequate electrophoretic resolution. These results confirm the proposal that variants of this K^+ channel group, known to exist in the nervous system, are heterooligomeric complexes of α- and β-subunits. Although different areas of rat brain contain proteins of similar sizes reactive with mAb 5, these are absent from heart, liver, pancreas, kidney, testes, and spleen, highlighting the selectivity of this antibody.

K^+ channels play important roles in excitable and nonexcitable cells (Cook & Quast, 1990). Four major groups of K^+ channels have been distinguished (voltage-activated, Ca^{2+}-dependent, ATP-sensitive, and receptor-operated), but few have been investigated at the molecular level due to the lack of suitable probes. With the successful application of α-dendrotoxin (α-DTX)[1] and its homologue toxin I, as selective ligands for certain variants of voltage-dependent, aminopyridine-sensitive K^+ channels, considerable progress has been made in their biochemical characterization [reviewed by Dolly (1991) and Meves (1991)]. High-affinity binding sites for ^{125}I-labeled α-DTX (^{125}I-α-DTX) and toxin I have been characterized in rat (Black et al., 1986; Rehm et al., 1988), chick (Black & Dolly, 1986), bovine (Parcej & Dolly, 1989), and guinea pig (Tibbs et al., 1989) brain. Heterogeneity of the α-DTX acceptors has been unveiled by β-bungarotoxin (Black & Dolly, 1986; Pelchen-Matthews & Dolly, 1989), another selective inhibitor of voltage-dependent K^+ channels (Petersen et al., 1986; Rowan & Harvey, 1989; Bräu et al., 1990).

Recently, α-DTX acceptors have been purified from bovine (Parcej & Dolly, 1989) and rat synaptic membranes (Rehm & Lazdunski, 1988); electrophoretic analysis revealed two major subunits with apparent M_r of 78 000 (α) and 39 000 (β). Microsequencing of the N-terminus of the larger subunit provided conclusive evidence for it being a K^+ channel constituent (Scott et al., 1990) because the 27 residues determined were identical to those present in BK 2, RBK 2, and RCK 5, a K^+ channel subunit cloned from rat brain by cross-hybridization with a *Drosophila Shaker* probe (McKinnon, 1989; Stühmer et al., 1989; Christie et al., 1990). Expression of RCK 5 in *Xenopus* oocytes yielded a voltage-activated K^+ current, which was highly sensitive to α-DTX (Stühmer et al., 1989). Cross-hydridization studies have revealed several other members of the RCK family in rat brain that are highly homologous in structure, but distinct in their electrophysiological properties and susceptibility to K^+ channel toxins (Stühmer et al., 1989; Swanson et al., 1990). RCK-specific mRNAs are coexpressed in several regions of the brain (Beckh & Pongs, 1990), suggesting that RCK proteins might also assemble together; further evidence for this possibility was obtained by coexpression of mRNAs for various RCKs in Hela cells and *Xenopus* oocytes, resulting in the formation of heteromultimeric channels with properties distinct from those of the individual homomultimers (Ruppersberg et al., 1990).

To help decipher the authentic structures of the several variants of this K^+ channel family known to exist in the nervous system (Dolly, 1991), probes directed at a variety of sites on these proteins are needed. Monoclonal antibodies (mAbs) have provided powerful tools for probing the molecular architecture of other cation channels (Ahlijanian et al., 1990; Elmer et al., 1990; Bahouth et al., 1991). Availability of such reagents would, therefore, be extremely useful for investigating structure/activity relationships and the channels' precise topographical arrangement in neuronal membranes, important goals that remain to be achieved. Thus, mAbs were raised, for the first time, against native α-DTX acceptors purified from bovine cerebrocortical membranes. A panel of seven mAbs was obtained that recognize exclusively this protein in a detergent extract of synaptic plasma membranes, by

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[1] Abbreviations: PBS, phosphate-buffered saline; α-DTX, α-dendrotoxin; ^{125}I-α-DTX, ^{125}I-labeled α-DTX; mAb, monoclonal antibody; TBS, 20 mM Tris, 150 mM NaCl, pH 7.5; ip, intraperitoneal injection; iv, intravenous injection; NC, nitrocellulose; BSA, bovine serum albumin; Ig, immunoglobulin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; M_r, relative molecular mass.

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interacting with the protein core of the glycosylated α-subunit. In fact, one mAb can detect isoforms of the α-subunit while coimmunoprecipitation of α- and β-subunits reaffirmed that these K^+ channels are heterooligomeric complexes.

EXPERIMENTAL PROCEDURES

Materials. The NSO myeloma cell line was obtained from the European Collection of Animal Cell Cultures, Porton Down, England, and $Na^{125}I$ from I.C.N. Poly(ethylene glycol) 4000, Myoclone plus fetal calf serum, and Dulbecco's modified Eagle's medium were from Gibco while green mamba venom was provided by J. Leakey (Kenya). Antibody isotyping kit was obtained from The Binding Site (Birmingham, England), and Thesit (equivalent to Lubrol PX) was from Boehringer Mannheim. Goat anti-mouse IgG alkaline phosphatase conjugate and Enzymobeads were purchased from Bio-Rad; anti-mouse IgG (whole molecule)–agarose and all other reagents were from Sigma Chemical Co.

Purification and Radioiodination of α-DTX-Sensitive K^+ Channels from Bovine Cerebral Cortex. The channels were solubilized and purified from synaptic plasma membranes of bovine cerebral cortex, as detailed previously (Parcej & Dolly, 1989), with improvements introduced later (Scott et al., 1990; Parcej et al., 1992). α-DTX was purified from the venom of *Dendroaspis angusticeps* and radiolabeled as described previously (Black et al., 1986); its saturable binding to Thesit-solubilized acceptors was quantified by a gel filtration/centrifugation assay (Parcej & Dolly, 1989). Radioiodination of the purified K^+ channels was accomplished using immobilized lactoperoxidase–glucose oxidase (Enzymobeads), as detailed in Parcej et al. (1992); samples were kept at 4 °C and used within 7–10 days. Unless specified differently, the purified α-DTX acceptor preparation was kept in a buffer used for purification [buffer A: 25 mM imidazole hydrochloride, pH 8.2/100 mM KCl/1 mM EDTA/0.2 mM benzamidine/0.5 mM PMSF/0.05% (w/v) Tween 80].

mAb Production. For the primary immunization, Balb/c mice received 5 pmol of active, purified K^+ channel immobilized on nitrocellulose (NC) membrane; sections were implanted under the skin behind the ears. Booster immunizations with 5–10 pmol of the immunogen in phosphate-buffered saline (PBS) were given subcutaneously in incomplete Freund's adjuvant at 2–4-week intervals. For the most successful fusion, final boosts were administered, beginning 5 days before fusion, as follows: day 1, intraperitoneal injection (ip) of 40 pmol and intravenous injection (iv) of 15 pmol; day 2, 40 pmol (ip) and 30 pmol (iv); day 3, 70 pmol (ip); day 5, fusion. Relative titer of sera from immunized mice were monitored by dot assay using the native, purified α-DTX acceptors as antigen. Mouse spleen cell were fused with mouse myeloma NSO cells, which do not synthesize immunoglobulin chains (Galfre & Milstein, 1981), following the procedure detailed by Newell et al. (1988) with a modification introduced by Orlik and Altaner (1988), namely, pretreatment of the cells before fusion with 0.25% (v/v) poly(ethylene glycol) for 90 min at 37 °C in a CO_2 incubator. Beginning 10 days after fusion, clones were screened by a dot-immunobinding assay for the production of antibody against purified α-DTX acceptor. Cells from the positive wells were cloned and recloned by either the limiting dilution method or serial dilution. The clones were subsequently grown as ascitic tumors in Balb/c mice by injection of (0.5–1.0) × 10^7 cells growing in logarithmic phase, having previously primed the animals with incomplete adjuvant. mAbs were subtyped by immunodiffusion using antisera directed against the mouse Ig heavy chains IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM.

Dot-Screening Assay. Hybridoma supernatants were screened against the native purified α-DTX–acceptor by an immuno-dot assay (Hawkes et al., 1982), except that the antigen (0.1–0.2 pmol of α-DTX binding sites/dot) was applied onto NC filters in a Bio-Dot apparatus (Bio-Rad Inc.). Nonspecific protein-binding sites were blocked by incubation of the filters in TBS solution (150 mM NaCl/20 mM Tris-HCl, pH 7.5) containing 3% (w/v) bovine serum albumin (BSA) for 1 h; the latter was then cut into equal squares and transferred to a 96-well titer plate. Supernatant from hybridoma culture (100 μL/well) was added and incubated overnight at 4 °C. After washing, the filters were blocked again (as above) and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1000 dilution in TBS) for 2 h at room temperature. The filters were developed with *p*-nitro blue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloroindolyl phosphate (0.05 mg/mL) in developing buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5). Nonimmune serum and mAb raised against an unrelated protein were used as negative controls while serum from the immunized mice served as a positive control.

Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) but using 8% gels. The latter were silver stained or autoradiographed or the samples were electrophoretically transferred to NC, following the procedure of Aebersold et al. (1986). The resultant membranes were blocked, incubated with antibodies, and developed as detailed above for the dot-immunobinding assay.

Enzymatic Deglycosylation of α-DTX Acceptors. Samples containing 10 pmol of α-DTX acceptors were treated with neuraminidase and peptide *N*-glycosidase F as described by Scott et al. (1990); control incubations devoid of enzymes were run in parallel. The deglycosylation was terminated by adding SDS sample buffer, and the samples were boiled prior to being subjected to SDS–PAGE.

Measurement of the Effect of mAbs on α-DTX Binding to Its Acceptor. mAbs were preincubated overnight with Thesit extract of bovine cerebrocortical membranes before incubation of the mixture for 30 min with 3 nM ^{125}I-α-DTX, in the presence or absence of an excess of unlabeled α-DTX in buffer A. Saturable binding of α-DTX to its acceptor was quantified as mentioned earlier.

Immunoprecipitation Assay. The ability of the antibodies to bind α-DTX–acceptor complex was assessed by indirect immunoprecipitation. Purified acceptor or crude detergent extract (0.1 pmol of α-DTX-binding sites/assay) from synaptic plasma membranes of bovine cerebral cortex was incubated with 3 nM ^{125}I-α-DTX in the presence or absence of an excess of unlabeled α-DTX in buffer A for at least 30 min. To this mixture, diluted ascitic fluid was added and incubated overnight at 4 °C. The quantity of immunoglobulins in all tubes from one particular experiment was made equal by inclusion of the appropriate amount of nonimmune serum. Goat anti-mouse IgG coupled to agarose beads (30 μL of suspension/assay) was then added and incubated (final volume of 250 μL) with mixing for 3–5 h at 4 °C. Samples were sedimented and the pellet was washed twice at 4 °C in buffer A, before counting in a γ-counter. All determinations were carried out in triplicate and repeated at least twice.

A direct immunoprecipitation assay using ^{125}I-labeled acceptor was also performed. The radioiodinated acceptor (40 000–60 000 cpm/assay) was incubated overnight with antibody at 4 °C and then processed as for indirect immunoprecipitation. To analyze the immunoprecipitates, the beads

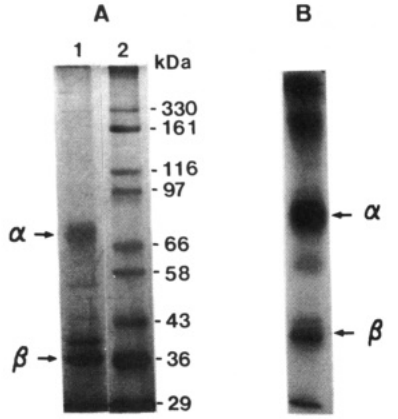

FIGURE 1: Polypeptide composition of the purified α-DTX acceptor used as immunogen. SDS–PAGE was performed using 8% gels, under reducing conditions. (A) Native acceptor (2 pmol) silver stained (track 1) and protein standards (track 2) whose M_r values are shown in descending order: thyroglobulin, α_2-macroglobulin, β-galactosidase, phosphorylase *b*, bovine serum albumin, catalase, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase. (B) Radioiodinated sample of the pure acceptor detected by autoradiography. Arrows indicate the mobilities of the acceptor α- and β-subunits.

were extracted with SDS sample buffer and subjected to SDS–PAGE.

Preparation of Crude Membranes from Different Tissues. A crude membrane fraction was prepared from freshly dissected rat organs by homogenization with a Polytron at medium speed for 60 s in 0.32 M sucrose/10 mM imidazole hydrochloride, pH 7.4/2 mM EDTA/0.1 mM PMSF/0.2 mM benzamidine. After centrifuging the homogenates at 1000*g* for 10 min, the pellets were discarded and the supernatant was centrifuged at 20000*g* for 30 min. The crude membranes were resuspended in wash buffer (homogenization buffer without sucrose) and repelleted before resuspension in the latter buffer; protein concentrations were determined colorimetrically using BSA as standard (Markwell et al., 1978). After solubilization of the membranes in 4% (w/v) Thesit in extraction buffer (Parcej et al., 1992), detergent extracts were assayed for ^{125}I-α-DTX binding and, where possible, immunoprecipitation was carried out. For immunoblot analysis, crude membrane samples were solubilized directly in SDS sample buffer prior to PAGE.

RESULTS

Purity of the K+ Channel Preparation Used for Immunization. A preparation of K+ channels purified from bovine cerebrocortical membranes was used as immunogen for the production of mAbs. Its purity was established from the presence of two major protein bands [M_r ≈78 000 (broad) and 39 000 (sharp) on 8% gels] revealed in SDS–PAGE gels by silver staining (Figure 1A). Likewise, detection of the ^{125}I-labeled protein by autoradiography (Figure 1B) confirmed the existence of the established α- and β-subunits in a heterooligomeric structure. With this sensitive autoradiographic method, two additional bands of higher M_r were detected; these apparently arise from acceptor aggregation (see later). After the initial immunizations of mice with purified protein, the sera at this stage were found to bind the antigen in a dot-immunobinding assay, even when diluted 1000-fold or more (cf. Table I). Despite the apparent effectiveness of this immunization protocol, hybridomas obtained from these mice failed to produce detectable levels of secreted antibodies against the α-DTX acceptor. To overcome the latter problem, recommended protocols (Stahli et al., 1980; Ratcliffe et al., 1990) were adopted, in which higher doses of antigen were administered repeatedly before fusion (detailed in Experimental Procedures), resulting in successful production of hybridomas secreting anti-acceptor antibodies (Table I and below).

Preparation and Characterization of mAbs against α-DTX-Sensitive K+ Channels. Hybridoma culture supernatants were first screened with a dot-binding assay for antibodies capable of complexing to the **native** antigen. The improved immunization protocol yielded, initially, 20 clones secreting specific mAbs, derived from 249 hybridomas, generated from 1200 wells. Eleven of these stopped secreting during the early stages of subcloning. Although the hybridomas producing the antibodies described in Table I (mAbs 1–9) originated from single clones present in the primary cultures, further cloning by limiting and serial dilution was performed to ensure monoclonality. mAbs 8 and 9 showed signs of instability in the later stages of cloning and, thus, were not further characterized. mAb 1 was found to be an IgM while the others were IgG_1, as revealed by double immunodiffusion against subclass-specific anti-mouse immunoglobulins (Table I).

mAbs Recognize the N-Deglycosylated K+ Channel α-Subunit. Reactivity of the mAbs toward the **denatured** antigen was determined by immunoblot analysis of purified K+ channels whose subunits were separated under reducing conditions by SDS–PAGE. After transfer and exposure to each of the antibodies, the major immunoreactive product was an intense but broadly running band whose M_r corresponds precisely to the larger glycosylated α-subunit of the K+ channel (Figure 2A); in control samples performed with preimmune serum or supernatant of unrelated IgG_1 mAb no reactivity was observed. None of the mAbs recognized the β-subunit; this is not unexpected since the polyclonal antiserum also failed to detect it, reaffirming that the β-subunit is not derived from the α-subunit but represents a distinct protein. The faint band in SDS–PAGE (M_r ≈36K; Figure 2A, track P) seen in overstained blots or when the purified acceptor was overloaded onto gels appears to be a proteolytic product of the α-subunit and not the β-subunit, since it migrates slightly faster than the latter. Moreover, this weakly immunoreactive band seems, unlike the β-subunit (Scott et al., 1990), to be N-glycosylated as its electrophoretic mobility increased after treatment with neuraminidase and peptide *N*-glycosidase F (Figure 2B). Trace amounts of high M_r species (>160K) were seen in some blots but only when grossly overstained (Figure 2A, track P, and Figure 2B, mAb 5); these are thought to represent aggregated α-subunit (see later). As little as 10–20 fmol of α-DTX binding sites could be detected with the more reactive mAbs (mAbs 5 > 4 > 7) when incubated with undiluted hybridoma supernatant.

As the α-subunit is heavily glycosylated (Rehm, 1989; Scott et al., 1990), we were prompted to ascertain if the antibodies were directed to the protein or carbohydrate moieties. Thus, the purified acceptor was treated with neuraminidase, an exosialidase, and peptide *N*-glycosidase F that removes N-linked oligosaccharides. When the products were analyzed by immunobloting, their increased mobilities on SDS–PAGE gels provided evidence that deglycosylation of the α-subunit had occurred (Figure 2B). All the other antibodies also stained the native and enzymically-treated K+ channels (data not shown). Hence, these data show that the epitopes recognized by the mAbs are resistant to extensive deglycosylation [using conditions established in Scott et al. (1990)] and, thus, probably

Table I: Characteristics of Antibodies Raised against α-DTX-Sensitive Voltage-Dependent K^+ Channels from Bovine Cerebral Cortex

antibody	class	^{125}I-α-DTX binding in membrane extract (% of control)	detection limit on dot assay (dilution, x-fold)		immunoprecipitation: apparent titers[a] (dilution, x-fold)	
			supernatant	ascitic fluid	^{125}I-α-DTX–acceptor complex[b]	^{125}I-labeled purified acceptor[b]
mAb 1	IgM	88 ± 11	5×10^2	2×10^5	1.2×10^3	ND[d]
mAb 2	IgG_1	103 ± 9	10^3	$>10^6$	8×10^2	7×10^3
mAb 3	IgG_1	77 ± 5	10^3	$>10^6$	1.2×10^3	ND
mAb 4	IgG_1	100 ± 7	$>10^3$	$>10^6$	1.8×10^3	4×10^3
mAb 5	IgG_1	89 ± 5	$\gg 10^3$	$\gg 10^6$	5×10^4	3×10^5
mAb 6	IgG_1	106 ± 7	5×10^2	10^6	$<3 \times 10^2$	ND
mAb 7	IgG_1	102 ± 4	$>10^3$	$>10^6$	6×10^2	10^4
mAb 8	ND	ND	10^3	ND	ND	ND
mAb 9	ND	ND	10^3	ND	ND	ND
polyclonal[c]		99 ± 3		$>10^3$ [c]	ND	ND

[a] Quantity capable of precipitating 50% of the respective maximum amounts of acceptor that could be sedimented by each antibody. [b] Values taken from Figures 3A and 4A. [c] Antiserum from the mouse whose spleen was used in the fusion. [d] ND, not determined.

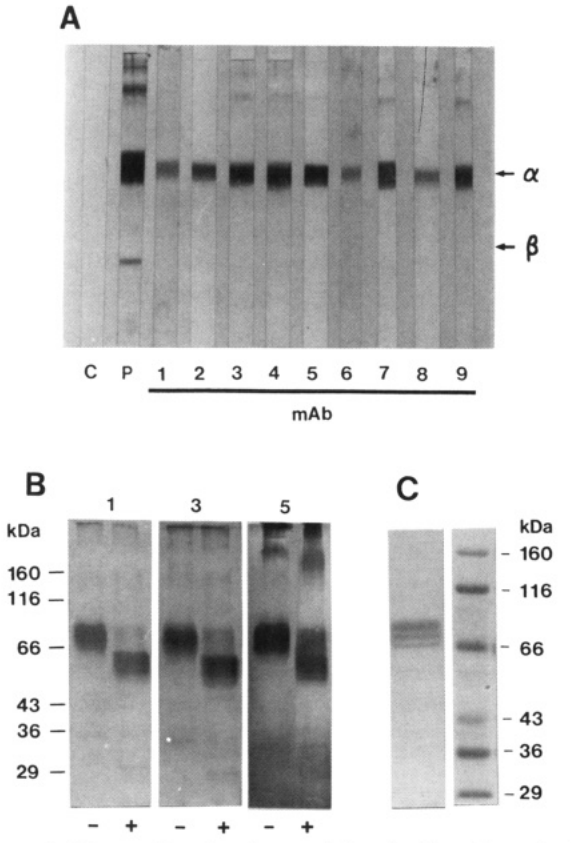

FIGURE 2: Western blots showing reactivity of mAbs with α-subunit(s) of the K^+ channels. Samples were subjected to 8% SDS–PAGE under reducing conditions and transferred to NC membranes. Blots were probed with hybridoma supernatants and developed with anti-species second antibody conjugated to alkaline phosphatase. (A) Purified α-DTX acceptor from bovine cerebrocortical membranes probed with the following: C, hybridoma supernatant of an unrelated IgG_1 mAb; P, mouse polyclonal serum (diluted 1:250); 1–9, mAbs 1–9. Arrows indicate the mobility of α- and β-subunits determined from the protein-stained pattern (not shown) of a separate gel track. (B) Samples of pure α-DTX acceptor in the native state (–) or after (+) deglycosylation by treatment with neuraminidase and peptide *N*-glycosidase F, reacted with mAbs 1, 3, or 5. (C) Detergent extract of bovine cerebral cortex membranes probed with mAb 5 and developed as in (A). In (B) and (C) the mobilities are shown for protein standards detailed in Figure 1 except for the omission of thyroglobulin and phosphorylase *b*.

reside on the protein core.

Being concerned about the possibility that some change might have occurred on the K^+ channel complex during the extensive purification, a detergent extract of synaptic plasma membranes from bovine cerebral cortex was investigated using the mAbs. Staining of the α-subunit was detected only with mAb 5, probably due to the low concentration of this protein in the solubilized membrane preparation. mAb 5, intriguingly, reacted specifically with three distinguishable bands (Figure 2C) that migrate closely within the limits of that corresponding to the diffuse α-subunit band (Figure 2A,B). These defined bands could also be seen in the deglycosylated purified receptor (Figure 2B) or crude membrane (data not shown) when blotted with mAb 5. Thus, the broad and diffuse band of the purified acceptor seen on silver straining gels (Figure 1A), or recognized by the mAbs, may represent microheterogeneity of the protein due to isoforms of the α-subunit, though proteolysis cannot be totally excluded. The sole presence of α bands in immunoblots using detergent extract of bovine synaptic membranes (Figure 2C) indicates that the additional bands seen with the purified α-DTX acceptor (Figure 2A, track P; Figure 2B, mAb 5) are due to aggregated α-subunit of the K^+ channel and its possible proteolytic products.

Dissimilar Reaction of mAbs with Native K^+ Channels. Since none of the mAbs inhibited appreciably the binding of ^{125}I-α-DTX to a Thesit extract of bovine synaptic membranes (Table I), this allowed toxin-labeled acceptor to be used in immunoprecipitation studies with the ascitic fluids. Concentration dependencies of the precipitation of crude acceptor complexed to ^{125}I-α-DTX (Figure 3A) yielded the relative titers for each mAb shown in Table I. Under optimal conditions, three mAbs (5 > 4 > 7) specifically precipitated the majority of the added complex, with lesser amount being sedimented by two others (2 and 3) and an even smaller fraction by mAbs 1 and 6 (Figure 3B; solid bars). Incomplete precipitation of the oligomeric acceptors observed with saturating concentrations of some mAbs may indicate specificity for subpopulations (e.g., mAb 7; see Figure 3A) of the α-subunit (see below), though in other cases low affinity could be a contributory factor.

Immunoprecipitation of purified K^+ channels, instead of crude material, was also performed. Using optimized dilutions, the proportion precipitated by mAbs 2 and 3 approximated those seen with the crude extract (Figure 3B, diagonal bars); three mAbs (4, 5, and 7) precipitated a lower fraction of purified acceptor, possibly due to certain subtypes being recovered at reduced levels in the purification procedure. mAbs 1 and 6 removed a higher proportion than those in the crude material; this may result from an enrichment of the K^+ channels in the purified sample and, thus, implies a lower affinity interaction.

Coimmunoprecipitation of Both Subunits of the K^+ Channel by mAbs. All seven mAbs were able to immunoprecipitate

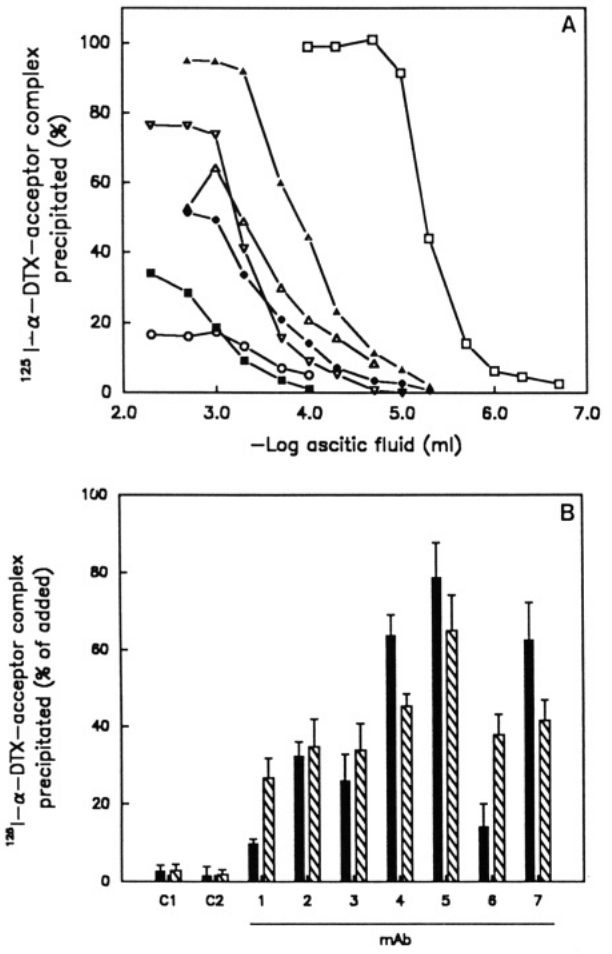

FIGURE 3: Immunoprecipitation of ^{125}I-α-DTX–acceptor complex. (A) A detergent extract of bovine brain membranes (≈0.1 pmol/0.25 mL), labeled to saturation with 3 nM ^{125}I-α-DTX, was incubated with varying concentrations of the different ascitic fluids. The total amount of antibody in each tube was kept constant by addition of the appropriate quantity of normal mouse serum. Immune complexes were sedimented with anti-species antibody coupled to agarose beads (see Experimental Procedures). To make corrections for nonspecifically bound ^{125}I-α-DTX, equivalent control reactions were carried out except for the inclusion of 500 nM α-DTX in the initial incubation; the values obtained for these represented less than 7% of the total radioactivity and were subtracted to give the amount specifically bound, as plotted. The relative quantities of radioactivity associated with the pellets were plotted relative to the maximum amount of specifically bound ^{125}I-α-DTX precipitated by mAb 5 (≈80% of the K+ channel complex added). Values are the mean of 2–3 experiments with triplicate points; SD was less than 8%. mAbs: 1, ○; 2, ●; 3, △; 4, ▲; 5, □; 6, ■; 7, ▽. (B) Purified (diagonal bars) and detergent-extracted (solid bars) α-DTX acceptors were complexed to ^{125}I-α-DTX as outlined in (A) and incubated with optimal amounts of ascitic fluids (2 μL for mAbs 1, 2, 4, and 7; 1 μL for 3, 0.1 μL for 5). All other experimental details were as described in (A) except that results were expressed relative to the total amount of specifically bound ^{125}I-α-DTX used.

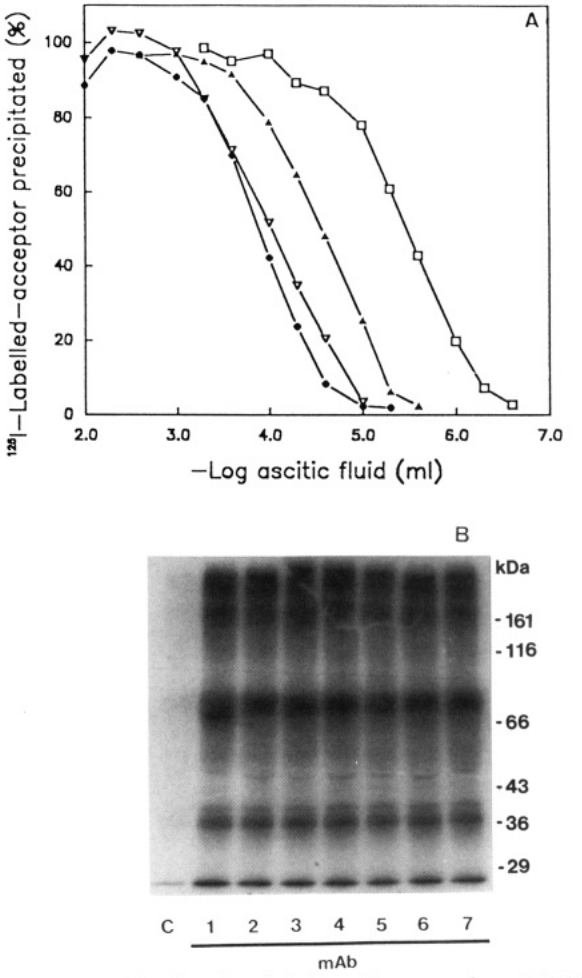

FIGURE 4: Precipitation of radioiodinated acceptor for α-DTX by mAbs. (A) Samples of ^{125}I-labeled acceptor (≈50 000 cpm/incubation; final volume of 0.2 mL) were reacted with varying concentration of ascitic fluids of mAb 2 (●), 4 (▲), 5 (□), or 7 (▽). The total amount of antibody in each tube was kept constant by inclusion of the requisite amount of normal mouse serum. Immune complexes were sedimented as detailed in Figure 3. In all cases, the values obtained for control samples, containing an irrelevant mAb, have been subtracted from the totals to yield the amounts precipitated specifically by each mAb. The latter were plotted relative to the maximum amount of radioactive acceptors precipitated by mAb 5. Values are the mean of 2 experiments with triplicate points, and SD was less than 5%. (B) Autoradiogram from SDS–PAGE of ^{125}I-labeled acceptor immunoprecipitated by each of the numbered mAbs or control (C). Immunoprecipitates obtained as in (A) were extracted from the beads with SDS sample buffer prior to SDS–PAGE/autoradiography. Positions of the standard protein markers (detailed in Figure 2) are marked.

^{125}I-labeled K+ channel, with specific recoveries ranging from 35% to 60%. The concentration dependencies of these precipitations (Figure 4A) yielded the relative titers shown in Table I; although these were somewhat higher than obtained when larger amounts of crude antigen were used, their rank order remains similar. It is noticeable, particularly with mAbs 2 and 7, that the amount of purified acceptors precipitated was lower when the latter were complexed to ^{125}I-α-DTX rather than directly radioiodinated; this may reflect noncompetitive inhibition by toxin of antibody binding. In order to ascertain if α and β polypeptides of the α-DTX acceptor are tightly associated, the native protein purified from bovine cortex was precipitated, after radiolabeling, with mAbs and the precipitates were analyzed by SDS–PAGE (Figure 4B). Both subunits were present in immunoprecipitates of all 7 mAbs and absent from the one obtained with an unrelated mAb (Figure 4B).

Tissue Specificity of mAb 5 Reactivity. Due to the high sensitivity of mAb 5 in detecting the K+ channels in crude membranes from bovine brain, it was exploited in screening other tissues of the rat for the presence of equivalent K+ channel proteins by immunoblotting and immunoprecipitation. Saturable α-DTX binding was not detectable in detergent (Thesit) extracts of liver, heart, kidney, spleen, testes, or pancreas; thus, immunoprecipitation could not be evaluated. Additionally, no specific staining of the α-subunit was evident in immunoblots performed with these various organs of the rat (Figure 5); the faint bands visible on the gel, migrating faster than the α-subunit, were considered irrelevant because a control mAb gave a similar background. These findings

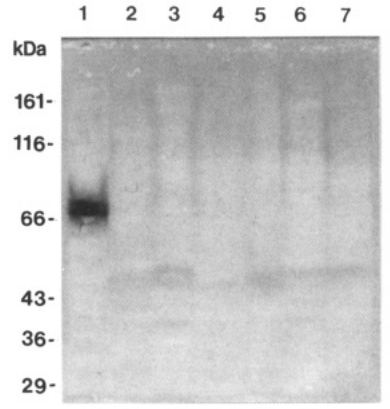

FIGURE 5: Tissue selectivity of mAb 5 reactivity. A crude membrane fraction was isolated from various rat tissues and dissolved in SDS sample buffer prior to PAGE (8% gel); 100 μg of protein/track was used. Proteins were then transferred to NC membrane and probed with mAb5. Lanes: 1, whole brain; 2, liver; 3, heart; 4, kidney; 5, spleen; 6, testes; 7, pancreas. Electrophoretic mobilities of standard proteins (as in Figure 2) are shown.

suggest that the mAb 5 recognizes an epitope in the α-subunit that is characteristic of brain tissue. In the case of the rat brain extract, it is notable that ≈50% of the ^{125}I-α-DTX acceptor complex was precipitated by this mAb, demonstrating a high degree of homology between the α-subunits in these two species. Moreover, a quantitative variation of staining observed in immunoblots with a detergent-solubilized extract from different regions of rat brain (data not shown) indicates that cortex, hippocampus, midbrain, and medulla pons possess a higher content of this immunoreactive K^+ channel than olfactory bulb and cerebellum.

DISCUSSION

This study reports, for the first time, the production and characterization of a series of mAbs against α-DTX-sensitive voltage-dependent K^+ channels. The failure to produce specific hybridomas in the initial fusions reflects the experience of many laboratories when small amounts of proteins were used in the immunization. However, this difficulty was overcome by following the conventional preimmunizations with multiple injections of higher doses of antigen before fusion. Stähli et al. (1980) have shown that the specific efficiency (proportion of positive hybridomas produced per fusion) depends on the fraction of freshly-stimulated, large lymphocytes present; maintaining high concentrations of circulating antigen up to the day of fusion promotes continued blast cell proliferation.

The mAbs described here exhibit a wide range of apparent titers and reactivity; most importantly, their usefulness in future research applications is highlighted by the ability to immunoprecipitate selectively the α-DTX acceptors from a detergent extract of bovine synaptic plasma membranes. All seven mAbs recognize the protein core of the α-subunit but none seemed to be reactive toward the K^+ channel β-subunit, demonstrating that the β-subunit is a distinct protein and not a proteolytic fragment of the larger subunit. In addition, coimmunoprecipitation of both subunits provides evidence that they are associated in the K^+ channel complex. These results agree with the recent report of Trimmer (1991) in which a 38-kDa polypeptide was found to be associated with drk1, a mammalian K^+ channel of the *Shab* subfamily, when antibodies raised against sequences from the latter were used to precipitate a solubilized extract of radiolabeled brain membranes. However, the relationship of this protein to the previously established β-subunit (Rehm & Lazdunski, 1988; Parcej & Dolly, 1989) of mammalian α-DTX-sensitive K^+ channels was not proven. It should be noted that although the α/β-subunit oligomeric species represent the bulk of α-DTX acceptors in this preparation, sucrose gradient density sedimentation separated a minor, simpler form containing α-subunit only (Parcej et al., 1992). The absence of mAbs against the β-subunit could reflect very low immunogenicity, as noted for the auxiliary subunits of other cation channels (Casadei et al., 1984; Takahashi & Catterall, 1987; Leung et al., 1987) or could indicate that it is hidden in the mixed micellar form of the protein used for immunization.

The broad band recognized by the mAbs on immunoblots of α-DTX acceptor purified from bovine brain membranes seems to represent microheterogeneity due to isoforms of the α-subunit; mAb 5 was able to detect at least three bands in crude extract that migrate closely within the limits of the staining seen with the α-subunit of purified α-DTX acceptor. This observation agrees with the study of Scott et al. (1990), in which the major N-terminal sequence of α-subunit (see the introduction) was accompanied by a minor secondary sequence that differed primarily in the first nine residues and was not dissimilar to the N-terminal sequence of RCK 1, a homologous K^+ channel protein cloned from rat brain (Stühmer et al., 1989). Clearly, this demonstrated existence of naturally-occurring α-subunit variants in brain provides scope for the proposed mechanism (Parcej & Dolly, 1989) of creating diversity of neuronal K^+ channel subtypes, through assembly of different α-subunits together with the β-subunit to form heterooligomeric channel complexes.

The cross-reactivity of mAb 5 with α-subunits of bovine and rat brain highlights the strong immunological relationship among mammalian K^+ channels. The fact that this antibody does not detect α-subunits in nonneuronal tissue accords with the apparent absence of mRNA for RCK 1 and RCK 5 K^+ channels from organs other than nervous tissue (Beckh & Pongs, 1990). This result indicates that mAb 5 is probably directed to a variant region, e.g., carboxy-terminal of the K^+ channel α-subunits, and not to the highly conserved core regions comprising the proposed transmembrane segments (S1–S6 and P) (Stühmer et al., 1989; Yool & Schwartz, 1991; Hartmann et al., 1991; Yellen et al., 1991). The faint band (≈58K) seen in autoradiograms of SDS–PAGE gels of radiolabeled purified α-DTX acceptor (Figure 1B) is probably the α-subunit from the α-DTX-sensitive RCK 2 K^+ channel (Grupe et al., 1990), since it corresponds in size and, also, type-specific anti-RCK 2 polyclonal antibodies recognized a band in the same region (unpublished observation). Notably, none of the mAbs described here recognized this protein on immunoblots, suggesting that the mAbs bind to epitopes in the α-subunits that are absent or different in the RCK 2 protein. Likewise, as RCK 4 protein, an α-DTX-insensitive K^+ channel, has a higher M_r than the other RCKs (Stühmer, 1989), it should have been separated on SDS–PAGE gels and, thus, easily identifiable in immunoblots. However, mAb 5 did not stain any such protein in the crude or purified samples, suggesting that all the mAbs raised against native α-DTX acceptors recognize RCK 1 or 5 (or possibly 3) but not RCK 2 or 4 proteins. Thus, these mAbs could be useful in immunoaffinity chromatographic isolation of K^+ channel subtypes when used in conjunction with toxin I resins; other valuable applications will include localization of these proteins in the central nervous system, establishing their membrane topography and characterization of K^+ channel variants in expression systems.

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