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Primary structure of a β subunit of α-dendrotoxin-sensitive K^+ channels from bovine brain

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ABSTRACT **Voltage-dependent cation channels are large heterooligomeric proteins. Heterologous expression of cDNAs encoding the α subunits alone of K^+, Na^+, or Ca^{2+} channels produces functional multimeric proteins; however, coexpression of those for the latter two with their auxiliary proteins causes dramatic changes in the resultant membrane currents. Fast-activating, voltage-sensitive K^+ channels from brain contain four α and β subunits, tightly associated in a 400-kDa complex; although molecular details of the α-subunit proteins have been determined, little is known about the β-subunit constituent. Proteolytic fragments of a β subunit from bovine α-dendrotoxin-sensitive neuronal K^+ channels yielded nine different sequences. In the polymerase chain reaction, primers corresponding to two of these peptides amplified a 329-base-pair fragment in a λgt10 cDNA library from bovine brain; a full-length clone subsequently isolated encodes a protein of 367 amino acids ($M_r \approx 40{,}983$). It shows no significant homology with any known protein. Unlike the channels' α subunits, the hydropathy profile of this sequence failed to reveal transmembrane domains. Several consensus phosphorylation motifs are apparent and, accordingly, the β subunit could be phosphorylated in the intact K^+ channels. These results, including the absence of a leader sequence and N-glycosylation, are consistent with the β subunit being firmly associated on the inside of the membrane with α subunits, as speculated in a simplified model of these authentic K^+ channels. Importantly, this first primary structure of a K^+-channel β subunit indicates that none of the cloned auxiliary proteins of voltage-dependent cation channels, unlike their α subunits, belong to a superfamily of genes.**

Voltage-gated cation channels play a major role in the generation of electrical signals in the nervous system (1). Members of each of the major channel types—selective for Na^+, K^+, and Ca^{2+}—have been biochemically purified. A hallmark of these isolated channels is that they all consist of several subunits having different molecular mass although, in each case, expression of the cDNAs for the α subunits alone is sufficient to form active ion channels. Voltage-sensitive Ca^{2+} and Na^+ channels possess one α subunit of ≈200 kDa consisting of four repeat domains (2, 3). On the other hand, the isolated neuronal K^+ channels (see below) contain four α subunits (4); the predominant one has an apparent molecular mass of ≈78 kDa but different sized minor variants are also present (5). It is well documented that the properties of expressed currents are strongly influenced by β subunits in the case of Na^+ (6) or by β, γ, and α_2/δ subunits for Ca^{2+} channels (2, 7, 8). Furthermore, expression studies with cloned α-subunit cDNAs have not faithfully reproduced the distinct properties of native voltage-gated cation channels (1, 3, 9). Although a family of genes encoding α subunit isoforms (Kv 1.1–1.9) of voltage-dependent K^+ channels have been cloned (reviewed in ref. 10), unavailability of cDNA for the β subunit has precluded their coexpression, despite the latter being an integral constituent of the channels' macromolecular complex (see below).

The mamba snake venom polypeptide α-dendrotoxin (α-DTX) has been instrumental in studying a family of fast-activating, aminopyridine-sensitive K^+ channels that are concerned with the control of neuron excitability and neurotransmitter release (11). This probe inhibits most efficiently neuronal K^+ current variants exhibiting slow inactivation, whereas related fast-inactivating K^+ conductances are either insensitive or blocked by higher toxin concentrations (12). High-affinity binding sites for radioiodinated α-DTX (^{125}I-α-DTX) have been identified (11, 13) and localized (14, 15) in the mammalian central nervous system. α-DTX-sensitive K^+ channels, affinity purified from rat (16) and bovine (17) synaptic plasma membranes, contain α and β subunits. The N-terminal sequence of the former (18) is homologous to that of Kv 1.2, a K^+ channel α subunit cloned from rat or bovine brain (10, 19), which when expressed in *Xenopus* oocytes was highly sensitive to α-DTX. However, the currents produced upon expression of this, and most other clones tested (10), were not identical to those recorded in native cell membranes; this may be due to an absence of the β subunit that occurs in native α-DTX-sensitive K^+ channels in a stoichiometry of four α and four β subunits (4). Clearly, understanding the role of this auxiliary β subunit in K^+-channel activity is of utmost importance. In this paper, the complete and unique sequence of a voltage-gated K^+-channel β subunit was obtained by gene cloning, using internal sequence information from the isolated β-subunit protein.§

MATERIALS AND METHODS

Phosphorylation of Purified α-DTX-Sensitive K^+ Channels. K^+ channels were isolated from synaptic plasma membranes of bovine brain cortex (4, 17) and aliquots were incubated at 37°C with 50 μM [γ-^{32}P]ATP (5×10^6 dpm/nmol) in 20 mM Hepes, pH 7.4/50 mM KCl/10 mM $MgCl_2$/0.05% Tween 80 in the absence and presence of the catalytic subunit (6 units; ≈100 ng) of protein kinase A (PKA) prior to SDS/PAGE. To quantify the extent of phosphorylation (after 3 h), 3–5 pmol of initial ^{125}I-α-DTX binding was subjected to SDS/PAGE autoradiography, and the major labeled bands on the gel were excised and counted in a β spectrometer.

Generation of Peptides from the β Subunit for Microsequencing. An aliquot of the fluorescently labeled β subunit (400–500 pmol of ^{125}I-α-DTX binding), isolated as detailed for

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Abbreviations: α-DTX, α-dendrotoxin; PVDF, poly(vinylidene difluoride); PKA, protein kinase A.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X70662).

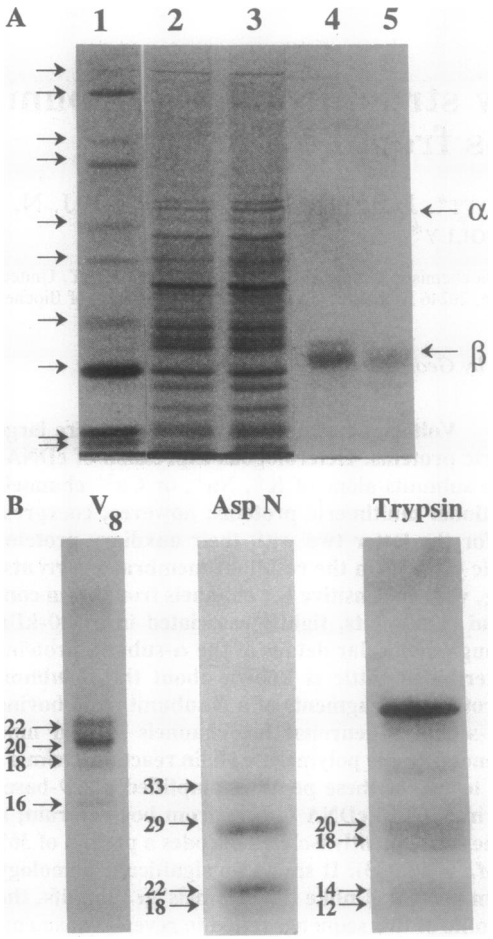
the α subunit (18), was incubated for 1 h at 37°C with V_8 protease in 67.5 mM sodium phosphate (pH 7.4) with a protein/enzyme ratio of 50:1, with trypsin in 0.1 M *N*-ethylmorpholine acetate buffer (pH 8.1) with a protein/enzyme ratio of 25:1 for 3 h at 37°C, or with endoproteinase Asp N in 10 mM Tris·HCl (pH 8.4) with a protein/enzyme ratio of 10:1 for 15 min at 37°C. The samples were boiled to inactivate the enzyme before separating the peptides by SDS/PAGE, electrophoretic transfer onto poly(vinylidene difluoride) (PVDF) in 10 mM CAPS buffer (pH 11.0) (20), and visualization by transillumination at 313 nm. The blotted peptides were then subjected to liquid-pulse or solid-phase microsequencing, depending on availability of instruments.

Microsequencing. Approximately 100 pmol of each membrane-immobilized peptide (as determined by amino acid analysis following acid hydrolysis of the membrane, after sequencing) was placed in the Blott reaction cartridge and subjected to automated liquid-pulse sequence analysis on an Applied Biosystems sequencer (model 477A), as detailed elsewhere (21). Alternatively, the piece of PVDF membrane containing the electroblotted polypeptide (100–150 pmol of protein as determined above) was subjected to the SequeNet entrapment procedure to immobilize the sample (22). The sample was then subjected to automated solid-phase Edman degradation on a MilliGen/Biosearch 6600 Prosequencer.

Cloning and Sequencing of the β Subunit. For nested PCR, ACSVARQ plus QFNLIPC and AEVVLGN plus AEVYAAG (see Fig. 1*C*) were used to design the following primers: GCIGARGTITAYGCIGCNGG and CAIGGDATIAGRTTRAAYTG/CAIGGDATYAARTTRAAYTG as the outer clamps and GCIGARGTIGTICTIGGNAA/GCIGARGTIGTITTRGGNAA plus TGICKIGCIACIGARCANGC/TGICKIGCIACRCTRCANGC as inner clamps. (N is A, G, C, or T; D is A, G, or T; R is A or G; K is T or G; and Y is T or C). These were incubated (see below) in the presence of 10 mM Tris·HCl, pH 9.0/50 mM KCl/1.5 mM $MgCl_2$/200 μM each dNTP, 5 μM each primer, and 2.5 units of *Taq* DNA polymerase. The template for the first PCR was 8×10^6 recombinants from a random-primed λgt10 cDNA library from bovine brain and for the second PCR 1/50th of the first PCR amplification. In both cases, 35 repeat cycles of the PCR were performed as follows: 1 min each at 94°C, 48°C, and 72°C for the first PCR; 1 min each at 94°C, 52°C, and 72°C for the second PCR. Subsequently, a 7-min extension at 72°C was carried out and the reaction was stopped at 4°C. The resulting PCR product was treated with 1 unit of T4 DNA polymerase for 45 min at 37°C in the presence of 500 μM each dNTP/50 mM Tris·HCl, pH 8.5/5 mM $MgCl_2$/5 mM dithiothreitol, following phosphorylation with 10 units of polynucleotide kinase for 45 min at 37°C under the same conditions. After separation on a 1.5% agarose gel, a 329-base-pair fragment was excised, purified with the Gene Clean II kit (Bio 101), and blunt-end ligated into pBluescript KS+ (Stratagene). Subsequent sequencing was carried out with the Sequenase DNA sequencing kit. The resultant PCR product was labeled with ^{32}P and was used to screen 1×10^6 plaques of a λgt10 bovine brain cDNA library. Positive clones were sequenced as for the fragment.

RESULTS

Purification of α-DTX-Sensitive K^+ Channels and Isolation of Their β Subunit. To obtain enough material for microsequencing, large scale purification of α-DTX acceptors ($\approx$500 pmol of ^{125}I-α-DTX binding isolated from $\approx$10 g of synaptic plasma membrane protein from bovine brain cortex) was performed and purity of the resultant K^+ channels, relative to the numerous proteins in the crude extract, was demonstrated by SDS/PAGE (Fig. 1*A*). This revealed the broad glycosylated α-subunit band together with the smaller β subunit (39 kDa); also, a 42-kDa protein was detected (see below). The β subunit was isolated by preparative SDS/



C

V_8	16	AlaCysSerValAlaArgGln PheAsnLeuIleProCysIleXGluGlnAla
		SerThrThrGlySerProAlaArgLeuXXArgGlnPro
	18	TyrHisMetPheGlnArgGluLysValGluValGlnLeuProGluXPhe
	20	MetTyrProGluAlaThrThrXLeuProLeuArgGlnLeu
		Ala CysSerValAlaArgGln PheAsnLeuIleProCysIle
	22	AlaValArgAlaMetIleGlyValIleAsnGlnAlaSerAlaMet
		AlaCysSerValAlaArgGln PheAsnLeuIlePro
Asp N	18	AspThrAlaGluValTyrAlaAlaGlyXAlaGluValValLeu-
		-GlyAsnIleIleXXXGly
	22	AspThrAlaGluValTyrAlaAlaGlyXAlaGluValValLeu-
		-GlyAsnIleIleXXXGly
		AspAsnGlyArgAsnLeuPheAspThrAlaSerArgTyrAlaTyrGln-
		-GlnLeuGluXXLeuLeuAsn
	29	AspThrAlaGluValTyrAlaAlaGlyXAlaGluValValLeu-
		-GlyAsnIleIleXXXGly
	33	AspGlnProGluProProAla
Trypsin	12	XXLeuLeuXGlyTyrTrpLeuPheAspXIleLeuSerGluGluGly-
		-XXGlnGlnAlaXLeuXGluLeuGlnAlaXXAlaGluXLeu
	14	XSerLeuValGlyTyrGlnTrpLeuPheAspGlyIleLeuSerGluGluGly-
		-XXGlnGlnAlaXLeuProGluLeuGlnAla

FIG. 1. Isolation and sequence of peptides from a β subunit of α-DTX acceptors. (*A*) SDS/PAGE [10% (wt/vol) gel] of K^+ channels at each stage of their purification (4) after silver staining. The β subunit was resolved in a preparative gel, excised, electroeluted, and analyzed as described. Lanes: 1, molecular size markers as detailed in ref. 4; 2, detergent-extracted synaptic plasma membranes; 3 and 4, breakthrough and eluate of toxin I-Sepharose column; 5, electroeluted β subunit. (*B*) Separation by SDS/PAGE of peptides produced by proteolysis of the purified β subunit with V_8 protease [10–20% (wt/vol) gradient gel], Asp N [12% (wt/vol) gel], and trypsin [15% (wt/vol) gel], followed by silver staining; sizes (kDa) of peptides are indicated. (*C*) Size (kDa) and sequence (major in boldface, minor secondary sequences in standard lettering; unknown indicated as X) of the purified peptides. Underlined sequences were used for design of two pairs of primers.

PAGE and shown to be free of contaminants (Fig. 1*A*). As initial attempts to sequence the β subunit were unsuccessful, probably due to a blocked N terminus, peptides were generated by limited proteolysis of the electroeluted protein using three specific proteases.

Isolation and Microsequencing of Proteolytic Fragments of the β Subunit from α-DTX-Sensitive K^+ Channels. After digestion of the β subunit with V_8 protease under optimized conditions, the resultant proteolytic fragments were resolved by SDS/PAGE (Fig. 1*B*), prior to electroblotting onto PVDF. Liquid-pulse microsequencing of these samples often yielded more than one residue at every cycle; major and minor sequences were assigned on the basis of the amounts of amino acids measured at each position taking into account relative recoveries of the various amino acids. The 18-kDa peptide yielded a single sequence while the 16-, 20-, and 22-kDa fragments produced two sequences apiece (Fig. 1*C*); the minor sequence in the two latter peptides corresponded to the N-terminal portion of the major sequence for the 16-kDa peptide. Cleavage with endoproteinase Asp N also generated four peptides; the three smaller fragments yielded identical signals (Fig. 1*C*) when subjected to solid-phase microsequencing while the fourth peptide gave a different sequence. Notably, a minor signal was also obtained for one of the three peptides (22 kDa). Although proteolysis with trypsin created four products, no signal was recorded with the two larger peptides, suggesting blockage of the N termini. However, the two other peptides yielded almost identical sequences after liquid-pulse microsequencing (Fig. 1*C*), although residues at certain positions were detected in only one or another of the peptides.

Cloning and Sequencing of a β Subunit Associated with K^+ Channels Susceptible to α-DTX. A 329-base-pair cDNA fragment was amplified by PCR, using primers based on the amino acid sequence of two peptides from the β subunit (see *Materials and Methods*). This PCR product was labeled with ^{32}P and was used to screen 1×10^6 plaques of a λgt10 bovine brain cDNA library. Six independent clones were isolated and the longest one, 1536 base pairs long, was sequenced. The 5′ nontranslated region contains two termination codons in different reading frames prior to the first initiation codon (assigned position 1). This would produce an uninterrupted protein sequence to position 1102, the first in-frame termination codon (Fig. 2*A*). The latter yields a protein sequence of 367 amino acids with a calculated M_r of 40,983. In the remaining 3′ untranslated sequence there is no polyadenylylation sequence (AATAAA), suggesting that the native sequence extends further. Notably, eight peptide sequences obtained by microsequencing of the purified β subunit are present (Fig. 2*A*), confirming the identity of the cloned subunit. Uncertain amino acids in the protein sequence could, in many cases, be identified after comparison with the primary structure of the cloned β subunit. However, it should be noted that one peptide could not be reliably aligned with the cloned sequence (see below). Importantly, a comparison of the predicted primary structure of the β subunit with sequences in the Swiss-Prot data base revealed no significant homologies, indicative of this β subunit in α-DTX-sensitive K^+ channels being an unusual and newly discovered protein. Hydropathy analysis of the amino acid sequence of the β subunit (Fig. 2*B*) did not identify any typical membrane-spanning regions (segments of at least 19 residues with an average hydropathy index > 1.6) (23) or the presence of a hydrophobic signal sequence. Indeed, the β subunit appears to be a peripheral membrane protein based on analysis by the method of Klein *et al.* (24), which correlates well with its dominant hydrophilic character (Fig. 2*B*). As judged by the probabilistic method of Garnier *et al.* (25), the β subunit may contain four major α-helical domains (residues 66–103, 126–140, 193–236, and 270–298), a postulation similar to that made

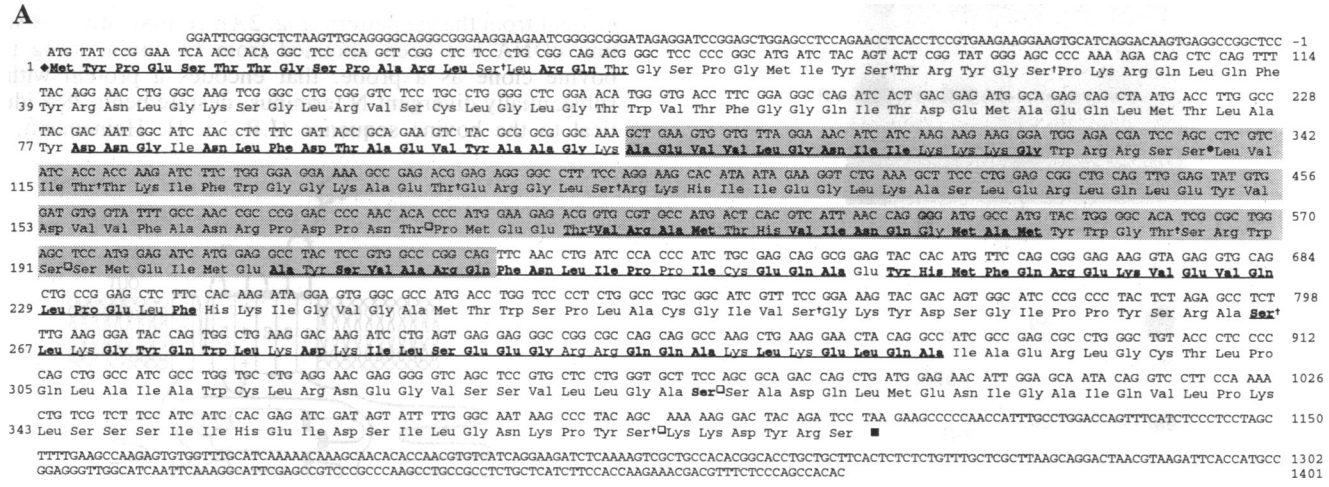


FIG. 2. Primary structure and hydrophobicity pattern of the K^+-channel β subunit cloned from bovine brain. (*A*) DNA and deduced amino acid sequence. cDNA fragment amplified by PCR is shown by shading; peptide sequences are shown by underlining, with those residues matching locations in the clone indicated in boldface. ◆ and ■, Initiation and termination codons that are flanked by untranslated regions. Phosphorylation sites are indicated by ● (PKA), † (protein kinase C), and □ (casein kinase II). (*B*) Hydropathy profile computed according to Kyte and Doolittle (23). Window size is 15 and profile is plotted at one-amino acid residue intervals.

for the cloned β subunit of the L-type voltage-dependent Ca^{2+} channel (7) from skeletal muscle (see below). There are 15 potential phosphorylation sites for different protein kinases present in the K^+-channel β subunit including 1, 10, and 4 loci for PKA, protein kinase C, and casein kinase II. Interestingly, all the potential phosphorylation sites occur outside the proposed α-helical domains except two predicted sites for casein kinase II at residues 127 and 132.

Direct Demonstration of Phosphorylation of K^+-Channel Subunits in the Native State. The purified K^+-channel preparation was incubated with [γ-^{32}P]ATP in the presence of PKA, followed by SDS/PAGE and autoradiography (Fig. 3*A*). This yielded a pattern similar to that obtained by silver staining except that an additional band (≈56 kDa) was visible; an equivalent minor protein was also detected by autoradiographic analysis of ^{125}I-labeled acceptor (5). The latter may be the bovine equivalent of Kv 1.6, another K^+-channel variant

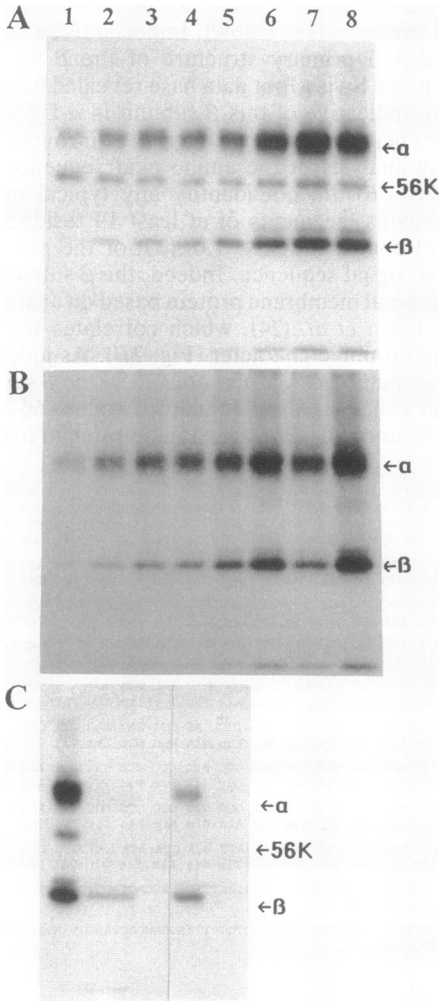


FIG. 3. Phosphorylation of α-DTX acceptors isolated from bovine cerebral cortex. Purified K^+ channels (1 pmol) were phosphorylated in the presence (*A*) or absence (*B*) of the catalytic subunit of PKA as described; aliquots were removed and subjected to SDS/PAGE on 10% (wt/vol) gels, followed by autoradiography. Lanes 1–8, samples taken after reaction times of 5, 10, 15, 20, 30, 45, 60, and 90 min, respectively. Note that gels showing phosphorylation by PKA and endogenous kinase were exposed to the x-ray film for 6 and 21 h, respectively. (*C*) Extent of phosphorylation after 90 min by the catalytic subunit of PKA and endogenous kinase in the presence of 10 mM $MgCl_2$ (lanes 1 and 4, respectively) or 10 mM $MnCl_2$ (lanes 2 and 5, respectively). Lane 3, lack of autophosphorylation (no added K^+ channels) of PKA catalytic subunit ($MgCl_2$-containing buffer). All lanes were exposed for 6 h. Arrows indicate positions of α, β, and 56-kDa subunits.

present in the purified preparation (28). Assuming the K^+ channels contain four α and four β subunits (4), an incorporation of 6.6 ± 1.3 and 3.3 ± 1.8 mol of phosphate per mol of subunit was calculated (n = 3) for α and β subunits, respectively. Incorporation of ^{32}P into α and β subunits by PKA followed a similar time course but was rather slow relative to phosphorylation of the 56-kDa protein. This sluggish incorporation into the two major subunits may indicate that the latter are already extensively phosphorylated *in vivo*, thereby restricting the PKA to a less preferable site. In the absence of added kinase, a low level of ^{32}P labeling of α and β subunits was also observed (Fig. 3*B*), indicating copurification with an endogenous kinase. Strikingly, phosphorylation of the 56-kDa band with the endogenous kinase was barely visible, suggesting it has a different specificity to PKA. Interestingly, Mn^{2+} proved to be a poor substitute for Mg^{2+} in supporting PKA and was unable to support the endogenous kinase activity (Fig. 3*C*). Although the latter activity has been observed (26) in an analogous preparation of these K^+ channels from rat brain, there was a noted absence of phosphorylation of the β subunit and it displayed the unusual feature of being able to use Mn^{2+} as a cofactor; apart from a species difference, the reason for this discrepancy remains unclear.

DISCUSSION

To allow cloning of cDNA for the β subunit of neuronal K^+ channels, 14 peptides were prepared from the purified protein and these yielded nine different sequences, two of which were used as primers for PCR that successfully amplified a fragment from a bovine brain cDNA library. Eight of the sequences were found in the deduced primary structure of the full-length clone obtained; thus, its identity was established conclusively. However, one of the peptides was absent, suggesting that isoforms of the β subunit may exist as is evident from the gel pattern (Fig. 2*A*); consistently, a β-subunit cDNA has been obtained from rat brain, using this bovine clone as a probe, that encodes a protein with a substantially divergent N terminus, despite being 78% identical to the bovine sequence (J.R., S. H. Heinemann, F.

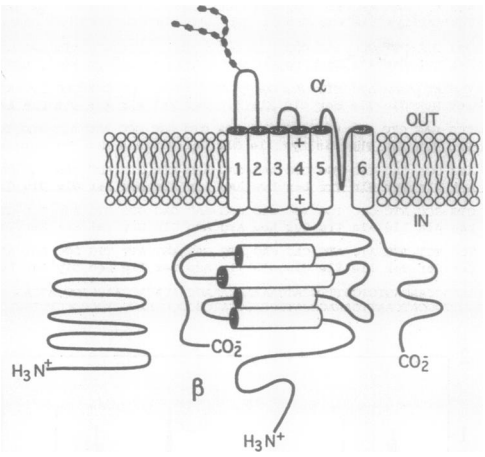


FIG. 4. A postulated model of the structures of α and β subunits comprising α-DTX-susceptible K^+ channels from mammalian brain. Schematic is based on information reported here for the β-subunit protein, together with data reported for bovine Kv 1.2 (α_5) subunit (19), incorporating structural features that have been well documented for homologous α-subunit variants (reviewed in ref. 10). Cylinders represent putative α-helical domains with adjacent segments indicated by lines, both drawn to scale. Note the helices of α and β subunits are transmembrane and intracellular, respectively, and that the areas of association between α and β subunits have not been identified.

Wander, C. Lorra, D.N.P., J.O.D., and O.P., unpublished work). The existence of multiple forms of β subunit could provide a further mechanism for the generation of K^+-channel diversity, with subtypes possessing different combinations of both β and α subunits. It is noteworthy that the predicted M_r of 40,983 for the β subunit corresponds, within experimental error, to the value of 39 kDa determined previously by SDS/PAGE (18). Furthermore, the absence from the cloned sequence of a consensus motif for N-glycosylation accords with our observation that the electrophoretic mobility of the β subunit remains unaltered after treatment with *N*-glycosidases. Its pronounced hydrophilicity and absence of typical membrane-spanning regions (Fig. 2*B*), leader sequence, and N-glycosylation, together with demonstrated phosphorylation sites (Fig. 3), are collectively suggestive of this subunit being located on the cytoplasmic side of the plasma membrane. Inability of high ionic strength to dissociate the α and β subunits during purification (17) and their coimmunoprecipitation (5) highlights a tight association of the β-subunit protein with the α subunits of the heterooligomeric complex. On the basis of numerous considerations, a hypothetical model (Fig. 4) is presented for the α-DTX-sensitive K^+ channels, which contrasts the topography of the β-subunit protein with the documented membrane-spanning arrangement of the α subunits that encompasses six putative α-helical transmembrane regions and a membrane-traversing β hairpin forming the cation pore (reviewed in ref. 10).

Clues to the function of this β-subunit protein in the K^+-channel complex have been gleaned by coexpression in *Xenopus* oocytes of the rat brain clone with cRNA encoding Kv 1.4 or Kv 1.1, α-subunit isoforms (27), when the macroscopic kinetics of channel inactivation were altered (J.R. *et al.*, unpublished work). These dramatic effects demonstrate the difficulties encountered in trying to compare artificially expressed channels with those present *in vivo* without providing all the constituents of the authentic macromolecules. This serious limitation of previous studies on expressed K^+ channels can now be overcome by use of the β-subunit cDNA obtained here. No significant sequence homology of the K^+-channel β subunit was detected with the recently cloned auxiliary subunits of other Na^+ or Ca^{2+} channels, suggesting that these proteins, unlike the pore-forming α-subunit constituents of voltage-dependent cation channels, are not part of a superfamily but may have evolved separately in order to influence the characteristics of each class of cation channel in a distinctive manner. However, the predicted secondary structure of the β subunit presented here resembles that of the recently cloned β subunit of the L-type Ca^{2+} channel (7, 8). Moreover, the effect of the latter on Ca^{2+} currents obtained by expression of an α_1 subunit alone, in *Xenopus* oocytes, is like that observed when K^+-channel α (Kv 1.4) and β subunits are coexpressed (see above). Surprisingly, the α_2/δ subunit of the Ca^{2+} channel (7) and the β_1 subunit of the Na^+ channel (6), which are structurally dissimilar to the β-subunit proteins of both the K^+ and Ca^{2+} channels (see above), also modulate channel kinetics in a manner analogous to those detailed for the latter. Therefore, these collective findings suggest that the correlation of the structures of these auxiliary components will require more extensive research. Nevertheless, this report of the primary structure of a K^+-channel β subunit will facilitate further investigation into the nature of K^+-channel diversity and roles in the central nervous system.

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