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Cross-Linking of β-Bungarotoxin to Chick Brain Membranes. Identification of Subunits of a Putative Voltage-Gated K^+ Channel†

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ABSTRACT: β-Bungarotoxin (β-Butx), a presynaptically active neurotoxin from snake venom, is thought to bind to a subtype of voltage-gated K^+ channels. ^{125}I-β-Butx was cross-linked to its high-affinity binding site in membrane fractions from chick brain by using the bivalent reagents 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and sulfosuccinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate. Two major adducts of apparent M_r 90 000–95 000 and 46 000–49 000 were obtained with both cross-linkers. Formation of both adducts was inhibited by the K^+ channel ligands dendrotoxin I and mast cell degranulating peptide. Our data indicate that the putative β-Butx-sensitive neuronal K^+ channel contains at least two different types of subunits of about 75 and 28 kDa.

K^+ channels regulate the membrane potential of many animal cells and represent a highly diverse family of transmembrane proteins. In excitable tissues, voltage-dependent K^+ channels control the duration of action potentials, cardiac pacemaking, and neurotransmitter secretion from presynaptic nerve endings (Hille, 1984). Through the use of mutants and specific neurotoxins, K^+ channel proteins have recently become amenable to molecular genetic and biochemical analysis [reviewed in Jan and Jan (1989) and Dolly (1988)]. In *Drosophila*, different A-type K^+ channel subunits have been shown to result from alternative splicing of the complex "*shaker*" gene locus (Pongs et al., 1988; Schwarz et al., 1988). In the same organism, a family of *shaker*-related genes has recently been identified which may encode other K^+ channel subtypes (Butler et al., 1989). For mammalian brain and muscle, evidence for the heterogeneity of K^+ channels mainly comes from electrophysiological and pharmacological data (Hille, 1984).

β-Bungarotoxin (β-Butx)[1] is a basic protein from snake venom composed of two disulfide-linked subunits of M_r 13 500 and 7000 (Kelly & Brown, 1974). The toxin exhibits Ca^{2+}-dependent phospholipase A_2 activity and potently inhibits

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[1] Abbreviations: β-Butx, β-bungarotoxin; Dtx-I, dendrotoxin I; MCDP, mast cell degranulating peptide; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; SSANPAH, sulfosuccinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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acetylcholine release at the neuromuscular junction (Abe et al., 1977). In the central nervous system, β-Butx is cytotoxic for cholinergic and γ-aminobutyric acid (GABA)-ergic neurons (Rehm et al., 1982). By electrophysiology, the mechanism of action of β-Butx at ganglionic and neuromuscular synapses has been shown to involve presynaptic K^+ channels of slow activation kinetics (Petersen et al., 1986; Dreyer & Penner, 1987). In chick brain, ^{125}I-labeled β-Butx binds to a membrane protein of M_r 430 000 which upon solubilization exhibits a marked K^+ dependence for conformational stability (Rehm & Betz, 1982, 1984). The β-Butx binding protein has been enriched about 300-fold from detergent extracts of chick brain membranes using chromatographic procedures and shown to interact with two putative A-type K^+ channel ligands, the facilitatory neurotoxins dendrotoxin I (Dtx-I, also called toxin I) and mast cell degranulating peptide (MCDP; Schmidt et al., 1988; Schmidt & Betz, 1988; Breeze & Dolly, 1989). On the basis of these data, the β-Butx binding protein has been proposed to represent a subtype of neuronal voltage-gated K^+ channels.

Both cDNA expression studies and cross-linking experiments indicate polypeptides of 64–77 kDa to constitute major subunits of K^+ channels in *Drosophila* (Pongs et al., 1988; Schwarz et al., 1988; Barbas et al., 1989) and mammals (Black & Dolly, 1986; Rehm et al., 1988). Purification of Dtx-I binding sites from rat brain has demonstrated an additional polypeptide of 38 kDa (Rehm & Lazdunski, 1988). With photoactivated cross-linking, a β-Butx binding component of ≈95 kDa has been identified in chick brain membranes (Rehm & Betz, 1983). Here, we have analyzed the subunit composition of the putative β-Butx-sensitive K^+ channel by employing different cross-linking methods. In addition to a large polypeptide of ≈75 kDa, a smaller subunit of ≈28 kDa is shown to be part of the toxin binding protein in chick brain.

Experimental Procedures

Materials. β-Butx was obtained from Miami Serpentarium (Miami, FL); Dtx-I and MCDP were kindly provided by Drs. H. Rehm and M. Lazdunski (Nice). β-Butx was labeled with ^{125}I to an initial specific activity of 1200–1800 Ci/mmol (Rehm & Betz, 1982).

Preparation of Membrane Fractions. Crude synaptic membranes were prepared from brains of newly hatched chicken by differential centrifugation as described (Rehm & Betz, 1982) with the following modifications. Instead of the final sucrose gradient step, the membranes were washed twice, each, in a 10-fold volume of (i) 3 mM potassium phosphate, pH 7.4, and 5 mM Na-EDTA; (ii) 20 mM K-HEPES, pH 7.4, 1 M KCl, and 5 mM Na-EDTA; and (iii) buffer A [20 mM K-HEPES, pH 7.4, 60 mM KCl, 2 mM Na-EDTA, and 0.01 % (w/v) NaN_3] by homogenization in a Potter homogenizer and subsequent centrifugation at 30000*g* for 20 min. The final membrane pellet was resuspended in buffer A (total volume 4 mL/g of membranes), frozen on liquid nitrogen, and stored at −70 °C. The protease inhibitors phenylmethanesulfonyl fluoride (final concentration 0.1 mM) and aprotinin (1.6 units/L) were freshly added before homogenization and hypotonic washes.

Cross-Linking of ^{125}I-β-Butx to Membranes. (A) Chemical Cross-Linking with the Water-Soluble Carbodiimide EDAC. Membrane fractions [250 μL, containing about 2.5 mg of protein, as determined by fluorescamine assay according to Schmidt and Betz (1988)] were supplemented with 1 mM $CaCl_2$, 5 mM $MnCl_2$, and 50 μM cytochrome *c* and incubated with 0.1–3.2 nM ^{125}I-β-Butx for 30 min at 22 °C. (In some experiments, membranes were pretreated with 2.5 mM dithiothreitol for 30 min at 37 °C. This incubation reduced retention of cross-linked membrane proteins on top of the stacking gel under nonreducing electrophoresis conditions. Where indicated, the membranes were additionally incubated with 40 mM iodoacetic acid for 30 min at 22 °C.) After dilution with 30 mL of ice-cold buffer B (buffer A containing 1 mM $CaCl_2$ and 5 mM $MnCl_2$ instead of EDTA) in 200 mM KCl, membranes were collected by centrifugation at 25000*g* for 20 min and resuspended in 0.5 mL of 1–100 mM EDAC (Bio-Rad) freshly dissolved in buffer B. After 30 min at 4 °C, the cross-linking reaction was quenched with 100 μL of 1 M glycine. The membranes were washed with 30 mL of ice-cold 5 mM EDTA, pH 7.4, and solubilized in 250 μL of 0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, and 0.01% (w/v) bromophenol blue. Routinely, half of the solubilized material was reduced with 10 μL of 1 M dithiothreitol for 10 min at 95 °C and separated on a 10% SDS–polyacrylamide gel (Laemmli, 1970). Remaining aliquots were incubated with 5 M urea, 100 μg of cytochrome *c*, and 10 μg of β-Butx for 30 min at 56 °C and subjected to electrophoresis on 3-mm-thick 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) under nonreducing conditions. After being fixed, stained with Coomassie Brillant Blue R 250 (Serva), and destained, the gels were dried on filter paper and exposed to Kodak X-Omat AR-5 film at −70 °C for 2–20 days using an intensifying screen. Molecular weight markers (Sigma) were either prestained subunits of triosephosphate isomerase (M_r 26 600), lactic acid dehydrogenase (M_r 48 500), pyruvate kinase (M_r 58 000), fructose-6-phosphate kinase (M_r 84 000), β-galactosidase (M_r 116 000), and α_2-macroglobulin (M_r 180 000) or unstained carbonic anhydrase (M_r 29 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), and subunits of phosphorylase B (M_r 97 400), β-galactosidase (M_r 116 000), and myosin (rabbit muscle, M_r 205 000). Molecular weights determined with either set of markers did not differ significantly.

(B) Photochemical Cross-Linking with the Heterobifunctional Reagent SSANPAH. After incubation with ^{125}I-β-Butx and washing (see above), the membrane pellets were resuspended in 2 mL of buffer B. SSANPAH dissolved in dried dimethyl sulfoxide (up to 100 μL per sample) was added in the dark to a final concentration of 10–1250 μM. After 15 min at 22 °C, unreacted reagent was quenched with 100 μL of 1 M Tris-HCl, pH 7.4. The samples were transferred to 4 wells, each, of a 24-well microtiter plate, and, after 5 min, cross-linking was initiated by photolysis with 5 flashes from a xenon flash lamp (Kiehm & Ji, 1977; Langosch et al., 1988). Subsequent washing and electrophoresis were performed as detailed above.

Results

Cross-linking of ^{125}I-β-Butx to chick brain membranes using the water-soluble carbodiimide EDAC produced two major labeled adducts which could be resolved upon 10% SDS–PAGE under reducing conditions (Figure 1). The larger adduct of 90 ± 3 kDa usually displayed some size heterogeneity, whereas the smaller one of 46 ± 1 kDa exhibited a more uniform appearance (n = 6). With rising EDAC concentrations (4–100 mM), both bands were increasingly labeled, their relative intensities staying, however, approximately constant (ratio 46 kDa/90 kDa ≈ 0.7–1.0). The adducts were not seen when cross-linker was omitted, or unlabeled β-Butx added in excess to the incubations. Control experiments showed that the total protein pattern of the membranes was unaffected at cross-linker concentrations up to 20 mM (compare Coomassie Blue staining shown in Figure 3). This is

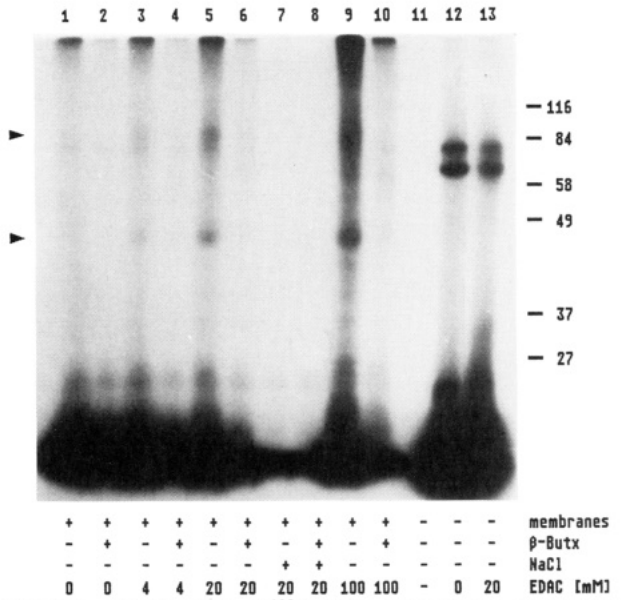

FIGURE 1: Cross-linking of ^{125}I-β-Butx (0.8 nM) to chick brain membranes using different concentrations of EDAC (as indicated below the figure). Controls included cross-linking in the presence of 170 nM unlabeled β-Butx and/or 0.5 M NaCl to reveal unspecific labeling, as well as omission of cross-linker (see figure). Lanes 12 and 13 contained incubations without membranes which after EDAC treatment were directly applied to the gel. SDS–PAGE was performed under reducing conditions. Positions of molecular weight markers ($M_r \times 10^{-3}$) run in lane 11 are indicated. Arrows point to the two major adducts produced.

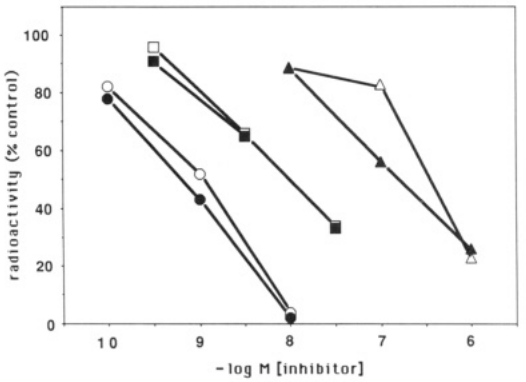

FIGURE 2: Pharmacology of ^{125}I-β-Butx cross-linking. The membranes were incubated with 0.8 nM ^{125}I-β-Butx in the presence of different inhibitory polypeptides, cross-linked using 20 mM EDAC, and analyzed by SDS–PAGE under reducing conditions. Autoradiograms were scanned in a laser densitometer, and relative peak heights of specific labeling (90-kDa adduct, open symbols; 46-kDa adduct, filled symbols) were plotted against inhibitor concentrations [β-Butx (○, ●); Dtx-I (□, ■); and MCDP (△, ▲)].

consistent with a low efficiency of incorporation (<3%) of the bound ^{125}I-β-Butx. Also, cross-linking of ^{125}I-β-Butx alone did not produce bands corresponding to those observed upon cross-linking in the presence of membranes (Figure 1, lanes 12 and 13). (Two radiolabeled bands of 84 and 66 kDa found in both cross-linked and untreated ^{125}I-β-Butx preparations corresponded to lactoperoxidase and bovine serum albumin added during the iodination procedure and not completely removed upon subsequent ion-exchange chromatography.)

Formation of the radiolabeled adducts of 90 and 46 kDa displayed the pharmacological characteristics expected for cross-linking to the previously described high-affinity β-Butx binding protein of chick brain membranes (Rehm & Betz, 1984; Schmidt et al., 1988). Figure 2 shows that the appearance of both labeled bands was inhibited by unlabeled β-Butx, Dtx-I, and MCDP. About 1 nM β-Butx, 10 nM

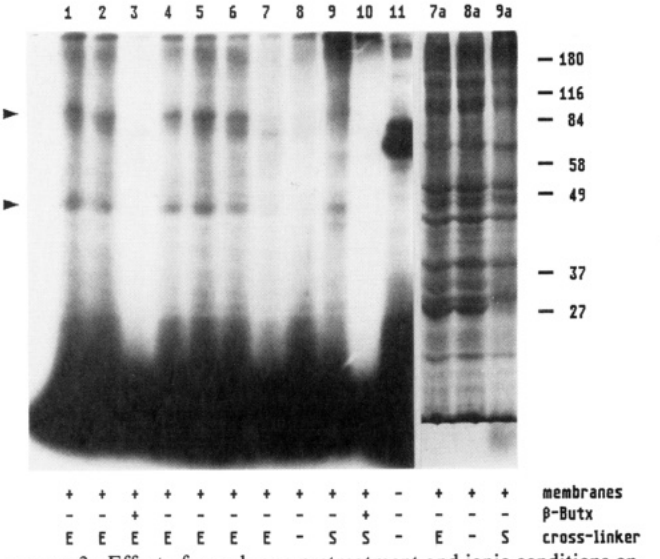

FIGURE 3: Effect of membrane pretreatment and ionic conditions on ^{125}I-β-Butx cross-linking. EDAC (E) and SSANPAH (S) were used as cross-linkers at concentrations of 20 and 0.5 mM, respectively. Membranes were pretreated with dithiothreitol except for sample 1. Sample 4 was additionally pretreated with 40 mM iodoacetamide for 30 min at 22 °C before addition of ^{125}I-β-Butx (0.8 mM), and sample 5 was incubated for 15 min with 80 mM iodoacetamide 30 min after addition of the radiotoxin. In sample 6, $MnCl_2$ was omitted during the toxin binding reaction, and in sample 7, $MnCl_2$ and $CaCl_2$ were replaced by 2 mM EDTA. SDS–PAGE was performed under reducing conditions. Lanes 1–11, autoradiograph; lanes 7a–9a, protein staining of corresponding lanes revealed by Coomassie Blue. The small difference seen in apparent molecular weight of the large adduct formed with prereduced and untreated membranes (lanes 1 and 2) was not significant when different experiments were compared.

Dtx-I, or 300 nM MCDP was required for a 50% reduction in labeling intensity of either polypeptide. These concentrations correspond closely to the previously reported K_i values of these toxins for reversible ^{125}I-β-Butx binding to chick brain membranes and detergent extracts (Schmidt et al., 1988; Schmidt & Betz, 1988). Also, specific cross-linking signals were barely detectable at ^{125}I-β-Butx concentrations <0.2 nM, optimally seen at radiotoxin concentrations of 0.8–1.0 nM (see Figures 1 and 3), and poorly resolved at >3 nM ^{125}I-β-Butx, largely due to high background radioactivity (not shown). These data are consistent with a K_d of 0.4 nM for reversible ^{125}I-β-Butx binding to chick brain membranes (Rehm & Betz, 1982).

Optimal binding of ^{125}I-β-Butx to brain membranes depends on the presence of divalent cations, in particular Ca^{2+} (Rehm & Betz, 1982; Schmidt et al., 1988). Accordingly, EDTA strongly reduced the formation of specific cross-linking products whereas omission of Mn^{2+} was without effect (Figure 3). Mn^{2+} is known to increase the apparent K_d of β-Butx binding to solubilized chick brain membranes (Schmidt & Betz, 1988).

The A-subunit of β-Butx has been implicated in binding of the toxin to its membrane receptor site (Rehm & Betz, 1982; Schmidt et al., 1988), although a role of the smaller B-subunit cannot presently be excluded. To reveal whether the two radiolabeled adducts obtained upon EDAC cross-linking contained one or both of the disulfide-linked toxin subunits, SDS–PAGE of cross-linked membrane preparations was also performed under nonreducing conditions. Unfortunately, however, the pattern of labeled bands seen after EDAC treatment on nonreducing gels was difficult to interpret, as (i) detection of specifically labeled adducts was impeded by high background radioactivity and (ii) multiple radioactive bands were visible in the 50- and 90-kDa ranges (not shown). The photoactivatable bivalent reagent SSANPAH was

therefore chosen for additional experiments. As shown in Figure 3 (lanes 9 and 10), this cross-linker (0.5 mM) produced results similar to those obtained with EDAC, i.e., two major radiolabeled adducts of comparable intensity. Although SSANPAH caused some unspecific cross-linking of membrane proteins (Figure 3, lane 9a), separation of the cross-linked membranes on nonreducing gels was much better than that of EDAC-treated material (not shown). Two radiolabeled adducts were again obtained, and no significant molecular weight differences were observed under these nonreducing electrophoresis conditions (calculated molecular weights of 96K ± 4K and 49K ± 2K, $n = 3$) as compared to conventional reducing SDS–PAGE (molecular weights of 95K ± 4K and 47K ± 2K, $n = 2$). Thus, the cross-linking adducts produced by SSANPAH apparently contained both subunits of β-Butx. The molecular weights of the toxin binding polypeptides then can be calculated as ≈75K and ≈28K, respectively.

Voltage-gated Na^+ and Ca^{2+} channels are known to contain subunits of M_r 27 000 and 38 000 which are disulfide-linked to other channel polypeptides (Catterall, 1988). To reveal whether accessible disulfide bridges are involved in ^{125}I-β-Butx binding and toxin receptor subunit association, membranes were pretreated for 30 min at 37 °C with 2.5 mM DTT (with or without subsequent incubation in the presence of iodoacetamide) before incubation and cross-linking with ^{125}I-β-Butx. This membrane pretreatment, however, had no major effect on the labeling and apparent molecular weights of the two major adducts seen upon SDS–PAGE under reducing (EDAC and SSANPAH; see Figure 3) and nonreducing (SSANPAH; not shown) conditions. These experments indicate that (i) the two labeled polypeptides identified here are not disulfide-linked to other proteins and (ii) accessible disulfide bonds are not important for β-Butx binding to its high-affinity binding site.

Discussion

In this study, two radiolabeled polypeptide adducts of 46 and 90 kDa were produced upon EDAC cross-linking of ^{125}I-β-Butx to chick brain membranes. Formation of these adducts displayed the pharmacological specificity expected for a K^+ channel associated β-Butx binding site both with respect to apparent radioligand affinity and inhibition by unlabeled β-Butx, Dtx-I, and MCDP [compare Schmidt et al. (1988)]. The two labeled bands did not change in relative intensities when concentrations of radioligand, inhibitors, or cross-linker were varied. Both adducts therefore most likely belong to the same high-affinity binding site of β-Butx. As EDAC generates peptide bonds without providing a spacer arm, the cross-linked proteins have to be in close proximity of the β-Butx binding region of its putative K^+ channel target. Considering the two band's identical dependence on EDAC concentration and the low efficiency of ^{125}I-β-Butx cross-linking under our experimental conditions, they probably correspond to independent polypeptides. Generation of the 90-kDa labeled band by homo- or heterooligomer formation of the 46-kDa adduct thus appears unlikely. On the basis of these data, we conclude that both adducts contained distinct polypeptides of the β-Butx binding protein which therefore is composed of at least two types of subunits.

Two comparable adducts were also obtained after cross-linking of ^{125}I-β-Butx using the photoactivatable reagent SSANPAH. Their molecular weights (95K ± 4K and 48K ± 3K) were not significantly affected by SDS–PAGE under reducing or nonreducing conditions. Thus, the cross-linking products seen here apparently contained both subunits of the radiotoxin, as has also been found in a previous cross-linking study (Rehm & Betz, 1983). Subtracting the M_r 20 500 of β-Butx, the molecular weights of the labeled polypeptide species can be calculated as ≈75K and ≈28K, respectively. It should be noted, however, that due to possible intramolecular bond formation molecular weight estimates after cross-linking are not very reliable. Indeed, the apparent molecular weights of the adducts formed after EDAC and SSANPAH treatment differed by up to 5 kDa. Some uncertainty thus remains about the true size of the putative K^+ channel subunits identified by our labeling procedure.

Although the precise nature of the polypeptides identified here by ^{125}I-β-Butx cross-linking has not been investigated, it is tempting to speculate that the 75-kDa polypeptide corresponds to the 55–77-kDa proteins identified by cDNA cloning and/or purification of *Drosophila* A-type and mammalian brain K^+ channels (Schwarz et al., 1988; Pongs et al., 1988; Tempel et al., 1988; Baumann et al., 1988; Rehm & Lazdunski, 1988). Also, cross-linking of Dtx-I and MCDP to chick and rat brain membranes has revealed polypeptides of M_r 75 000–77 000 (Black & Dolly, 1986; Rehm et al., 1988). An adduct corresponding to the 28-kDa polypeptide revealed here has, however, not been seen in previous cross-linking studies using other K^+ channel ligands, whereas a 38-kDa polypeptide was found in a purified Dtx-I receptor preparation isolated from rat brain (Rehm & Lazdunski, 1988). The function of these smaller polypeptides is presently unclear; for the expression of functional K^+ channels from cloned DNA, only the larger type of subunit appears to be required (Timpe et al., 1988; Iverson et al., 1988; Stühmer et al., 1988). Interestingly, however, multiple polypeptides in the M_r 25 000–40 000 range are invariably found in purified preparations of mammalian voltage-gated Na^+ and Ca^{2+} channel proteins (Catterall, 1988). In contrast to the 27- and 38-kDa Na^+ and Ca^+ channel subunits, however, the 75- and 28-kDa polypeptides of the β-Butx binding protein apparently are not involved in interchain disulfide linkages.

A previous study from our laboratory using the hydrophobic reagent succinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate had revealed an adduct of 116 kDa upon cross-linking of ^{125}I-β-Butx to chick brain membranes (Rehm & Betz, 1983). An adduct of similar size, however, was not obtained under cross-linking conditions employed here although in some experiments with SSANPAH a weak radiolabeled band of 115–120 kDa was seen (R. R. Schmidt, unpublished results). Such a band may represent a toxin-labeled heterooligomer containing the 75- and 28-kDa subunits, its formation being favored by treatment with the membrane-permeant succinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate reagent. This assumption is consistent with the relatively constant stoichiometry of ^{125}I-β-Butx labeling of both major adduct bands identified here and might indicate that similar amounts of the 75- and 28-kDa polypeptides are assembled in the native toxin receptor/channel complex. Sedimentation experiments and gel exclusion chromatography have indicated a $M_r \approx$ 430 000 for the β-Butx binding site solubilized from chick brain membranes (Rehm & Betz, 1984). This value has recently been confirmed for the Dtx-I receptor from rat brain (Black et al., 1988). Because of a striking sequence and structural homology of cloned *Drosophila* and rat K^+ channel sequences with the repeat domains of the quasi-tetrameric large subunits of voltage-gated Na^+ and Ca^{2+} channels, K^+ channels also are assumed to represent tetrameric transmembrane proteins (Catterall, 1988). We consequently suggest that the putative β-Butx-sensitive K^+ channel analyzed here contains four copies of the 75-kDa subunit together with multiple copies of the

28-kDa polypeptide, thus resulting in a macromolecular complex of calculated molecular weight close to 400 000. This not only is within the error range of previous molecular weight determinations (Rehm & Betz, 1984) but also is similar to current structural models of mammalian Na^+ and Ca^{2+} channels (Catterall, 1988). A conserved structural design thus indeed may be shared by the different voltage-gated channels of excitable membranes.

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