High-Conductance Calcium-Activated Potassium Channels in Rat Brain: Pharmacology, Distribution, and Subunit Composition†

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ABSTRACT: In rat brain, high-conductance Ca^{2+}-activated K^+ (BK) channels are targeted to axons and nerve terminals [Knaus, H. G., et al. (1996) *J. Neurosci. 16*, 955−963], but absolute levels of their regional expression and subunit composition have not yet been fully established. To investigate these issues, an IbTX analogue ([^{125}I]IbTX-D19Y/Y36F) was employed that selectively binds to neuronal BK channels with high affinity (K_d = 21 pM). Cross-linking experiments with [^{125}I]IbTX-D19Y/Y36F in the presence of a bifunctional reagent led to covalent incorporation of radioactivity into a protein with an apparent molecular mass of 25 kDa. Deglycosylation and immunoprecipitation studies with antibodies raised against α- and smooth muscle β-subunits of the BK channel suggest that the β-subunit that is associated with the neuronal BK channel is a novel protein. Quantitative receptor autoradiography reveals the highest levels of BK channel expression in the outer layers of the neocortex, hippocampal perforant path projections, and the interpeduncular nucleus. This distribution pattern has also been confirmed in immunocytochemical experiments with a BK channel-selective antibody. Taken together, these findings imply that neuronal BK channels exhibit a restricted distribution in brain and have a subunit composition different from those of their smooth muscle congeners.

In native tissues, voltage-gated and Ca^{2+}-activated K^+ (BK) channels are formed by tetrameric assembly of two distinct subunit components: the pore-forming α-subunit and an accessory β-subunit. Our current level of understanding regarding subunit composition of these ion channels is based primarily on identification of channel components using cross-linking and photoaffinity labeling protocols (*1*−*3*) followed by successful biochemical purification of native channel complexes (*4*−*6*). β-Subunits have been shown to influence the activity of the channel pore protein in several different fashions (*7*). Functional characterization of various β-subunit properties has been achieved by coexpression of these proteins with the pore-forming subunit using heterologous expression systems (*7*, *8*).

BK channels purified from aortic or tracheal smooth muscle membranes are composed of two noncovalently linked subunits: a pore-forming 125 kDa α-subunit and a glycosylated 31 kDa β-subunit (*6*, *9*). The α-subunit is a representative of the *Slo* family of potassium channels (*10*−*12*). It is predicted to contain seven transmembrane segments (S_0−S_6), a pore region located between S_5 and S_6 (*13*), and a large cytoplasmic tail where a locus, termed the "Ca^{2+} bowl", has been shown to contribute to determining some of the Ca^{2+} sensitivity properties of the channel. The β-subunit is a structurally unrelated polypeptide which modifies both the Ca^{2+} sensitivity and pharmacological properties of the α-subunit (*7*). To date, only one gene encoding the smooth muscle β-subunit has been cloned from mammalian sources (*14*−*16*). Although high expression levels of the β-subunit have been observed in various smooth muscle tissues (*14*, *15*, *17*), this subunit is virtually undetectable in human or canine brain (*16*). Two structurally unrelated proteins that interact with the *Drosophila* homologue of the BK channel (*dSlo*) have recently been cloned (*18*, *19*). These proteins influence multiple properties of *dSlo*, such as levels of expression at the level of the plasma membrane, as well as the functional activity of the channel.

In this study, we report the pharmacological characterization of neuronal BK channels through application of the selective BK channel inhibitor [^{125}I]IbTX-D19Y/Y36F,[1] and use this ligand in autoradiographic protocols combined with immunocytochemical studies to determine those regions in

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[1] Abbreviations: BSA, bovine serum albumin; ChTX, charybdotoxin; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; IbTX, iberiotoxin; IC_{50}, concentration causing half-maximal inhibition; [^{125}I]-IbTX-D19Y/Y36F, mono-[^{125}I]iodotyrosine-iberiotoxin-D19Y/Y36F; k_{+1}, association rate constant; k_{-1}, dissociation rate constant; K_d, dissociation constant; K_i, inhibition constant; n_H, pseudo-Hill slope; NxTX, noxiustoxin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, tetraethylammonium.

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the CNS that display channel expression. Cross-linking studies with [^{125}I]IbTX-D19Y/Y36F and rat brain membranes using a bifunctional cross-linking reagent, combined with immunoprecipitation studies, strongly suggest that a novel β-subunit is associated with the neuronal BK channel complex. These results imply that neuronal BK channels display a unique subunit composition which differs from that of their smooth muscle counterparts.

EXPERIMENTAL PROCEDURES

Materials. All tissue culture media and the LipofectAMINE reagent were from GIBCO, Life Technology (Vienna, Austria). COS-1 cells (culture CRL1650) were obtained from American Type Culture Collection (Rockville, MD). Recombinant *N*-glycanase was from Boehringer Mannheim. The FMOC (9-fluorenylmethoxycarbonyl) lysine core solid-phase peptide support was from NovaBiochem. Prestained molecular mass standards and Immobilon polyvinylidene difluoride membranes were obtained from Bio-Rad. Disuccinimidyl suberate (DSS) was from Pierce. Glass fiber filters (GF/C) were obtained from Whatman. Hyperfilm-βmax and [^{125}I]Microscales were purchased from Amersham. Cyanogen bromide-activated Sepharose was obtained from Pharmacia (Uppsala, Sweden). Triton X-100, protein A−Sepharose, paxilline, nitroblue tetrazolium, polyethyleneimine, penitrem A, affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indoyl phosphate, and bovine serum albumin were purchased from Sigma. Aflatrem and dehydrosoyasaponin-1 (DHS-I) were obtained from the Natural Product Chemistry Department at Merck Research Laboratories (Rahway, NJ). Charybdotoxin (ChTX) and iberiotoxin (IbTX) were produced by recombinant means as previously described (*20*). IbTX-D19Y/Y36F was expressed, purified, and labeled with ^{125}I as described previously (*21*). All other reagents were obtained from commercial sources and were of the highest purity grade commercially available.

Membrane Preparation and [^{125}I]IbTX-D19Y/Y36F Radioligand Binding Assays. Membranes derived from COS-1 cells transiently transfected with either α- or α- and β-subunits of the BK channel were prepared as previously described (*22*). HEK293 cells stably expressing BK channel α- or α- and β-subunits were obtained from O. Pongs (Zentrum für Molekulare Neurobiologie, Hamburg, Germany) and grown in Dulbecco's modified Eagle's medium in the presence of fetal calf serum and genetecin (0.8 mg/mL) until a confuency of 70−80% was obtained. Cells were scraped from the cell culture flasks and subjected to centrifugation for 5 min at 1000*g*; the supernatant was discarded and the pellet frozen at −20 °C. The pellet was thawed on ice in 10 mM Tris-HCl (pH 7.4) and 0.1 mM phenylmethanesulfonyl fluoride and stored on ice for 15 min. The cells were homogenized using a glass homogenizer, and the resulting homogenate was subjected to centrifugation for 15 min at 500*g*. The remaining supernatant was clarified by centrifugation for 45 min at 100000*g*. The supernatant was discarded, and the membrane pellet was resuspended in 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl. Aliquots were quick-frozen and stored at −80 °C. Purified rat synaptic membrane vesicles were prepared using well-established procedures as previously described (*21*). The interaction of [^{125}I]IbTX-D19Y/Y36F with membranes was monitored as previously described (*21*).

Antibody Production and Affinity Purification. Polyclonal serum was raised against residue positions 1118−1135 of *mSlo* (*10*) using the peptide sequence STANRPNRPKSRESRDKQ (anti-$\alpha_{1118-1135}$). Polyclonal serum raised against residues 85−102 (anti-β_{85-102}) and 118−132 (anti-$\beta_{118-132}$) of the rat BK β-subunit protein used the peptide sequences GRWAMLYHTEDTRDQNQQ and VDVKKVRANFYKHHN, respectively. Antibodies were raised and affinity purified on their respective antigen columns as described previously (*20*).

Cross-Linking of [^{125}I]IbTX-D19Y/Y36F to Rat Brain Synaptosomal Plasma Membranes. Rat brain synaptosomal plasma membranes were incubated with 80−120 pM [^{125}I]-IbTX-D19Y/Y36F for 12 h at room temperature until equilibrium was achieved. Membranes were collected by centrifugation at 20000*g* and washed three times, and the final pellet was resuspended in 50 mM sodium phosphate and 150 mM NaCl (pH 9.0). The bifunctional cross-linking reagent, DSS, was dissolved in dimethyl sulfoxide and added repetitively in five aliquots up to a final concentration of 2 mM. Incubation was continued for 2 min at room temperature, and the cross-linking reaction was stopped by addition of Tris-HCl (pH 7.5) up to a final concentration of 50 mM. Samples were washed three times by centrifugation with 50 mM sodium phosphate and 150 mM NaCl (pH 9.0), finally resuspended in sample buffer [5% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol], and separated by 13 or 15% SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The gels were dried and subjected to autoradiography overnight on a Fuji BAS 1000 Phospho Imager, or exposed to Kodak XAR film at −80 °C.

Isolation of [^{125}I]IbTX-D19Y/Y36F-Labeled BK Channel β-Subunits. Covalent labeling of rat brain membranes with [^{125}I]IbTX-D19Y/Y36F is described above. Membrane protein (5−10 mg) was resuspended in 2 mL of sample buffer (5% SDS and 1% β-mercaptoethanol) and loaded onto a 12% SDS−PAGE gel. The sample was separated on a Bio-Rad model 591 prep cell (6 cm running gel, gel volume of ∼50 mL) at a constant power of 12 W. Sample collection was started after the dye front had eluted. Elution of [^{125}I]IbTX-D19Y/Y36F-labeled fractions was monitored with a γ-radiation counter. The toxin-labeled BK channel β-subunit was collected and concentrated 10-fold using a Centriprep-10 device (Amicon, Inc., Witten, Germany), followed by dialysis for 4 h at 4 °C against 100 mM Tris-HCl and 0.5% Triton X-100 (pH 7.4).

Immunoblot analysis was performed as previously described (*20*).

Deglycosylation of [^{125}I]IbTX-D19Y/Y36F-Labeled BK Channel β-Subunits. Samples were denatured by heating for 5 min at 50 °C in the presence of 0.5% SDS and 50 mM β-mercaptoethanol, and then adjusted to 1.5% Triton X-100. Deglycosylation was started by addition of 1 IU of recombinant *N*-glycanase F. After incubation at 37 °C for 12 h, the reaction was stopped by addition of SDS sample buffer.

Immunoprecipitation Experiments. For all immunoprecipitation studies, affinity-purified anti-α and anti-β antibodies were prebound to protein A−Sepharose by incubating 100 μL of the affinity-purified antibody and 100 μL of the packed gel in 100 μL of RIA buffer [20 mM Tris-HCl, 150

mM NaCl, and 0.5% (w/v) Triton X-100 (pH 7.4)] for 60 min under gentle rotation. The gel was washed three times with 2 mL of RIA buffer before the addition of either solubilized cross-linked rat brain synaptosomal plasma membranes, cross-linked bovine smooth muscle membranes, solubilized [^{125}I]IbTX-D19Y/Y36F-labeled rat brain synaptosomal plasma membranes, or isolated cross-linked β-subunits. Incubation was continued for 12 h at 4 °C under gentle rotation, and the gel was washed three times with 2 mL of RIA buffer. The amount of radioactivity associated with the gel was determined in a γ-radiation counter. SDS−PAGE sample buffer was added to samples containing covalently labeled β-subunits. Samples were heated to 37 °C for 2 h in the presence of 1.5% β-mercaptoethanol, and were then subjected to SDS−PAGE employing 15% polyacrylamide gels.

Analysis of Radioligand Binding Data and Protein Determination. All radioligand binding data were analyzed as described elsewhere (*21*). The concentration of membrane protein was determined according to a method published previously (*23*) using bovine serum albumin as a standard.

Receptor Autoradiography of Neuronal BK Channels with [^{125}I]IbTX-D19Y/Y36F. Rats were anesthetized with carbon dioxide and sacrificed by rapid decapitation. Brains were quickly removed and frozen in isopentane at −40 °C. Twenty micrometer coronal or sagital cryostat sections were thaw-mounted onto gelatin-coated glass slides and stored at −20 °C. The sections were incubated with 60−80 pM [^{125}I]IbTX-D19Y/Y36F in 20 mM Tris-HCl (pH 7.4), 0.1% BSA, and 10 mM NaCl for approximately 12 h at 22 °C. The level of nonspecific binding was determined in a series of adjacent sections by inclusion of 10 nM IbTX in the incubation solution. Thereafter, sections were washed for a total of 4 h with ice-cold washing buffer [20 mM Tris-HCl (pH 7.4) and 150 mM NaCl], changing the washing buffer four times. Finally, the sections were briefly rinsed in cold distilled water and dried in a stream of cold air. Dried sections were exposed to Hyperfilm-βmax for 2−5 days at 22 °C. Autoradiograms were developed with Kodak D19 developer and fixed with Kodak AL4. To facilitate quantification, radiolabeled standards ([^{125}I]Microscales) were included in some exposures. For quantification of toxin binding, the regions of interest were excised after film exposure to determine the absolute levels of [^{125}I]IbTX-D19Y/Y36F binding, as well as protein concentrations. The values obtained were correlated with the [^{125}I]Microscale determinations.

Immunohistochemistry of BK Channels in Rat Brain. All experiments were performed as described previously (*20*) by employing the indirect peroxidase/anti-peroxidase technique (*24*).

RESULTS

[^{125}I]IbTX-D19Y/Y36F Binds to BK Channels in Rat Brain Synaptic Plasma Membrane Vesicles. Previously, an analogue of iberiotoxin radiolabeled with tritium ([^{3}H]IbTX-D19C) was used to specifically label rat brain BK channels (*20*), but the low specific activity of this ligand precluded its use in autoradiographic and certain biochemical studies of BK channels. In this study, we have characterized the interaction of [^{125}I]IbTX-D19Y/Y36F, an iberiotoxin analogue which selectively binds to BK channels (*21*), with

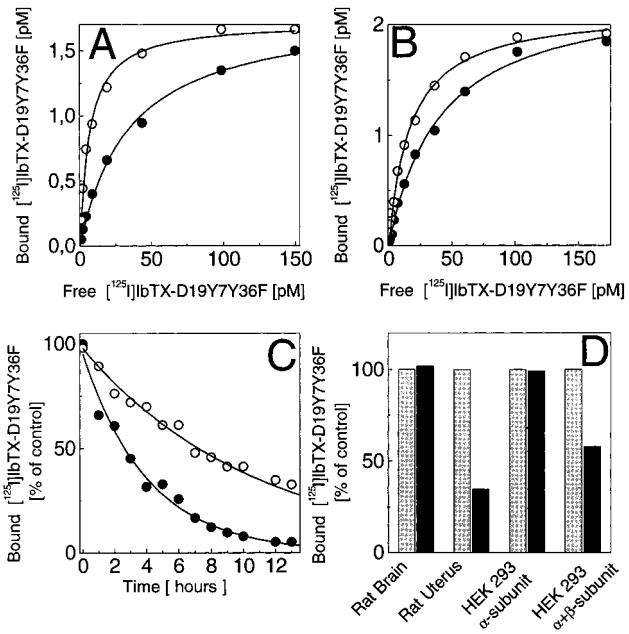

FIGURE 1: Characteristics of [^{125}I]IbTX-D19Y/Y36F binding. (A) Binding of [^{125}I]IbTX-D19Y/Y36F to rat brain and uterus smooth muscle membranes. Rat brain synaptosomal (●) or uterine sarcolemmal membrane vesicles (○) were incubated with increasing concentrations of [^{125}I]IbTX-D19Y/Y36F in a medium consisting of 20 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 0.1% BSA at 22 °C for 60 h. The extent of nonspecific binding was determined in the presence of 10 nM ChTX. Specific binding data were fittted as described in Experimental Procedures: (●) K_d = 32.1 pM and B_{max} = 0.295 pmol/mg of protein and (○) K_d = 7.23 pM and B_{max} = 0.108 pmol/mg of protein. (B) Binding of [^{125}I]IbTX-D19Y/Y36F to COS-1 membranes. Membranes, prepared from COS-1 cells transiently transfected with either the BK channel α-subunit (●) or α- and β-subunits (○), were incubated with increasing concentrations of [^{125}I]IbTX-D19Y/Y36F in the medium described for panel A at 22 °C for 24 h. Fitting of specific binding data yielded a K_d of 14.9 pM and a B_{max} of 8.35 pmol/mg of protein for membranes containing α- and β-subunits and a K_d of 29.7 pM and a B_{max} of 4.94 pmol/mg of protein for membranes containing the α-subunit. (C) Toxin dissociation kinetics. Rat brain synaptosomal (●) or smooth muscle plasma membrane vesicles (○) were incubated with 5 pM [^{125}I]IbTX-D19Y/Y36F for 24 h at room temperature. Dissociation of bound ligand was initiated by addition of 10 nM IbTX, and samples were incubated at room termperature for different periods of time. Fitting of the experimental data to monoexponential kinetics yielded k_{-1} values of 0.00138 and 0.0042 min^{-1} for either smooth muscle or rat brain membrane receptors, respectively. (D) Modulation of [^{125}I]IbTX-D19Y/Y36F binding by DHS-1. Membrane vesicles prepared from different sources were incubated with 4 pM [^{125}I]IbTX-D19Y36F in the absence (gray columns) or presence (black columns) of 10 μM DHS-1 for 24 h at room temperature. Inhibition of toxin binding was assessed relative to an untreated control.

synaptic plasma membranes derived from rat brain. This radioligand labels a single class of binding sites (K_d = 21 pM; n = 5) that display a maximum density of 0.30 pmol/mg of protein (Figure 1A). These values are similar to those reported for the interaction of [^{3}H]IbTX-D19C with the same type of membranes (*20*). The kinetic binding parameters that were determined for [^{125}I]IbTX-D19Y/Y36F confirm the equilibrium binding data ($k_{+1} = 2.1 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 4.9 \times 10^{-5}$ s^{-1}; calculated K_d of 23 pM, Figure 1C; association kinetics not shown; n = 4). We also investigated the binding properties of this ligand in rat uterine smooth muscle membranes. In this tissue, [^{125}I]IbTX-D19Y/Y36F displayed an equilibrium binding constant of 7 pM (Figure

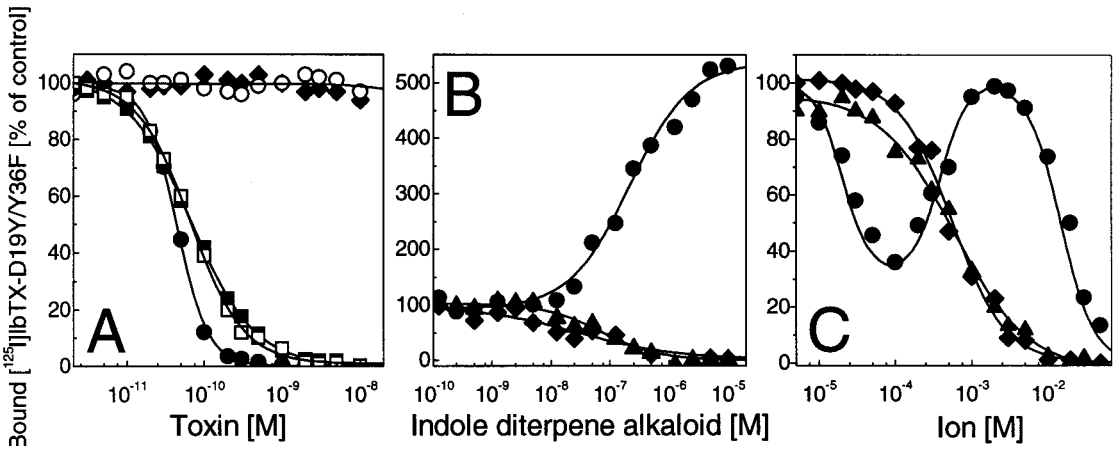

FIGURE 2: Interaction of [^{125}I]IbTX-D19Y/Y36F with rat brain membranes. (A) Inhibition of [^{125}I]IbTX-D19Y/Y36F binding to rat brain membranes by peptidyl toxins. Rat brain synaptosomal plasma membrane vesicles were incubated with [^{125}I]IbTX-D19Y/Y36F in the absence or presence of increasing concentrations of either IbTX (■), IbTX-D19Y/Y36F (□), ChTX (●), MgTX (◆), or α-DaTX (○) at 22 °C for 12 h. Inhibition of binding by these peptides was assessed relative to an untreated control. For IbTX, IC_{50} = 68 pM and n_H = 1.05. For IbTX-D19Y/Y36F, IC_{50} = 67 pM and n_H = 1.06. For ChTX, IC_{50} = 45 pM and n_H = 2.2. (B) Effect of indole diterpenes. Rat brain membranes were incubated with 1.7 pM [^{125}I]IbTX-D19Y/Y36F in an assay volume of 8 mL for 60 h at room temperature in the absence or presence of increasing concentrations of either paxilline (●), aflatrem (▲), or penitrem A (◆). Modulation of binding was assessed relative to an untreated control. For paxilline, EC_{50} = 216 nM and n_H = 0.85, with a maximal stimulation of 536%. For aflatrem, IC_{50} = 72.8 nM and n_H = 0.89. For penitrem A, IC_{50} = 29.8 nM and n_H = 0.58. (C) Effect of ions. Rat brain plasma membrane vesicles were incubated with 2.0−4.1 pM [^{125}I]IbTX-D19Y/Y36F in the absence or presence of increasing concentrations of either $CaCl_2$ (◆), KCl (●), or TEA (▲). Inhibition of binding was asssessed relative to an untreated control. For $CaCl_2$, IC_{50} = 0.58 mM and n_H = 0.95. For TEA, IC_{50} = 0.55 mM and n_H = 1.2. For KCl, $IC_{50}(I)$ = 85 μM, $EC_{50}(II)$ = 7 mM, and $IC_{50}(III)$ = 90 mM.

1A). This binding constant is virtually identical to the one measured in bovine aortic smooth muscle membranes (*21*). Thus, [^{125}I]IbTX-D19Y/Y36F labels neuronal BK channels with an approximately 3-fold lower affinity than it does smooth muscle BK channels. The difference in toxin affinities in these two tissues can be ascribed to differences in the respective [^{125}I]IbTX-D19Y/Y36F dissociation kinetics that were observed (Figure 1C).

It has previously been shown that the β-subunit of the BK channel influences the affinity of [^{125}I]ChTX for the pore-forming α-subunit (*22*, *25*). BK channels which are composed of α- and β-subunit complexes display an almost 50-fold higher affinity for [^{125}I]ChTX when compared to channels formed solely by association of α-subunits. To reveal possible differences in BK channels from different tissues, we compared toxin binding affinities from neuronal and smooth muscle channels (Figure 1A) with those of COS-1 cells expressing either the BK channel α-subunit or an α- and β-subunit complex (Figure 1B). [^{125}I]IbTX-D19Y/Y36F binds to membranes expressing only α-subunits with an equilibrium dissociation constant of 30 pM (a value very close to that observed in rat brain membranes), while membranes containing α- and β-subunit complexes displayed a K_d of 14 pM (a value closer to the K_d measured in smooth muscle membranes).

Stimulation of BK channel activity in smooth muscle by the glycosylated triterpene, DHS-1, requires the presence of the β-subunit (*7*). On the basis of this observation, we determined whether DHS-I could be used to characterize neuronal tissue for the presence of a smooth muscle-like β-subunit. Rat brain membranes were incubated with [^{125}I]-IbTX-D19Y/Y36F, in the absence or presence of DHS-1, until equilibrium was established (Figure 1D). In parallel experiments, plasma membranes from smooth muscle tissues in which BK channels are known to be composed of both rat α- and β-subunits (rat uterus), or those prepared from HEK293 cells expressing α- and β-subunits, or the α-subunit alone, were also investigated (Figure 1D). DHS-1, at a concentration of 10 μM, inhibited [^{125}I]IbTX-D19Y36F binding to smooth muscle membranes and HEK293 cells expressing α- and β-subunits (54 and 37% of the control level of binding, respectively; Figure 1D). However, toxin binding to plasma membranes derived from either rat brain, or from HEK293 cells expressing only α-subunits, was unaffected by DHS-1.

Other pharmacological properties of the [^{125}I]IbTX-D19Y/Y36F interaction with neuronal BK channels appear to be similar to those found with smooth muscle BK channels. Thus, peptides known to selectively interact with voltage-gated K^+ channels [e.g., MgTX and α-dendrotoxin (α-DTX)] do not affect [^{125}I]IbTX-D19Y/Y36F binding, while ChTX and IbTX block toxin binding as expected (Figure 2A). Moreover, a number of indole diterpenes (e.g., paxilline and aflatrem) modulate [^{125}I]IbTX-D19Y/Y36F binding to rat brain membranes with the same profile (Figure 2B) that has previously been described in experiments with smooth muscle membranes (*21*), consistent with the idea that these agents interact exclusively with the pore-forming subunit of the BK channel complex (*26*). Several ions such as K^+, Ca^{2+}, and TEA, which are known to interact with BK channels by binding to sites located along the ion conduction pathway, also modulate [^{125}I]IbTX-D19Y/Y36F binding to rat brain membranes (Figure 2C), displaying the same profiles as those observed with smooth muscle membranes (*21*, *27*). Taken together, these data support the notion that [^{125}I]IbTX-D19Y/Y36F is a selective, high-specific activity radioligand for neuronal BK channels.

Subunit Composition of Neuronal BK Channels. To gain some insight into the subunit composition of BK channels in rat brain, we performed cross-linking studies using [^{125}I]-IbTX-D19Y/Y36F and the bifunctional cross-linking reagent, DSS (Figure 3). In rat brain membranes, [^{125}I]IbTX-D19Y/

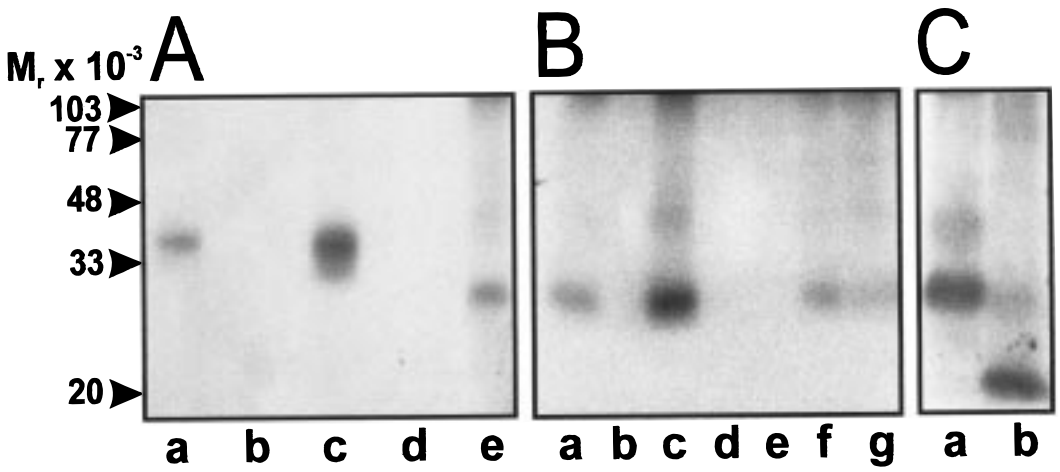

FIGURE 3: Covalent incorporation of [^{125}I]IbTX-D19Y/Y36F into the neuronal β-subunit of the BK channel. (A) Membrane vesicles derived from either bovine aortic **(**lanes a and b**)** or tracheal **(**lanes c and d**)** smooth muscle or rat brain synaptosomal plasma membrane vesicles **(**lane e**)** were incubated with 60 pM [^{125}I]IbTX-D19Y/Y36F in the absence **(**lanes a, c, and e**)** or presence **(**lanes b and d**)** of 10 nM ChTX until equilibrium was achieved. Samples were subjected to reaction with DSS and separated by SDS−PAGE as described in Experimental Procedures. The migration of standards is denoted by arrows. (B) Rat brain synaptosomal plasma membrane vesicles were incubated with 60 pM [^{125}I]IbTX-D19Y/Y36F alone **(**a**)** or in the presence of 3 nM IbTX **(**b**)**, 3 μM paxilline (c), 1 mM TEA **(**d**)**, 10 nM ChTX **(**e), 3 nM MgTX **(**f**)**, or 1 mM KCl **(**g**)**. Samples were subjected to reaction with DSS and separated by SDS−PAGE. (C) Deglycosylation studies. [^{125}I]IbTX-D19Y/Y36F-cross-linked brain membranes were denatured by heating for 5 min at 50 °C in the presence of 0.5% SDS and 50 mM β-mercaptoethanol. The samples were then adjusted to 1.5% Triton X-100, and deglycosylation was started by addition of 1 IU of recombinant *N*-glycanase F. After incubation at 37 °C for 12 h, the reaction was stopped by addition of SDS−PAGE sample buffer **(**lane b**)**. In lane a, samples were treated identically, but in absence of enzyme.

Y36F is specifically cross-linked to a 25 kDa polypeptide (after subtracting the molecular mass contributed by the toxin itself). This is in marked contrast to the situation with smooth muscle membranes where incorporation of radioactivity occurs into a 31 kDa polypeptide (Figure 3A). Labeling of a 31 kDa polypeptide was never observed in the rat brain preparations. Covalent incorporation of radioactivity into rat brain membranes was modulated by agents which are known to affect either [^{125}I]ChTX (*1*) or [^{125}I]IbTX-D19Y/Y36F (*21*) cross-linking to smooth muscle BK channels. The cross-linking reaction was also affected by agents known to modulate reversible [^{125}I]IbTX-D19Y/Y36F binding to neuronal BK channels (Figure 2). For instance, ChTX, IbTX, TEA, and aflatrem all decreased the extent of toxin incorporation, while paxilline stimulated covalent labeling of the polypeptide (Figure 3B). Structurally related toxins which do not interact with BK channels [e.g., MgTX (*28*) or α-DTX (*29*)] did not affect the cross-linking reaction (Figure 3B). These findings indicate that the 25 kDa polypeptide (which we term the neuronal β-subunit) is intimately associated with the BK channel complex.

To exclude the possibility that differing extents of glycosylation account for the observed differences in apparent molecular masses between the smooth muscle and neuronal BK channel β-subunits, deglycosylation studies in which *N*-glycosidase F was used were performed with cross-linked rat brain membranes (Figure 3C). In parallel experiments, we also subjected an [^{125}I]IbTX-D19Y/Y36F-labeled bovine aortic smooth muscle preparation to deglycosylation since the biochemical properties of this BK channel subunit are well-characterized (*6*). Together, these experiments indicate that both smooth muscle and neuronal BK channel β-subunits possess ~10 kDa of complex carbohydrates attached through N-linkages to each respective protein. These data suggest that the neuronal BK channel β-subunit is also a membrane-associated protein. Upon deglycosylation, the apparent molecular masses of the smooth muscle and neuronal BK channel β-subunits decrease from 35 to 25 kDa, and from 29 to 20 kDa, respectively. After subtracting the molecular mass contributed by covalently linked [^{125}I]IbTX-D19Y/Y36F, we estimate an apparent molecular masses of 21 kDa for the deglycosylated smooth muscle β-subunit (in agreement with previously published data; *6*) and 16 kDa for the neuronal BK channel β-subunit.

To confirm that this newly identified polypeptide is an integral component of neuronal BK channels, the nature of the channel complex was probed in immunoprecipitation experiments. Since none of the previously raised antibodies were able to quantitatively precipitate [^{125}I]IbTX-D19Y/Y36F-labeled receptors from digitonin-solubilzed rat brain membranes (*30*), we raised a battery of additional sequence-directed antibodies against the BK channel α-subunit. One antiserum (anti-$\alpha_{1118-1135}$) yielded a sufficiently high antibody titer to be characterized in greater detail. The respective recognition sequence is comprised of the last 18 residues of the α-subunit C-terminal domain. Anti-$\alpha_{1118-1135}$ specifically recognized the rat brain α-subunit in Western blots (Figure 4A). In immunoprecipitation experiments in which detergent-solubilzed [^{125}I]IbTX-D19Y/Y36F-labeled synaptic membranes were used, this antibody quantitatively immunoprecipitated the receptor complex (Figure 4B). In addition, as shown in Figure 4C, anti-$\alpha_{1118-1135}$ also precipitated the neuronal BK channel β-subunit that was covalently linked with toxin. Similar results were obtained with anti-$\alpha_{913-926}$. These data imply that a tight interaction must exist between this accessory subunit and the α-subunit of the neuronal BK channel.

We next asked the question of whether either brain-specific cleavage of a signal peptide or proteolysis at the C- or N-terminus of a smooth muscle-type β-subunit that might occur during membrane isolation could account for the

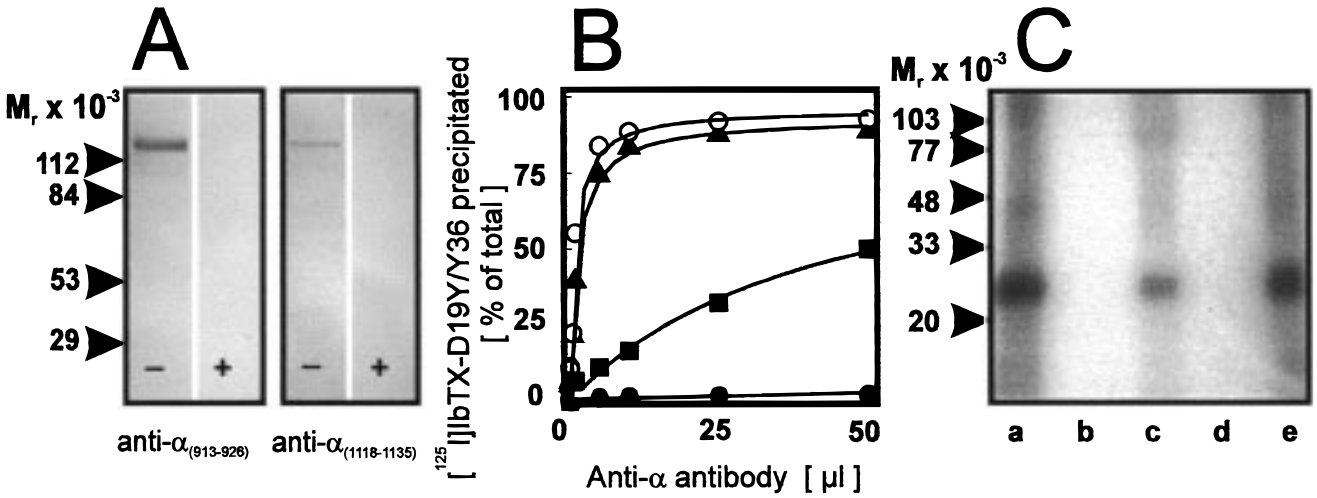

FIGURE 4: Characterization of rat BK channels using α-subunit antibodies. (A) Immunoblot analysis of the α-subunit of the BK channel. Membranes prepared from HEK293 cells stably transfected with the rat BK channel α-subunit were separated by SDS−PAGE and immunoblotted using either anti-$\alpha_{913-926}$ or anti-$\alpha_{1118-1135}$ in the absence (−) or presence (+) of the corrresponding competing peptide (1 μM). Positions of the molecular mass standards are denoted by arrows. (B) Digitonin-solubilized rat brain receptors labeled with [^{125}I]-IbTX-D19Y/Y36F were immunoprecipitated with increasing concentrations of either anti-$\alpha_{913-926}$ (■), anti-$\alpha_{1118-1135}$ (○), affinity-purified anti-$\alpha_{1118-1135}$ (▲), or preimmune serum of anti-$\alpha_{1118-1135}$ (●), as described in Experimental Procedures. Data from a single experiment are shown. (C) Immunoprecipitation of rat brain BK channel β-subunits covalently labeled with [^{125}I]IbTX-D19Y/Y36F by anti-$\alpha_{1118-1135}$. Rat brain membranes were covalently labeled with [^{125}I]IbTX-D19Y/Y36F in the presence of DSS, and solubilized with digitonin. Solubilized starting material (a) was subjected to immunoprecipitation with 15 (c) or 50 μL (e) of affinity-purified anti-$\alpha_{1118-1135}$ or the respective amount of preimmune serum (b and d). Samples were resuspended in SDS−PAGE sample buffer and subjected to electrophoresis using 12% acrylamide gels. The migration of molecular mass standards is shown. Starting material (SM), preimmune sera (PI), and anti-α sera (lane IM) were the conditions that were examined.

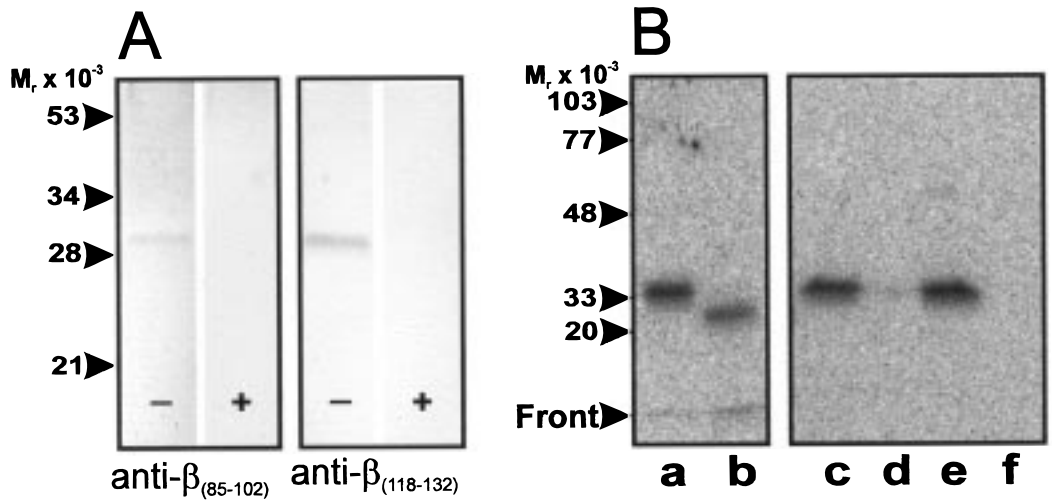

FIGURE 5: Characterization of rat BK channels using β-subunit antibodies. (A) Immunoblot analysis of the β-subunit of the BK channel. Membranes prepared from HEK293 cells stably transfected with the smooth muscle β-subunit of the rat BK channel were separated by SDS−PAGE under reducing conditions and immunoblotted using either anti-β_{85-102} or anti-$\beta_{118-132}$ in the absence **(**−**)** or presence **(**+**)** of the corresponding competing peptide (1 μM). The positions of the molecular mass standards are denoted. (B) Immunoprecipitation of isolated [^{125}I]IbTX-D19Y/Y36F covalently labeled BK channel β-subunits with anti-β_{85-102} and anti-$\beta_{118-132}$. BK channel β-subunits derived from either smooth muscle (a) or neuronal (b) membranes were isolated by preparative SDS−PAGE after covalent incorporation of [^{125}I]-IbTX-D19Y/Y36F in the presence of DSS. Equivalent amounts of either isolated smooth muscle **(**c and e**)** or neuronal **(**d and f**)** BK channel β-subunits were subjected to immunoprecipitation with either anti-β_{85-102} **(**c and d**)** or anti-$\beta_{118-132}$ **(**e and f**)**. Samples were resuspended in SDS−PAGE sample buffer and subjected to electrophoresis using 12% acrylamide gels. The migration of molecular mass standards is shown.

observed difference in the apparent M_rs of the β-subunits from the two tissues. For this, we used previously characterized (*31*) as well as newly raised antibodies directed against the rat smooth muscle BK channel β-subunit. The ability of these antibodies to recognize rat tissue-derived β-subunits was investigated in Western blots. As shown in Figure 5A, anti-β_{85-102}, as well as anti-$\beta_{118-132}$, immunostained the smooth muscle β-subunit. Given these data, we subjected isolated [^{125}I]IbTX-D19Y/Y36F-labeled β-subunits to immunoprecipitation experiments using the same antibodies. As shown in Figure 5B, anti-β_{85-102} and anti-$\beta_{118-132}$ quantitatively precipitated the smooth muscle β-subunit. However, the neuronal β-subunit was not recognized by these antibodies. These data clearly indicate that smooth muscle and neuronal β-subunits are distinct structural entities and that the difference in apparent the M_rs cannot be explained by either brain-specific cleavage of a signal peptide or proteolysis of the subunit.

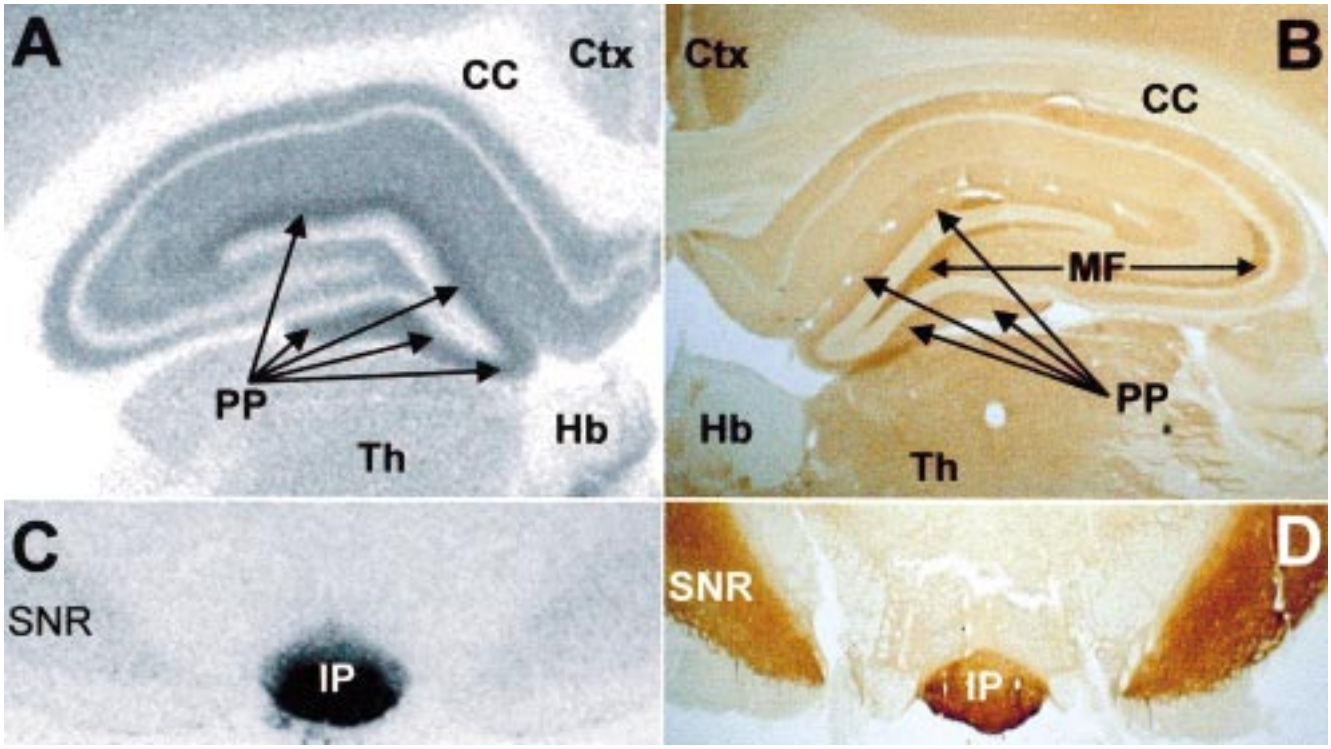

FIGURE 6: Distribution of [^{125}I]IbTX-D19Y/Y36F binding sites and expression of BK channels in rat brain. Rat brain coronal sections were processed for either autoradiography with [^{125}I]IbTX-D19Y/Y36F **(**A and C**)** or immunocytochemistry **(**B and D) by employing the sequence-directed antibody anti-$\alpha_{913-926}$. Both techniques reveal BK channel expression in hippocampal perforant path projections (PP), the terminal field of mossy fibers (MF), substantia nigra pars reticulata (SNR), the interpeduncular nucleus (IP), neocortex (CTX), and the thalamic nuclei (Th). The habenula (Hb), corpus callosum (CC), and the hippocampal granule and pyramidal cell layers are devoid of BK channels.

Together, the data indicate that rat BK channels are composed of two subunits (α and β). The α-subunit possesses an apparent molecular mass of ~125 kDa in both smooth muscle and brain (*20*, *30*). The respective deglycosylated β-subunits possess a molecular mass of either 21 kDa in smooth muscle or 16 kDa in brain. It is predicted that the neuronal β-subunit will be a unique protein.

Neuronal BK Channel Distribution. Given that [^{125}I]IbTX-D19Y/Y36F is a ligand that is suitable for characterizing neuronal BK channels, we used this probe to determine the distribution of BK channels in rat brain by receptor autoradiography. Tissue sections were also processed in parallel for antibody staining to compare directly the distribution of immunoreactivity and labeled receptors (Figure 6). For these immunostaining experiments, we used anti-$\alpha_{913-926}$, a sequence-directed antibody against the pore-forming α-subunit which has previously been shown to immunostain neuronal BK channels (*20*).

[^{125}I]IbTX-D19Y/Y36F-labeled receptors were present in widespread parts of the brain, including the cerebral neocortex, neostriatum, hippocampus, thalamus, substantia nigra pars reticulata, interpeduncular nucleus, and cerebellum (Figure 6A,C), but three brain regions exhibited very high levels of BK channel expression. A high BK channel density was observed in the outer neocortical layers. This distribution pattern was in good agreement with previous in situ hybridization data and antibody staining experiments (*20*). A second and well-defined brain region with high BK channel content was the hippocampal formation (arrowheads in Figure 6A). Receptor autoradiography revealed the highest levels of protein expression exist in the middle and outer (but not the inner) molecular layer of the dentate gyrus, and in the mossy fiber pathway. Antibody staining confirmed this distribution pattern of BK channels for the second excitatory hippocampal neuron (see Figure 6). All other layers of the hippocampus, such as the stratum oriens and the stratum radiatum, exhibited lower but still significant levels of [^{125}I]IbTX-D19Y/Y36F binding. In previous studies, we identified the medial habenula as the region with the highest *Slo* mRNA expression levels, although the corresponding immunoreactivity was not found in this nucleus, but instead was concentrated over the interpeduncular nucleus and the tractus retroflexus which connects these two structures (*20*). As shown in panels B and D of Figure 6, immunocytochemistry demonstrated about equal levels of immunoreactivity in the substantia nigra pars reticulata and in the interpeduncular nucleus. However, receptor autoradiography performed in a parallel section indicated that BK channel density is significantly higher in the interpeduncular nucleus, in accordance with the in situ hybridization data (*20*). It is possible that differences in staining patterns between these two techniques are due to the detection systems, with the receptor autoradiography data being linear over a much larger concentration range. However, we cannot discard the possibility that differences between immunostaining and receptor autoradiography patterns may be due to the existence of [^{125}I]IbTX-D19Y/Y36F-insensitive BK channels that are otherwise labeled by the antibody. Evidence for the existence of such toxin-insensitive channels in rat brain has been reported (*32*). The outer layer of the neocortex, the hippocampal perforant path projection, and the interpeduncular nucleus exhibited similar binding site densities after autoradiographic quantification (12−15 fmol/mg of protein). These numbers are slightly lower than those reported after

quantitative autoradiography with either [^{125}I]apamin, [^{125}I]-ChTX, or [^{125}I]iodoglyburide (*33*), but are in good agreement with autoradiographically determined densities of ion channels in general. Other brain regions expressing very low BK channel densities (e.g., the corpus callosum and the brain stem) were found to have less than 10% of this site density.

DISCUSSION

Neuronal BK Channels. The results presented in this study provide new information concerning the subunit composition and regional distribution of rat neuronal BK channels. These channels were studied with the aid of the selective radiolabeled BK channel blocker, [^{125}I]IbTX-D19Y/Y36F, and a panel of sequence-specific antibodies directed against either the α- or *β*-subunit of the smooth muscle BK channel. [^{125}I]-IbTX-D19Y/Y36F binds to rat neuronal BK channels with high affinity ($K_d \approx 20$ pM), and the binding reaction displays properties similar to those previously described for this ligand's interaction with smooth muscle BK channels. It does appear, however, that this ligand possesses a 3-fold lower affinity for neuronal BK channels than for the corresponding channels in smooth muscle. Interestingly, a qualitatively similar effect is observed after expression of BK channel subunits in COS-1 cells; the affinity of [^{125}I]IbTX-D19Y/Y36F for the α- and *β*-subunit complex is 2-fold higher when compared to that of BK channels comprised of only α-subunits. These data suggest that neuronal BK channels do not possess a "smooth muscle-like" *β*-subunit. It has previously been shown that coexpression of α- and *β*-subunits leads to a 50-fold increase in the affinity for [^{125}I]ChTX (*22*, *25*). Thus, the effects of the *β*-subunit on the affinity of [^{125}I]IbTX-D19Y/Y36F for the α-subunit are reminiscent of those observed with [^{125}I]ChTX, although the absolute shift in K_d is not as large.

An independent way of detecting the presence of a smooth muscle-like BK channel *β*-subunit in brain is by investigating the effect of the BK channel agonist, DHS-1, since smooth muscle BK channel modulation by this agent requires the presence of this accessory subunit (*7*, *17*). DHS-1 is a partial inhibitor of [^{125}I]ChTX binding to smooth muscle membranes which are known to contain the α and *β* BK channel complex (*34*). Consistent with this, [^{125}I]IbTX-D19Y/Y36F binding to rat smooth muscle membranes is also DHS-1-sensitive. However, in rat brain membranes, DHS-1 has no effect whatsoever on [^{125}I]IbTX-D19Y/Y36F binding, and in HEK293 cells stably transfected with either α- or α- and *β*-subunits, inhibition of [^{125}I]IbTX-D19Y/Y36F binding by DHS-1 is only observed with channels comprised of α- and *β*-subunits. Thus, it appears that the characteristics of toxin interaction with rat neuronal BK channels are consistent with the absence of a smooth muscle-like *β*-subunit as part of the brain channel complex.

Distribution of Neuronal BK Channels. The distribution of BK channels in rat brain was also investigated by receptor autoradiography, and these results were compared with those derived from immunocytochemical studies in which the sequence-directed BK channel antibody, anti-$\alpha_{913-926}$, was used (*20*). The overall distribution of [^{125}I]IbTX-D19Y/Y36F binding sites in rat brain is qualitatively identical to the immunocytochemical profile of BK channels, but quantitatively, the absolute levels of channel expression in various regions of rat brain appear to differ significantly. This discrepancy between autoradiography and immunocytochemistry data could be due to the different detection systems employed in these experiments, with the autoradiography data being linear over a wider concentration range. Alternatively, antibodies may recognize BK channels that do not bind [^{125}I]IbTX-D19Y/Y36F. Evidence for the existence of such channels has been reported in experiments where BK channel activity was monitored after reconstituting rat brain membranes in artificial lipid bilayers (*32*).

Characterization of a Novel Neuronal BK Channel β-Subunit. The presence of a novel BK channel *β*-subunit in rat brain has been revealed through covalent incorporation of [^{125}I]IbTX-D19Y/Y36F into rat brain membranes in the presence of a bifunctional cross-linking reagent. A heavily glycosylated 25 kDa polypeptide has been identified as the specific substrate of this reaction. About 10 kDa of the mass of this protein is contributed by N-linked glycosylation, indicating that it is a membrane protein. This first biochemical characterization of a neuronal BK channel *β*-subunit suggests that it is an entity that is distinct from the previously characterized protein in smooth muscle where the mass of the core structure is 22 kDa. Such a prediction has been further supported by experiments in which antibodies directed against the smooth muscle type *β*-subunit failed to immunoprecipitate the neuronal *β*-subunit, implying that these two proteins do not share significant sequence homology. Immunoprecipitation was also not observed after deglycosylation of the protein, discarding the possibility of any interference of sugars with the antibody recognition site. The neuronal BK channel *β*-subunit detected in this study is also different from that of two other polypeptides cloned and characterized from *Drosophila* head libraries that interact with the fly BK channel α-subunit (*18*, *19*).

Additional evidence that the newly identified *β*-subunit is an integral component of neuronal BK channels has been obtained from other immunoprecipitation experiments. Thus, antibodies directed against the α-subunit of the BK channel precipitate the covalently labeled *β*-subunit, indicating that both subunits must form part of a tightly associated complex which is stable in the presence of detergents. A Lys residue(s) of the *β*-subunit must be in close proximity to another Lys in [^{125}I]IbTX-D19Y/Y36F when the toxin is bound in the outer vestibule of the pore since the maximum length of the spacer arm of the cross-linking agent that is used is 11.4 Å.

In summary, with the use of [^{125}I]IbTX-D19Y/Y36F, the distribution of BK channels in rat brain has been determined. In addition, a novel neuronal BK channel subunit has been identified and shown to be tightly associated in a complex with the pore-forming α-subunit. Future studies will be directed at identifying this protein and determining its role in BK channel function.

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NOTE ADDED IN PROOF:

The existence of a new BK channel β-subunit has recently been reported (Wallner M., Pratap M., and L. Toro; Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: a transmembrane β subunit homolog. *Proc. Natl. Acad. Sci. U.S.A.*, in press) that when coexpressed with the BK channel α-subunit alters the biophysical and pharmacological properties of the channel. It remains to be determined whether the β-subunit described in this manuscript is related to this newly identified protein.

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