Neuronal α -Bungarotoxin Receptors Are $\alpha 7$ Subunit Homomers

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Nicotinic acetylcholine receptors in the nervous system are heterogeneous with distinct pharmacological and functional properties resulting from differences in post-translational processing and subunit composition. Because of nicotinic receptor diversity, receptor purification and biochemical characterization have been difficult, and the precise subunit composition of each receptor subtype is poorly characterized. Evidence is presented that α -bungarotoxin (Bgt)-binding nicotinic receptors found in pheochromocytoma 12 (PC12) cells are pentamers composed solely of $\alpha 7$ subunits. Metabolically labeled, affinity-purified Bgt receptors (BgtRs) consisted of a single 55 kDa band on SDS gels, which was recognized by anti- $\alpha 7$ antibodies

on immunoblots. Isoelectric focusing separated the 55 kDa band into multiple spots, all recognized by anti- $\alpha 7$ antibodies and, therefore, each a differentially processed $\alpha 7$ subunit. Cell-surface BgtR subunits, cross-linked to each other and ^{125}I -Bgt, migrated on gels as a ladder of five bands with each band a multiple of an $\alpha 7$ subunit monomer. Similar characteristics of BgtRs from rat brain suggest that they, like PC12 BgtRs, are $\alpha 7$ pentamers containing differentially processed $\alpha 7$ subunits.

Key words: α -bungarotoxin; nicotine; neuronal nicotinic receptors; PC12 cells; rat brain; protein structure; post-translational modification

Nicotinic receptors are ionotropic neurotransmitter receptors in the CNS. Although less numerous than glutamate receptors, neuronal nicotinic receptors have been implicated in many important functions including memory formation and nociception. They are the receptors responsible for nicotine addiction and also are involved in a number of pathologies including epilepsy and Parkinson's disease (Gotti et al., 1997; Lindstrom, 1997; Lena and Changeux, 1998). Nicotinic receptors are members of a family of ionotropic receptors that includes GABA_A, glycine, and serotonin (5HT₃) receptors (Unwin, 1993; Karlin and Akabas, 1995). Multiple neuronal nicotinic receptor subtypes exist with distinct pharmacological and functional properties (for review, see Sargent, 1993; Lindstrom et al., 1995; McGehee and Role, 1995). These different subtypes are composed of at least 11 different subunit isoforms, $\alpha 2$ – $\alpha 9$ and $\beta 2$ – $\beta 4$. As with virtually all ion channels, the subunit composition of various nicotinic receptor subtypes is poorly characterized, and a major challenge is to determine the differences in subunit composition that underlie differences in subtype pharmacology and function.

In this study, we have examined the subunit composition of the neuronal nicotinic receptors that bind α -bungarotoxin (Bgt). High-affinity Bgt receptors (BgtRs) are Ca²⁺-permeable channels found throughout the nervous system (Zorumski et al., 1992; Alkondon and Albuquerque, 1993; Seguela et al., 1993; Zhang et al., 1994; Castro and Albuquerque, 1995). Ca²⁺ entry through activated BgtRs causes presynaptic enhancement of neurotransmitter release (McGehee et al., 1995; Alkondon et al., 1996; Gray et al., 1996), neurite retraction (Chan and Quik, 1993; Pugh and

Berg, 1994), apoptosis (Berger et al., 1998), and also neuron survival (Messi et al., 1997). By the use of Bgt affinity chromatography, BgtRs were the first neuronal nicotinic receptors purified and appeared to be composed of two or more subunits of different molecular weight (Betz et al., 1982; Conti-Tronconi et al., 1985; Kemp et al., 1985; Whiting and Lindstrom, 1987). BgtRs from various preparations contained $\alpha 7$ subunits (Schoepfer et al., 1990; Vernallis et al., 1993) but not $\alpha 3$, $\alpha 5$, $\beta 2$, or $\beta 4$ subunits (Chen and Patrick, 1997; Rangwala et al., 1997). Moreover, expression of $\alpha 7$ subunits in *Xenopus* oocytes resulted in functional BgtRs (Couturier et al., 1990; Seguela et al., 1993), raising the possibility that BgtRs are $\alpha 7$ homomeric receptors.

Adding to the confusion about the subunit composition of BgtRs was the observation that heterologous expression of $\alpha 7$ subunits in many different cell lines results in little to no expression of Bgt-binding sites (Cooper and Millar, 1997; Rangwala et al., 1997). Recently, we reported that $\alpha 7$ subunits in cells expressing BgtRs folded into two different conformations and that surface receptors contained both conformations (Rakhilin et al., 1999). In contrast, $\alpha 7$ subunits in cells not expressing BgtRs folded into a single conformation. Our findings suggested that two $\alpha 7$ subunit conformations in a receptor are required for receptor function. In this study, we demonstrate that pheochromocytoma 12 (PC12) BgtRs are composed solely of $\alpha 7$ subunits and that BgtRs from rat brain appear to have the same subunit composition. Even though BgtRs are homomers of $\alpha 7$ subunits, BgtR subunits are heterogeneous, displaying multiple charged forms and at least two different conformations.

MATERIALS AND METHODS

Metabolic labeling and solubilization. PC12 N21 cells, also referred to as PC12-C cells (Blumenthal et al., 1997), were a gift from Dr. Richard Burry (Ohio State University). The cells were cultured in DMEM plus 5% heat-inactivated horse serum and 10% fetal bovine serum (Hyclone, Logan, UT). Cells stably expressing $\alpha 7/5\text{HT}_3$ BgtRs were established as described previously (Rangwala et al., 1997) and maintained in DMEM plus 10% calf serum and 0.6 mg/ml G418. All cells were cultured at 37°C in the presence of 5% CO₂. To label PC12 cells metabolically, cultures

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were grown to ~60% confluence in 10 cm plates. The cells were washed on the plates with PBS and then incubated for 10 min in methionine-free DMEM at 37°C. Culture medium was replaced with methionine-free DMEM with 333 μ Ci/ml [35 S]methionine/[35 S]cysteine (EXP-35S; NEN) for 1 hr at 37°C. After the labeling period, cells were incubated in culture medium at 37°C for 1 hr. Cells were washed with PBS, pelleted at 5000 $\times$ g for 2 min, and resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% Na $_2$ S $_2$ O $_8$, and 1% Triton X-100) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride and chymostatin, pepstatin, leupeptin, and tosyl-lysine chloromethyl ketone each at 10 μ g/ml). *N*-ethylmaleimide (NEM; 2 mM) was also added to the lysis buffer as indicated. After 1 hr at 4°C, the lysate was centrifuged for 30 min at 10,000 $\times$ g. Supernatants were rotated overnight at 4°C in the presence of Sepharose 4B to “preclean” the samples. Samples were centrifuged for 30 min at 10,000 $\times$ g, and the pellets were discarded.

BgtR cross-linking. Confluent 6 or 10 cm cultures, washed in PBS and pelleted at 5000 $\times$ g for 2 min, were resuspended in PBS with 4–10 nM [125 I]-Bgt and rotated for 2 hr at room temperature. [125 I]-Bgt-bound BgtRs on the surface of intact cells were treated with the indicated concentrations of 3,3'-dithiobis-sulfosuccinimidylpropionate (DTSSP; spacer arm = 12 Å; Pierce, Rockford, IL) or 3 mM sulfodisuccinimidyl tartrate (sDST; spacer arm = 7.4 Å; Pierce) in PBS for 1 hr at room temperature. The reaction was stopped by the addition of 10 mM Tris, pH 7.4, for 15 min, and cells were then washed in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, and 0.02% Na $_2$ S $_2$ O $_8$. Cells were then solubilized as described above.

Affinity isolation of BgtRs. Bgt was conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Solubilized BgtRs were incubated with Bgt-Sepharose at 4°C for 6 hr. The beads were pelleted and washed two times for 5 min with lysis buffer containing 500 mM NaCl and 0.1% SDS and one time with lysis buffer. The solubilized [125 I]-Bgt-labeled, cross-linked receptors were incubated with polyclonal anti-Bgt conjugated to protein A-Sepharose and were rotated overnight at 4°C. Tubes were centrifuged 30 sec at 8000 $\times$ g, and the pellets were washed three times before counting and electrophoresis.

Sucrose gradient sedimentation. Solubilized BgtRs (300 μ l) were layered onto a 5 ml 5–20% sucrose gradient in lysis buffer and sedimented as described previously (Rangwala et al., 1997), and 300 μ l fractions were taken. For measurement of Bgt binding, 4 nM [125 I]-Bgt was added to unlabeled fractions and incubated for 2 hr at room temperature. [125 I]-Bgt-bound receptors were precipitated overnight at 4°C with concanavalin A-Sepharose (Sigma). The beads were washed three times with lysis buffer and counted in a gamma counter. Linearity of the gradient was confirmed by measuring the osmolality of each fraction. Catalase (11 S), [125 I]-Bgt-bound α 7SHT $_3$ receptors (9 S), and alkaline phosphatase (5.4 S) were used as standards.

Electrophoresis and immunoblot analysis. Proteins were separated on linear 4–8% gradient SDS-PAGE. Except where indicated, samples were treated with 10 mM dithiothreitol (DTT) for reducing SDS-PAGE. Molecular weights (M_r) were determined on linear gradient gels by plotting the log M_r of the standards versus the log of the total percent acrylamide at the migration point (Lambin, 1978). Molecular weights are reported as the mean $\pm$ SD. [35 S]-labeled or [125 I]-Bgt-labeled gels were dried and exposed to film. For immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Towbin et al., 1979). After transfer, the nitrocellulose was treated with 3% bovine serum albumin (BSA) in wash buffer (10 mM Tris, pH 7.4, 0.05% Tween 20, and 150 mM NaCl). Membranes were washed briefly in wash buffer and then treated overnight with the primary antibody directed against a 20 amino acid C-terminal epitope of the α 7 subunit (goat polyclonal anti- α 7; Santa Cruz Biotechnology). The blots were washed and incubated with secondary antibody (rabbit anti-goat-HRP; Pierce) at the appropriate dilution for 1 hr. After washing, membranes were treated with an enhanced chemiluminescent reagent (ECL; Amersham) according to the manufacturer's protocol and exposed to film. Two-dimensional gel electrophoresis was performed as described previously (O'Farrell, 1975). In the first dimension, affinity-purified BgtRs were run on 12 $\times$ 0.4 cm tube isoelectric-focusing (IEF) gels containing 2% ampholytes [1.6% at isoelectric point (pI) 6–8; 0.4% at pI 3–10; Bio-Rad]. Samples separated on IEF gels were then run on gradient SDS-PAGE as the second dimension.

Rat brain membranes. Membranes were prepared as described previously (Chen and Patrick, 1997). Briefly, adult rats were decapitated, and the entire brain was dissected and placed in ice-cold 50 mM NaPO $_4$, pH 7.4, 50 mM NaCl, 2 mM EDTA, and 2 mM EGTA plus protease inhibitors.

Figure 1. Affinity-purified BgtRs are composed of α 7 subunits. BgtRs were affinity-purified from PC12 cells using Bgt-Sepharose and were analyzed on 4–8% gradient SDS-PAGE. BgtRs were metabolically labeled and processed for fluorography (left) or subjected to immunoblot analysis with α 7-specific antibodies (right). Metabolically labeled BgtR subunits migrated as a single band at 55 kDa (lane 2), identical to the position of α 7 subunits identified on immunoblots (lane 5). A mean value of 55 $\pm$ 1 kDa ($\pm$ SD) was obtained for the labeled band in the figure together with the results from three other experiments. Alkylation of the subunits with the sulfhydryl-specific agent NEM produced two closely spaced bands centered at 55 kDa for both labeled subunits (lane 3) and α 7 subunits on immunoblots (lane 6). No additional bands were observed in the 20–40 kDa range when affinity-purified BgtRs were analyzed on 7.5% gels, and no bands were observed when 100 μ M nicotine was present during the Bgt-Sepharose precipitation (lanes 1, 4). The positions of molecular markers are shown on the left: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; and actin, 45 kDa.

Brain tissue was minced and homogenized in a Teflon-glass Dual homogenizer. Homogenates were centrifuged at 100,000 $\times$ g for 1 hr. Pellets were taken through one more cycle of homogenization and centrifugation. The resulting pellets were resuspended in lysis buffer plus protease inhibitors and stored at -80° C until needed.

RESULTS

BgtRs from PC12 cells contain only α 7 subunits

The PC12 cell line variant N21 (Burry, 1993) expresses high levels of functional BgtRs (Blumenthal et al., 1997; Rangwala et al., 1997) and five different neuronal nicotinic subunits: α 3, α 5, α 7, β 2, and β 4 (Blumenthal et al., 1997). We used this cell line to determine the subunit composition of the endogenous neuronal BgtRs. PC12 cells, unlike brain preparations, allowed metabolic labeling of BgtR subunits and separation of surface BgtRs from intracellular pools. BgtR subunits from PC12 cells were metabolically labeled and affinity purified using Bgt-Sepharose (Fig. 1). Labeled BgtR subunits migrated on SDS-PAGE as a single band (Fig. 1, lane 2) with an apparent molecular weight of 55 $\pm$ 1 kDa. The same band was recognized by α 7 subunit-specific antibodies on Western blots (Fig. 1, lane 5) and thus contains α 7 subunits. The molecular weight of 55 kDa is slightly larger than that predicted by the open reading frame of the α 7 subunit gene (54.2 kDa) (Seguela et al., 1993) and is in good agreement with other molecular weight estimates for rat α 7 subunits (Blumenthal et al., 1997; Chen and Patrick, 1997). We have shown previously that

alkylation of $\alpha 7$ subunits by a sulfhydryl alkylating agent such as NEM causes $\alpha 7$ to migrate as two closely spaced bands, with each band a differently processed form of $\alpha 7$ (Rakhilin et al., 1999). This effect of BgtR subunit alkylation was observed for both the labeled subunits (Fig. 1, lane 3) and the subunits recognized by $\alpha 7$ subunit-specific antibodies (Fig. 1, lane 6). After alkylation using NEM, BgtR subunits separated into the two processed $\alpha 7$ subunit forms centered at 55 kDa with more $\alpha 7$ found in the slower-migrating band. Precipitation of $\alpha 7$ by Bgt-Sepharose was completely prevented by 100 μ M nicotine (Fig. 1, lanes 1, 4), which blocks all Bgt binding to these receptors (Rangwala et al., 1997).

Because affinity-purified BgtRs in Figure 1 contained both intracellular as well as surface Bgt-binding sites, we tested whether purified BgtR subunits were found in assembled complexes by size fractionation on sucrose gradients. In Figure 2A, labeled BgtR subunits were precipitated with Bgt-Sepharose from the indicated sucrose gradient fractions. The 55 kDa subunit band sedimented predominantly in a single peak at 10 S, which is where fully assembled, surface BgtRs migrate (Rangwala et al., 1997). The 55 kDa subunit band in the 10 S peak was also recognized by $\alpha 7$ subunit-specific antibodies on Western blots (Fig. 2B). The 125 I-Bgt-bound subunits, both surface and intracellular, sedimented predominantly in a single peak centered at 10 S (Fig. 2C). A small percentage (8%) of the 125 I-Bgt-binding sites (Fig. 2C) that appears to be a population of partially assembled $\alpha 7$ was observed at 5–6 S. Thus, partially assembled BgtR complexes contribute only minimally to the signal in Figures 1 and 2, and we conclude that we are purifying predominantly fully assembled BgtRs.

The PC12 BgtR is a pentamer

To characterize surface BgtRs from PC12 cells further, experiments were performed cross-linking surface receptor subunits to each other and to 125 I-Bgt. 125 I-Bgt-bound surface receptors were cross-linked with the indicated concentrations of the cell-impermeant reagent DTSSP (Fig. 3A), and the cross-linked receptors were immunoprecipitated with anti-Bgt antibodies. At lower concentrations of DTSSP, the cross-linked surface receptors migrated on gels as a ladder of five bands with the largest amount of 125 I-Bgt cross-linked to the lowest band on the gels. The apparent molecular weight of the lowest band on the gels was 58 ± 2 kDa. The molecular weight of the cross-linked subunit monomer was smaller than expected on the basis of the apparent molecular weight of the $\alpha 7$ subunit monomer (55 kDa) plus that of Bgt (8 kDa). As shown by the position of the other four bands on the gel (Fig. 3C), each band migrates as a multiple of the monomer band, consistent with cross-linked $\alpha 7$ subunit dimers, trimers, tetramers, and pentamers. As the DTSSP concentration was increased, there was a progressive shift from monomer to pentamer, and no bands larger than the pentamer band were observed. The immunopurified, cross-linked pentamers had an apparent molecular weight of 293 ± 8 kDa (Fig. 3C), which is the same as that of the fully cross-linked PC12 BgtRs before purification (Rangwala et al., 1997) and indicates that there was no significant proteolysis of the BgtR subunits during purification.

Additional experiments were performed to compare the cross-linking of PC12 BgtR subunits with the cross-linking of a BgtR established to be a homomer. Receptors composed of chimeric subunits containing the N-terminal half of the $\alpha 7$ subunit fused to the C-terminal half of the 5HT₃ receptor subunit (Eisele et al., 1993) ($\alpha 7/5HT_3$ subunits) form homomeric BgtRs when expressed in mammalian cell lines (Corringer et al., 1995; Rangwala

Figure 2. Affinity-purified BgtRs are fully assembled. *A*, Metabolically labeled BgtRs were solubilized in the absence of NEM and size-fractionated on 5–20% linear sucrose gradients before affinity purification. Labeled subunits were precipitated from each gradient fraction, 1 (top) to 16 (bottom), and analyzed on SDS-PAGE as in Figure 1. The majority of the labeled subunits migrated at 10 S (fractions 12, 13). Arrows at the top mark the peak fractions of the standards: alkaline phosphatase, 5.4 S; 125 I-Bgt-bound $\alpha 7/5HT_3$ receptors, 9 S; and catalase, 11 S. Arrows on the right indicate the positions of molecular weight standards: actin, 45 kDa, and BSA, 66 kDa. The arrowhead indicates the position of $\alpha 7$ subunits. *B*, Immunoblot analysis performed on BgtRs size-fractionated on 5–20% linear sucrose gradients is shown. BgtRs, precipitated from each gradient fraction with Bgt-Sepharose, were prepared on SDS-PAGE as described in *A*, followed by immunoblot analysis with anti- $\alpha 7$ -specific antibodies. Standards used are identical to those in *A*. *C*, 125 I-Bgt-binding sites were size-fractionated on 5–20% linear sucrose gradients. After sedimentation on sucrose gradients, BgtRs in each gradient fraction were bound with 125 I-Bgt and precipitated using concanavalin A-Sepharose. The majority of the sites (92%) sedimented at 10 S (fractions 12, 13), whereas a smaller number (8%) sedimented at ~6 S (fractions 5, 6).

et al., 1997). 125 I-Bgt-bound surface $\alpha 7/5HT_3$ receptors expressed in tsA201 cells were cross-linked with the indicated concentrations of DTSSP (Fig. 3B). Again, a ladder of five bands was generated by cross-linking the receptors at the lower DTSSP concentrations. The lowest band had an apparent molecular weight of 54 ± 2 kDa. As seen with cross-linked $\alpha 7$ subunits, this molecular weight is lower than expected for an $\alpha 7/5HT_3$ subunit (52 ± 1 kDa) cross-linked to 125 I-Bgt. The similar pattern observed by cross-linking PC12 BgtRs and $\alpha 7/5HT_3$ homomers provides further evidence that PC12 BgtRs are pentamers consisting only of $\alpha 7$ subunits.

Figure 3. Affinity-purified BgtRs contain five subunits of equal molecular weight. *A, B*, Cross-linking of surface ^{125}I -Bgt-bound BgtRs is shown. BgtRs on the surface of PC12 cells (*A*) or from the surface of cells expressing $\alpha 7/5\text{HT}_3$ chimeras (*B*) were bound with ^{125}I -Bgt and cross-linked with the indicated concentrations of DTSSP. Cross-linked complexes were solubilized in the presence of NEM, immunoprecipitated with anti-Bgt antibodies conjugated to protein A-Sepharose, and run on nonreducing SDS-PAGE. Arrowheads on the right indicate the positions of subunit monomers, dimers, trimers, tetramers, and pentamers, whereas arrows on the left in *A* indicate positions of the molecular weight markers (see Fig. 1). *C*, Molecular weights of the cross-linked complexes were estimated and plotted as a function of the number of subunits in the cross-linked PC12 (■) and $\alpha 7/5\text{HT}_3$ (○) complexes. Molecular weight values are the mean $\pm$ SD from four experiments. The lines represent a least-squares linear regression fit to each data set. *D*, Cross-linking of surface ^{125}I -Bgt-bound BgtRs with the shorter-arm reagent sDST is shown. BgtRs on the surface of PC12 cells (left lane) or from cells expressing $\alpha 7/5\text{HT}_3$ chimeras (right lane) were bound with ^{125}I -Bgt and cross-linked with sDST. Samples were treated as described in *A* except they were solubilized without NEM. Arrowheads on the left and right represent the positions of oligomers for PC12 BgtR and $\alpha 7/5\text{HT}_3$ subunits, respectively. Arrows show the migration of molecular weight markers.

A ladder of five bands was also obtained by cross-linking ^{125}I -Bgt-bound surface PC12 and $\alpha 7/5\text{HT}_3$ receptors with a shorter cross-linking reagent, sDST (Fig. 3*D*). As shown in Figure 3*D*, the ladder of cross-linked PC12 receptor subunits is identical to that of the $\alpha 7/5\text{HT}_3$ homomers except that each rung migrates proportionately slower because of the slightly larger molecular weight of $\alpha 7$ monomers. The difference in apparent molecular weight between $\alpha 7$ and $\alpha 7/5\text{HT}_3$ monomers is consistent with the molecular weights calculated from the open reading frame of the

Figure 4. Two-dimensional gel analysis of affinity-purified BgtRs. *A*, PC12 BgtRs were metabolically labeled, solubilized without NEM, precipitated with Bgt-Sepharose in the presence (*top*) or absence (*bottom*) of 100 μM nicotine, and run on two-dimensional gels. The 55 kDa band observed on SDS-PAGE in Figure 1 separated into six different spots with pIs between 5.5 and 5.7 (labeled 1–6) and a seventh spot at 5.9 (labeled 7). Arrows on the bottom mark the positions of the standards for the IEF dimension (left to right): actin, pI 5.0–5.1; BSA, pI 5.4–5.6; carbonic anhydrase, pI 5.9–6.0; and conalbumin, pI 6.0–6.6. Each arrowhead marks the position of 55 kDa in the SDS-PAGE dimension, and *a* marks the position of actin. *B*, PC12 BgtRs were precipitated with Bgt-Sepharose in the presence (*top*) or absence (*bottom*) of 100 μM nicotine and analyzed by immunoblot after being run on two-dimensional gels. All seven spots observed on two-dimensional gels for metabolically labeled BgtR subunits were stained by anti- $\alpha 7$ antibodies. Standards used are identical to those in *A*.

two subunits. Surface receptor cross-linking with sDST differed from DTSSP cross-linking in that the receptors could not be completely cross-linked into pentamers by higher sDST concentrations and the cross-linking into dimers, trimers, tetramers, and pentamers was prevented by sulfhydryl alkylation during solubilization (data not shown). Previously, we demonstrated that $\alpha 7/5\text{HT}_3$ and PC12 $\alpha 7$ subunits form disulfide-bonded dimers, trimers, tetramers, and pentamers during solubilization if solubilized without alkylating reagents (Rakhilin et al., 1999) (also see Fig. 5). Thus, in Figure 3*D*, it appears that sDST only cross-linked bound ^{125}I -Bgt to receptor subunits and that the cross-linking of subunits into dimers, trimers, tetramers, and pentamers occurred during the subsequent solubilization.

Heterogeneity of BgtR $\alpha 7$ subunits

The affinity-purified 55 kDa band was further analyzed using two-dimensional gels to test whether proteins in addition to $\alpha 7$ subunits are present in the 55 kDa band. The labeled BgtR subunits that had migrated as a single 55 kDa band separated into multiple spots in the IEF dimension (Fig. 4*A*). Importantly, each

Figure 5. Rat brain BgtRs. BgtRs were affinity-purified from solubilized rat brain membranes and compared with PC12 BgtRs using immunoblot analysis with anti- $\alpha 7$ antibodies. Rat brain (left) and PC12 (right) BgtRs were solubilized in the absence or presence of NEM as indicated and run on reducing (+DTT) or nonreducing (–DTT) SDS-PAGE. The positions of oligomers (arrowheads) and molecular weights of the standards (arrows) are shown (Fig. 3). Results are representative of three separate experiments.

spot was recognized by $\alpha 7$ -specific antibodies (Fig. 4*B*) and, therefore, represents $\alpha 7$ subunits with different pIs. Six of the $\alpha 7$ subunit spots were closely spaced on the gels with pIs between 5.5 and 5.7, whereas a seventh spot was more basic with a pI of 5.9 (Fig. 4). The cause of the charge heterogeneity has not been determined. $\alpha 7$ subunits are subjected to post-translational disulfide bonding (Rakhilin et al., 1999), phosphorylation (Moss et al., 1996), and glycosylation (Chen et al., 1998). Two-dimensional gel analysis can resolve peptides possessing a single-charge difference; therefore, one or any combination of these modifications could give rise to multiple $\alpha 7$ forms.

The subunit composition of rat brain BgtRs is similar to that of PC12 BgtRs

Experiments were performed to compare BgtRs from rat brain with PC12 BgtRs. Rat brain membranes were prepared and BgtRs were affinity purified from the solubilized membranes using Bgt-Sepharose. When rat brain receptors were solubilized without NEM, a single 55 kDa band was recognized by $\alpha 7$ subunit-specific antibodies (Fig. 5, lane 3) and comigrated precisely with PC12 affinity-purified $\alpha 7$ subunits (Fig. 5, lane 7). Like PC12 BgtRs, rat brain BgtRs contain differently processed forms of $\alpha 7$ subunits. Alkylation of the solubilized rat brain $\alpha 7$ subunits with NEM caused the subunits to separate into two closely spaced bands centered at 55 kDa (Fig. 5, lane 2), identical to the effect of NEM alkylation on PC12 $\alpha 7$ subunits (Fig. 5, lane 6). When PC12 BgtRs were solubilized without NEM and analyzed on nonreducing gels, the $\alpha 7$ subunits were cross-linked by disulfide bonds and appeared as a ladder of five bands corresponding to monomers, dimers, trimers, tetramers, and pentamers (Fig. 5, lane 8) as observed previously (Rakhilin et al., 1999). Under the same conditions, rat brain BgtR subunits were also cross-linked by disulfide bonds into a ladder of five bands in which each of the five

bands comigrated with the corresponding PC12 BgtR monomers, dimers, trimers, tetramers, and pentamers (Fig. 5, lane 4). In all experiments, 100 μ M nicotine blocked BgtR binding to Bgt-Sepharose (Fig. 5, lanes 1, 5). Thus, we conclude that rat brain BgtRs, like PC12 BgtRs, are $\alpha 7$ homomers and contain different conformations of the $\alpha 7$ subunit in a single receptor.

DISCUSSION

Multiple nicotinic receptor subtypes exist in the nervous system with distinct pharmacological and functional properties and differences in subunit composition (McGehee and Role, 1995; Gotti et al., 1997). Because of nicotinic receptor diversity, receptor purification and biochemical characterization have been difficult, and the precise subunit composition of each receptor subtype has not been characterized. Previous purification of neuronal BgtRs used predominantly brain preparations from which it was concluded that BgtRs are composed of anywhere from two to four different subunit isoforms. The most extensive studies have characterized chick brain BgtRs in which three bands with molecular weights of ~50, ~57, and ~67 kDa were observed (Conti-Tronconi et al., 1985; Gotti et al., 1991, 1992). Multiple bands were also observed with rat brain preparations (Betz et al., 1982; Kemp et al., 1985; Whiting and Lindstrom, 1987). The ~57 kDa band was shown to consist of $\alpha 7$ subunits (Schoepfer et al., 1990; Gotti et al., 1994) that bind Bgt (Hermans-Borgmeyer et al., 1988), but the identity of the other bands has not been determined. Although these data provide evidence of multiple BgtR subunits, there are features of the data that raise questions about this conclusion. Microsequencing the N terminus of the 50 kDa band demonstrated that it was identical to that of the $\alpha 7$ subunit (Conti-Tronconi et al., 1985), which indicates that the 50 kDa band is either a proteolytic fragment or an unprocessed form of the $\alpha 7$ subunit. Evidence favoring the latter interpretation is that chick BgtRs containing the 57 kDa band bind wheat germ agglutinin (WGA), a lectin that only recognizes mature glycans, whereas BgtRs containing the 50 kDa band do not bind to WGA (Hermans-Borgmeyer et al., 1988). Another explanation for multiple bands is that chick brain contains at least two BgtR subtypes because of the expression of $\alpha 8$ subunits (Schoepfer et al., 1990; Gotti et al., 1992, 1994; Keyser et al., 1993). $\alpha 8$ subunits, which are highly homologous to $\alpha 7$ subunits, are not found in mammals (Elgoyhen et al., 1994).

In this paper, we studied the neuronal BgtRs found endogenously in the PC12 cell variant N21. These cells express a homogenous population of functional BgtRs at high levels (Blumenthal et al., 1997; Rangwala et al., 1997). The advantage of PC12 cells as compared with the brain preparations used previously was that we could start with a single population of intact, living cells, which allowed us to label BgtR subunits metabolically and to separate surface BgtRs from intracellular pools. In contrast to previous studies, we found that BgtRs from PC12 cells are composed only of $\alpha 7$ subunits. Labeled, affinity-purified BgtRs migrated as a single 55 kDa band on SDS-PAGE gels. A combination of two-dimensional gel electrophoresis and immunoblotting with $\alpha 7$ subunit-specific antibodies demonstrated that the 55 kDa band consisted of only $\alpha 7$ subunits. Cross-linking surface BgtR subunits to each other and 125 I-Bgt further showed that BgtRs are pentamers with five subunits of equal molecular weight. Affinity-purified BgtRs from rat brain contained $\alpha 7$ subunits that migrated at a position on SDS-PAGE gels identical to the 55 kDa band from PC12 cells. Furthermore, rat brain BgtRs, like the PC12 BgtRs, formed intersubunit disulfide cross-links during

solubilization in the absence of sulfhydryl alkylation. The resulting cross-linked dimers, trimers, tetramers, and pentamers were identical in size to the corresponding cross-linked PC12 subunits and indicated that rat brain BgtRs are pentamers consisting of five $\alpha 7$ subunits.

The assembly of BgtRs involves much more than simply associating five $\alpha 7$ subunits into a pentamer. Most mammalian cells do not express functional BgtRs when transfected with $\alpha 7$ subunits (Cooper and Millar, 1997; Rangwala et al., 1997). In contrast, certain cells of neuronal origin, such as PC12 and SH-SY5Y cells, can produce functional BgtRs when transfected with $\alpha 7$ subunits (Puchacz et al., 1994; Blumenthal et al., 1997). In these cells, $\alpha 7$ subunits are folded into a second disulfide-bonded conformation, and surface receptors contain $\alpha 7$ subunits in both conformations (Rakhilin et al., 1999). These studies indicate that neuron-specific mechanisms are needed to fold $\alpha 7$ subunits into a different conformation that is required for functional BgtR assembly. Results from this paper confirm and extend these recent findings that BgtRs contain $\alpha 7$ subunits in different conformations. In PC12 cells, two different $\alpha 7$ subunit forms were apparent on SDS-PAGE, but only after sulfhydryl alkylation (Rakhilin et al., 1999) (also see Figs. 1, 5). We also found that affinity-purified $\alpha 7$ subunits from rat brain, like those from PC12 cells, separated into two distinct bands when alkylated (Fig. 5). Because the two $\alpha 7$ bands separated on SDS-PAGE only after they were alkylated, the separation does not seem to be caused by a difference in molecular weight. It is, therefore, unlikely that the separation on SDS-PAGE is caused by a truncation of the subunits or a different number of N-linked glycans attached to the subunits.

BgtR subunits exhibited even greater heterogeneity in the IEF dimension of two-dimensional gels (Fig. 4). On two-dimensional gels, $\alpha 7$ subunits separated into different forms with seven different pI values. Because $\alpha 7$ subunits are glycosylated (Gotti et al., 1992; Chen et al., 1998) and possess several phosphorylation consensus sites (Seguela et al., 1993), differences in oligosaccharide trimming or phosphorylation could cause the different $\alpha 7$ subunit pI values. The relation between these different $\alpha 7$ charged forms and the separation of alkylated $\alpha 7$ subunits on SDS-PAGE is unclear, but it is unlikely that a change in pI directly causes the separation because charge differences are typically masked on SDS gels. More likely, the processing of $\alpha 7$ subunits that alters the net charge of the subunits changes subunit conformation, and the separation on SDS-PAGE reflects different structural conformations. This scenario is consistent with our observations indicating that the differences observed on SDS-PAGE gels arise slowly over ~90 min and parallel the formation of Bgt-binding sites and disulfide bonds on $\alpha 7$ subunits (Rakhilin et al., 1999). The differences observed on SDS-PAGE gels, just like Bgt-binding site and disulfide-bond formation, occur only for $\alpha 7$ subunits expressed in cells of neuronal origin and thus appear to be required for BgtR function (Rakhilin et al., 1999). Altogether, our data suggest that the subunit changes in pI are caused by neuron-specific processing events such as phosphorylation or trimming of N-linked glycans that, in turn, initiate conformational changes involved in the formation of Bgt-binding sites, certain disulfide bonds, and a functional receptor.

Over a billion years ago, the family of nicotinic receptor subunits began to emerge by a series of gene duplications from a single common subunit (LeNovere and Changeux, 1995; Ortells and Lunt, 1995). The first branch of the family evolved into the subunits found in neuronal BgtRs, which include $\alpha 7$, $\alpha 8$, and $\alpha 9$ subunits. Of these three nicotinic subunits, only $\alpha 7$ subunits are

found in mammalian nervous tissue. $\alpha 9$ subunits are only present in cochlea and vestibular organs (Elgoyhen et al., 1994; Anderson et al., 1997), whereas no mammalian homolog for chick $\alpha 8$ subunits has been observed (Elgoyhen et al., 1994). In addition to its precursor being the first nicotinic subunit to diverge from the others, $\alpha 7$ subunits appear to have evolved without additional gene duplications. These characteristics of $\alpha 7$ subunit evolution suggest that among all the nicotinic receptor subtypes, neuronal BgtRs have the most features in common with the primordial nicotinic receptor. One such shared feature is the homomeric structure of both neuronal BgtRs and primordial nicotinic receptors. All other mammalian nicotinic receptors, with perhaps the exception of $\alpha 9$ -containing receptors, appear to be heteromeric receptors (Lindstrom, 1997). It is also possible that the subunits of the primordial nicotinic homomer were folded and processed into different conformations just as we have observed for BgtR $\alpha 7$ subunits. Such folding and processing, as we have suggested previously, may serve as a post-translational mechanism used to generate subunit diversity and may be critical for proper functioning of the receptors (Rakhilin et al., 1999). The different subunit conformations found in homomeric BgtRs could play a role similar to that of different subunit isoforms found within heteromeric nicotinic receptors. The additional proteins and/or factors needed to mediate the neuron-specific folding and processing of $\alpha 7$ subunits may provide an important regulatory role by determining when and where functional BgtRs are produced. However, there might be advantages to having functional nicotinic receptors that can be assembled without the accessory proteins that mediate cell-specific folding. Part of the evolutionary pressure for additional nicotinic subunit isoforms may have been to produce the different subunit conformations without the additional accessory proteins needed for proper folding.

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