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A major class of nicotinic receptors in the nervous system is one that binds α-bungarotoxin and contains the α7 gene product. PC12 cells, frequently used to study nicotinic receptors, express the α7 gene and have binding sites for the toxin, but previous attempts to elicit currents from the putative receptors have failed. Using whole-cell patch-clamp recording techniques and rapid application of agonist, we find a rapidly desensitizing acetylcholine-induced current in the cells that can be blocked by α-bungarotoxin. The current amplitude varies dramatically among three populations of PC12 cells but correlates well with the number of toxin-binding receptors. In contrast, the current shows no correlation with α7 transcript; cells with high levels of α7 mRNA can be negative for toxin binding and yet have other functional nicotinic receptors. Northern blot analysis and reverse transcription-PCR reveal no defects in α7 RNA from the negative cells, and immunoblot analysis demonstrates that they contain full-length α7 protein, although at reduced levels. Affinity purification of toxin-binding receptors from cells expressing them confirms that the receptors contain α7 protein. Transfection experiments demonstrate that PC12 cells lacking native toxin-binding receptors are deficient at producing receptors from α7 gene constructs, although the same cells can produce receptors from other transfected gene constructs. The results indicate that nicotinic receptors that bind α-bungarotoxin and contain α7 subunits require additional gene products to facilitate assembly and stabilization of the receptors. PC12 cells offer a model system for identifying those gene products.

Key words: nicotinic; acetylcholine receptors; α-bungarotoxin; PC12 cells; α7 gene product; α7 subunits; neuronal

One of the most abundant nicotinic acetylcholine receptors (AChRs) in the vertebrate nervous system is a species that binds α-bungarotoxin (αBgt) and contains α7 subunits (Sargent, 1993; Role and Berg, 1996). The receptor is a cation-selective ligand-gated ion channel with a high relative permeability to calcium and rapid desensitization (Couturier et al., 1990; Schoepfer et al., 1990; Zorumski et al., 1990; Alkondon and Albuquerque, 1993; Alkondon et al., 1994; Zhang et al., 1994). It is capable of diverse functions, including presynaptic modulation of transmitter release (McGehee et al., 1995; Gray et al., 1996), postsynaptic generation of synaptic currents (Zhang et al., 1994), and regulation of calcium-dependent cellular events (Pugh and Berg, 1994; Vijayaraghavan et al., 1995). Recent evidence also links it to a form of schizophrenia (Freedman et al., 1997).

Heterologous expression of the α7 gene in Xenopus oocytes and stably transfected cell lines produces functional αBgt-sensitive receptors with pharmacological properties similar to those of native receptors containing α7 subunits (Couturier et al., 1990; Anand et al., 1993; Gopalakrishnan et al., 1995; Quik et al., 1996). The α7 gene product may assemble into homomeric receptors, although a requirement for additional kinds of subunits has not been excluded. Heteromers of α7 and α8 subunits have been found among chick AChRs but represent a minority of the receptors containing α7 protein (Schoepfer et al., 1990; Keyser et al., 1993; Gotti et al., 1994). Interestingly, when Xenopus oocytes are injected with the α7 gene alone, expression of functional AChRs requires a cyclophilin acting either as a prolyl isomerase or as a chaperone (Helekar et al., 1994). It is not known whether neurons have a similar requirement for cyclophilin.

A continuing challenge to our understanding of receptors containing α7 subunits is that presented by the rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976). PC12 cells express the α7 gene product, along with other nicotinic receptor genes (Boulter et al., 1986, 1990; Rogers et al., 1992; Henderson et al., 1994). Although the cells can assemble receptors that bind αBgt, no electrophysiological response or ion flux has been attributed to the receptors despite attempts for nearly two decades (Patrick and Stallcup, 1977a, b; Amy and Bennett, 1983; Kemp and Edge, 1987; Daly et al., 1991; Rogers et al., 1991). In contrast, autonomic neurons, which express a similar complement of AChR genes (Listerud et al., 1991; Corriveau and Berg, 1993; Mandelzys et al., 1994), assemble substantial numbers of functional αBgt-sensitive AChRs that contain α7 subunits and lack the other known AChR gene products present (Vernallis et al., 1993; Zhang et al., 1994). The one indication of functional αBgt-sensitive AChRs on PC12 cells comes from studies showing that αBgt blocks the effects of nicotine on neurite outgrowth from the cells (Chan and Quik, 1993). We reexamine here the question

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of whether PC12 cells express AchRs that contain α7 subunits and generate αBgt-sensitive currents.

MATERIALS AND METHODS

PC12 cells. PC12 cells were obtained from M. Quik (McGill University) (PC12-A), S. Rogers (University of Utah) (PC12-B), and R. T. Boyd (Ohio State University) (PC12-C). The cell lines were grown at 37°C in a humidified incubator under 8% CO2 and passed when 70–80% confluent (trypsin was used to pass the PC12-A cells). The cultures were replaced after 2–3 months of passage by thawing a fresh aliquot of frozen cells. Each strain was maintained in culture medium of the composition used at the source laboratory: PC12-A received DMEM with 4.5 g/ml glucose, 5% heat-inactivated fetal bovine serum (Gibco-Product, Calabasas, CA), and 10% heat-inactivated donor horse serum (Gemini); PC12-B received DMEM with 1 g/ml glucose, 10% heat-inactivated fetal bovine serum (Characterized; HyClone, Logan, UT), and 5% heat-inactivated donor horse serum (Gemini); PC12-C received RPMI 1640 medium with 5% heat-inactivated fetal bovine serum (Gemini) and 10% heat-inactivated donor horse serum (Gemini). All media contained 2 mm glutamine and 50 U/ml penicillin and streptomycin. In some experiments the culture medium was supplemented with 50–100 ng/ml nerve growth factor (7 S NGF, Promega, Madison, WI) for 3–5 days before assaying the cells.

Electrophysiology. For electrophysiological experiments, cells were plated at low density on 35 mm tissue culture dishes and used the following day. For PC12-A cells the dishes were first coated with poly-l-lysine (Sigma, St. Louis, MO). Currents were recorded using the whole-cell patch-clamp configuration controlled by an Axopatch 200A amplifier (Axon Instruments) as described previously (Hamill et al., 1981; Zhang and Berg, 1995). The extracellular solution contained (in mM) 140 NaCl, 3 KC1, 2 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPE, pH 7.4 (with NaOH). The pipette solution contained (in mM) 140 CsCl, 1 MgCl2, 10 EGTA, 10 glucose, and 10 HEPE, pH 7.2 (with CsOH). Electrode resistances were ~3 MΩ. Series resistance was always ~6 MΩ and was 80% compensated. Cells were clamped at −60 mV, and 500 μm ACh was rapidly applied as follows. Solutions were delivered from a linear array of glass tubing (370 μm inner diameter, 470 μm outer diameter; Polymerotechnics, Phoenix, AZ) mounted on a microbimorph element (Motor-Matrox, Bedford, OH). Solution flow was gravity-fed and controlled by a set of solenoid valves (General Valve, Fairfield, NJ). The valves and the bimorph were all controlled by a Master-8 programmable stimulator (A.M.P.I., Jerusalem, Israel). Using this system, the time of solution exchange at an open pipette was 0.5–3 msec as measured by the change in junction potential. Data were filtered at 10 kHz and digitized at either 0.7 or 2 kHz with the pClamp software (Axon Instruments). Currents were analyzed using Axograph (Axon Instruments). Maximum activation rates were calculated using the Axograph subroutine and normalized to peak amplitude.

For recordings from αBgt-treated cells, the toxin (Biotinex Inc, St. Cloud, FL) was recorded at 60 nM for 1–3 hr at 37°C, and 18 nm αBgt was also recorded at 60 nM for 1–3 hr at 37°C. The recording solution contained BSA (Sigma; fraction V, cold alcohol precipitation) was included in the recording solution, because it increased the amplitude of the nicotine responses that could be elicited from cells after multiple passages in culture. Exposure to BSA had no effect on the αBgt sensitivity of the response; therefore, results obtained in the presence and absence of BSA were combined in the present analysis. The mechanism of the BSA effect is unknown, although it may be similar to the increased nicotinic response seen in chick ciliary ganglion neurons after acute exposure to BSA (Gurantz et al., 1993).

Monoclonal antibodies. Monoclonal antibodies (mAbs) 318 and 319 were raised against a fusion protein corresponding to the large putative cytoplasmic loop of the chick α7 gene product (Schoepfer et al., 1990); mAb 318 cross-reacted with the α7 protein in SDS-PAGE, Western blots, whereas mAb 318 does not (Del Toro et al., 1994). Anti-α7 antibody (PC12-C) with the anti-4/7 mAb 319 and 319 and quantified with 125I-αBgt or 1–5 nm 1H-epibatidine (DuPont NEN, Boston, MA) for 1 hr at room temperature. The reaction was stopped by the addition of 4 ml of 10 mM Tris buffer, pH 7.5, containing 0.05% Triton X-100 (Tri-X-100) and immediate filtration through Whatman (Maidstone, UK) GF/B filters treated with 0.5% polyethyleneimine. The filters were washed twice with Tri-X-TX and counted in a gamma counter to quantify 125I-αBgt or in a scintillation counter to measure 1H-epibatidine.

In the second method, detergent extracts were assayed in two-site solid-phase radioimmunoassays (RIAs) as described previously (Conroy and Berg, 1995). Briefly, subunit-specific mAbs were used to immunoprecipitate from detergent extracts in microtiter wells, and bound receptors were quantified with radiolabeled probes and either gamma or scintillation counting. AchRs containing α7 protein were retained with the anti-α7 mAb 318 or 319 and quantified with 125I-αBgt or in a scintillation counter to measure 1H-epibatidine.
guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). RNA was quantified by measuring optical density at 260 nm; RNA integrity was routinely checked by formaldehyde agarose gel electrophoresis.

**RNase protection.** 32P-labeled riboprobes used for RNase protection experiments were generated by “runoff transcription” from appropriately linearized constructs using methods described previously (Corriveau and Berg, 1993). The α3 probe construct was made by subcloning a fragment encoding amino acids 346–441 from a full-length rat α3 cDNA (Boulier et al., 1986) into pGEM-T (Promega). For the β2 probe construct, a 370 base pair fragment was subcloned into pGEM-T from a full-length rat β2 cDNA to generate a probe encompassing amino acids 334–457 (Deneris et al., 1988). Portions of the rat α5, α7, and β4 cDNAs were isolated by reverse transcription (RT)-PCR from PC12 total RNA, and the fragments were directly cloned into PGM-T. The β4 probe encodes amino acids 543–442, the α2 probe encodes amino acids 339–405 (Boulter et al., 1990), and the α7 probe encodes amino acids 305–438 (Seguela et al., 1993). The undigested and protected probe sizes, respectively, in base pairs are as follows: α3, 320 and 283; α5, 315 and 203; α7, 507 and 402; β2, 470 and 370; and β4, 372 and 301.

**Results**

**Discovery of αBgt-sensitive ACh responses.** Whole-cell patch-clamp recording was used to examine ACh-induced currents in three populations of PC12 cells obtained from three different laboratories. Agonist was applied by a rapid delivery system to optimize detection of quickly desensitizing currents. ACh at 500 μM elicited two kinds of responses that could be distinguished by their apparent kinetics of activation, their rates of desensitization, and their sensitivity to αBgt. One was a rapidly activating current that quickly desensitized and could be blocked by αBgt. The other was a more slowly activating current that decayed less quickly and was insensitive to the toxin. The relative proportions of the two kinds of responses differed dramatically among the three PC12 populations tested (Fig. 1, Table 1). PC12-A cells displayed only the rapidly desensitizing response that was completely blocked by 60 nM αBgt (Fig. 1A). PC12-B cells, in contrast, had only the slowly desensitizing response that was insensitive to blockade by αBgt (Fig. 1B). PC12-C cells had both kinds of responses (Fig. 1C).

In the case of mixed responses, one method of detecting the rapidly desensitizing toxin-sensitive component is to compare the proportion of the current remaining 30 msec after the peak in the presence and absence of αBgt. By this criterion PC12-C cells have a toxin-sensitive component, whereas PC12-B cells do not (Table 1). A more sensitive method for revealing even a small amount of the toxin-sensitive response in the presence of a large insensitive response is to take advantage of the observed rapid activation kinetics of the toxin-sensitive component. This was done by calculating the maximum rate of current activation for each cell and normalizing it to the peak amplitude of the response. Comparing these values in the presence and absence of αBgt again reveals the rapidly activating αBgt-sensitive current in PC12-C cells but fails to detect such a component in PC12-B cells (Table 1).

**Correlation between αBgt-sensitive responses and αBgt binding sites.** The differential effect of αBgt on the two classes of ACh-evoked currents suggested that they arose not from different gating modes of a single class of receptor but rather from two pharmacologically distinct receptor subtypes. Accordingly, binding studies were performed to distinguish AChR classes pharmacologically and to correlate them with the currents observed. 125I-αBgt was used to quantify αBgt binding sites both on the cell surface.
and in cell extracts. \(^3\)H-Epibatidine was used to quantify other classes of nicotinic receptors in the extracts. Epibatidine binds with high affinity to many nicotinic receptor subtypes but not those containing rat or chick \(\alpha_7\) subunits (Gerzanich et al., 1995) (W. Conroy and D. Berg, unpublished observations). Binding to the cell surface was not attempted with \(^3\)H-epibatidine, because it seems to cross the cell membrane.

PC12-A cells had a small but significant number of \(\alpha\)Bgt binding sites on the cell surface, whereas PC12-C cells had substantially more (Fig. 2). Both had even greater numbers in cell extracts, implying the existence of intracellular pools of the receptors. PC12-B cells, in contrast, had no detectable sites on the surface and few, if any, in the cell extracts. This pattern of expression could not be explained by the different culture media used for the three populations. Growing the PC12-B cells in culture medium normally used for PC12-C cells did not result in the expression of \(\alpha\)Bgt binding sites. Conversely, PC12-C cells grown in PC12-B medium continued to express \(\alpha\)Bgt binding sites (data not shown).

NGF increases the excitability of PC12 cells (Dichter et al., 1977; Mandel et al., 1988) and can induce long-term expression of a sodium channel gene after even a brief exposure (Toledo-Aral et al., 1995). Because NGF also increases AChR expression in at least some populations of PC12 cells (Dichter et al., 1977; Henderson et al., 1995), its effects on PC12-B cells were tested to determine whether it could induce the appearance of \(\alpha\)Bgt-sensitive AChRs. Exposure of the cells to 100 ng/ml NGF for 4 d failed to induce any morphological changes or to arrest cell growth and division. Consistent with their nonresponsive phenotype, PC12-B cells in the presence of NGF did not express detectable levels of \(\alpha\)Bgt binding (two experiments gave values of 0.0 \pm 2.0 and 0.2 \pm 1.0 fmol of \(\alpha\)Bgt binding/mg of protein). NGF treatment stopped cell division in PC12-C cells and caused them to extend neurites, as previously reported for the parent line (Greene and Tischler, 1976), but it had no effect on the expression of \(\alpha\)Bgt binding sites by the cells (two experiments gave 277 \pm 29 and 371 \pm 12 fmol/mg protein compared with the mean of about 280 fmol/mg protein for untreated control cells as shown in Fig. 2).

The distribution of \(\alpha\)Bgt binding sites among the three cell populations correlates with the presence of \(\alpha\)Bgt-sensitive currents evoked by ACh in the cells. The other class of receptors, namely those binding epibatidine, is likely to be responsible for the \(\alpha\)Bgt-insensitive currents evoked by ACh. Extracts from all three strains had significant numbers of such receptors, but only the PC12-B and PC12-C cells displayed the slow, toxin-insensitive response (see Discussion).

**Lack of correlation between \(\alpha\)Bgt-sensitive responses and \(\alpha_7\) mRNA**

Because AChRs that bind \(\alpha\)Bgt in autonomic neurons have been shown to contain the \(\alpha_7\) gene product (Schoepfer et al., 1990; Vernallis et al., 1993), experiments were performed to compare the levels of \(\alpha_7\) transcript among the three PC12 populations. RNase protection assays revealed the presence of \(\alpha_7\) mRNA in all three types of PC12 cells (Fig. 3A). In fact, PC12-B cells, which lack detectable \(\alpha\)Bgt binding sites, had by far the greatest abundance of \(\alpha_7\) transcripts (Fig. 3B). PC12-B cells also expressed substantial amounts of the other four AChR gene transcripts tested: \(\alpha_3\), \(\alpha_5\), \(\beta_2\), and \(\beta_4\). PC12-A and -C cells expressed the same five genes, with the exception of \(\beta_4\) in PC12-A. The lack of \(\beta_4\) transcripts in PC12-A cells might account for the absence of a...
Differences were detected between PC12-B and -C cells (Fig. 5). A full-length fragment was obtained in each case, and no size variation was observed in the two samples (Fig. 4). The results indicate that the defect preventing accumulation of the a7 transcript into mature mRNA is likely to result from a small truncation or deletion in the region in question as well as adjacent regions to determine whether such a deletion might account for the inability of PC12-B cells to produce competent aBgt-binding ACChRs. Primers were chosen to amplify fragments corresponding to nucleotide positions 18–1000 (a), 627–1000 (b), 627–1534 (c), and 1148–1534 (d) (Fig. 5A; numbering as in Seguela et al., 1993). Only the expected full-length fragment was obtained in each case, and no size differences were detected between PC12-B and -C cells (Fig. 5B). The small 627–1000 fragment includes the M2 region; even a deletion of 10–20 nucleotides should have been apparent. The RNase protections (covering positions 913–1314), the PCR analysis, and the Northern blots found no defects in the a7 mRNA from PC12-B cells.

Table 1. Effects of aBgt on the properties of ACh-evoked currents in PC12 cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Cm (pF)</th>
<th>Current (pA)</th>
<th>Current density (pA/pF)</th>
<th>I30/Ipeak</th>
<th>Activation rate (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12-A</td>
<td>148</td>
<td>10.9 ± 0.3</td>
<td>215 ± 30</td>
<td>19 ± 2</td>
<td>0.11 ± 0.01</td>
<td>256 ± 15</td>
</tr>
<tr>
<td>+ aBgt</td>
<td>40</td>
<td>11.4 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PC12-B</td>
<td>73</td>
<td>11.7 ± 0.3</td>
<td>1814 ± 179</td>
<td>154 ± 14</td>
<td>0.92 ± 0.01</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>+ aBgt</td>
<td>3</td>
<td>11.5 ± 0.4</td>
<td>2121 ± 247</td>
<td>192 ± 23</td>
<td>0.92 ± 0.01</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>PC12-C</td>
<td>117</td>
<td>14.6 ± 0.3</td>
<td>2418 ± 181</td>
<td>178 ± 14</td>
<td>0.74 ± 0.02</td>
<td>120 ± 5</td>
</tr>
<tr>
<td>+ aBgt</td>
<td>5</td>
<td>14.6 ± 0.4</td>
<td>1763 ± 146°</td>
<td>131 ± 12°</td>
<td>0.90 ± 0.01**</td>
<td>69 ± 2**</td>
</tr>
</tbody>
</table>

Whole-cell patch clamp was used to record responses evoked by 500 μM ACh in the presence and absence of 60 nM aBgt. The peak current in each case was divided by the capacitance (Cm) to obtain the current density for individual cells. I30/Ipeak is the current amplitude 30 msec after the peak current divided by the latter. The activation rate was calculated as the maximum of the derivative of the current normalized to the peak current. Currents <100 pA were not included in the calculations of activation rate. Values represent the mean ± SEM. The number of cells is indicated by n.

*, **Significantly different from paired values in the absence of aBgt; *p ≤ 0.02; **p ≤ 0.001, Student’s t test.

**Figure 2.** Differences among PC12 populations in their relative levels of ACheRs that bind aBgt and epibatidine. 125I-aBgt was used to measure binding sites on intact cells, whereas both 125I-aBgt and [3H]epibatidine were used to quantify sites in cell extracts. The results were normalized for total protein. Non-specific binding was determined by adding 1 μM aBgt (aBgt assays), 1 μM epibatidine (epibatidine assay), or 250 μM nicotine (both assays) to the binding reactions and was subtracted from the values shown. PC12-A and -C cells express significant amounts of aBgt binding sites both on the surface (solid bars) and in cell extracts (stippled bars), whereas PC12-B cells have few if any specific aBgt binding sites. All three populations have significant epibatidine binding (hatched bars). Data represent the mean ± SEM of triplicate determinations from four to seven independent experiments.

Slow aBgt-insensitive response, which was prominent in PC12-B and -C cells.

One explanation for the finding that PC12-B cells express a7 mRNA and yet fail to assemble aBgt-binding ACheRs is that the transcripts may be defective. A first test of this was performed by Northern blot analysis. Total RNA was extracted from both PC12-B and PC12-C cells and probed on Northern blots with a random-primed, 32P-labeled a7 probe. A single band of about 6.4 kb was observed in the two samples (Fig. 4). The results indicate that the defect preventing accumulation of aBgt-sensitive ACheRs in PC12-B cells is unlikely to result from a large truncation or deletion in the a7 transcript.

Bovine adrenal chromaffin cells express a truncated splice variant of the a7 transcript that lacks 87 nucleotides corresponding to an exon encoding the M2 domain and adjacent regions (Garcia-Guzman et al., 1995). When co-injected with full-length a7 transcript into Xenopus oocytes, the truncated splice variant inhibits the expression of functional ACheRs. A deletion of this size would probably not have been detected in the Northern analysis. Therefore, RT-PCR analysis was performed over the region in question as well as adjacent regions to determine whether such a deletion might account for the inability of PC12-B cells to produce competent aBgt-binding ACheRs. Primers were chosen to amplify fragments corresponding to nucleotide positions 18–1000 (a), 627–1000 (b), 627–1534 (c), and 1148–1534 (d) (Fig. 5A; numbering as in Seguela et al., 1993). Only the expected full-length fragment was obtained in each case, and no size differences were detected between PC12-B and -C cells (Fig. 5B). The small 627–1000 fragment includes the M2 region; even a deletion of 10–20 nucleotides should have been apparent. The RNase protections (covering positions 913–1314), the PCR analysis, and the Northern blots found no defects in the a7 mRNA from PC12-B cells.

**Presence of a7 protein in PC12 ACheRs**

In view of the transcript analysis, it became important to confirm that PC12 receptors capable of binding aBgt actually contain a7 subunits. This was demonstrated by using aBgt-Actigel to affinity purify the receptors from PC12 cell extracts and then analyzing the eluted protein on immunoblots. The blots were probed with the anti-a7 mAb A7–1 generated against a fusion protein corresponding to the large putative cytoplasmic loop of the rat a7 gene product. A single component of ~60 kDa was recognized by the mAb in extracts from PC12-C cells (Fig. 6A). It was comparable in size to the monomeric a7 species obtained from chick brain and ciliary ganglion (Schoepfer et al., 1990; Vernallis et al., 1993). Competition with 250 μM nicotine during the affinity purification caused the complete loss of the PC12 component, confirming the specificity of the purification. No specific bands were detected in extracts prepared from PC12-B cells. The results demonstrate that the aBgt-binding species in PC12 cells contains the a7 gene product.

Immunoblot analysis of immunopurified material was used to compare the total amounts of a7 protein in extracts prepared from PC12-B and -C cells. mAb 319 coupled to Actigel was used to immunoprecipitate a7 protein, whereas mAb A7–1 was used to detect it on the blots. Although a7 protein could be detected in both cases, PC12-C cells contained much more of it than did
Expression of AChR genes in PC12 populations

Northern analyses revealed that the PC12-B cells (Fig. 6B) express the lowest levels of AChR gene transcripts, whereas the PC12-C cells express the highest levels. The protected species obtained with each of the five probes was quantified on a Bio-Rad Molecular Imager. The results represent the mean ± SEM for three to seven determinations from a total of three or four RNA preparations and are normalized to the signal obtained for α3 transcript in PC12-A cells.

PC12-B cells (Fig. 6B). The size of the monomeric α7 protein in PC12-B and -C cells is the same, consistent with the PCR and Northern analyses finding no differences in transcript size.

Expression of AChRs from transfected genes

The small amounts of α7 protein in PC12-B cells suggested that the transcript was competent but that a posttranslational block prevented assembly of the subunits and permitted rapid turnover. The possibility of a posttranslational block was tested by transfecting cells with chicken AChR gene constructs and then assaying cell extracts for receptor expression. Because the anti-α7 mAb 318 is specific for chicken, it enabled the transfected α7 gene product to be distinguished from the endogenous rat homolog in solid-phase RIAs. The relative amounts of αBgt binding in such assays in which only the chick α7 gene product was measured indicated that PC12-B cells are two orders of magnitude less efficient than PC12-C cells at producing AChRs from a competent α7 cDNA construct (Fig. 7A).

For comparison, cells were also transfected with the chicken α4 and β2 genes and assayed by RIA using the anti-α4 mAb 289 to immunotether receptors and 3H-epibatidine to quantify them. In contrast to the results with the α7 construct, PC12-B cells were only a few-fold less efficient than PC12-C cells at producing transfected α4/β2 AChRs (Fig. 7A). A comparison was also performed using an α7/5-HT3 chimera receptor gene construct in which the N-terminal portion of the 5-HT3 receptor gene encoding the N-terminal putative extracellular portion of the protein was replaced with the equivalent region of the α7 gene. The encoded protein retains the ability to bind αBgt (Elsele et al., 1993). The construct was also engineered to encode a myc epitope on the C terminus so that the anti-myc mAb 9E10 could be used to immunotether the chimera receptor in RIAs. Again, PC12-B cells were only a few-fold less efficient than PC12-C cells at expressing the transfected chimera receptors (Fig. 7A).

A different way of analyzing the data is to calculate the ratio of different receptor types produced by transfected PC12-B cells and then to compare the ratio with that obtained from transfected PC12-C cells. This provides a way of normalizing results among experiments and reduces the inherent variation among transfections. Viewing the results in this way indicates that PC12-B cells are 10- to 20-fold less able to produce receptors from the transfected α7 construct than they are from either the transfected α4 and β2 constructs or from the transfected α7/5-HT3 chimera receptor construct compared with PC12-C cells (Fig. 7B). If all of the differences between PC12-B and -C cells in expressing either α4/β2 receptors or α7/5-HT3 receptors were considered nonspecific, e.g., attributed to differences in the efficiency of transfection,
the results would still demonstrate a substantial difference between the two cell populations in their ability to produce AChRs with \( \alpha 7 \) subunits. It is at least as likely, however, that all of the differences arise from the PC12-B defect, and that it is most severe in the case of the \( \alpha 7 \) gene product.

Tests for cyclophilin involvement

The possibility was considered that the PC12-B deficiency resulted from an altered or lost cyclophilin, because a cyclophilin has been shown to be necessary in \textit{Xenopus} oocytes for expression of functional AChRs from the \( \alpha 7 \) gene (Helekar et al., 1994). This was not a strong likelihood in the present case, however, because PC12-B cells show little deficit in expressing the \( \alpha 7/5 \)-HT\(_3\) chimeric receptors, whereas \textit{Xenopus} oocytes are as dependent on cyclophilin for 5-HT\(_3\) receptors as they are for receptors composed of \( \alpha 7 \) subunits (Helekar et al., 1994).

A requirement for cyclophilin was tested in the present experiments by exposing PC12-C cells for 24 hr to cyclosporin A, which inhibits cyclophilin, and then assaying cell extracts to determine whether the cells had reduced levels of AChRs. Cyclosporin A at 10 \( \mu M \) had no effect on the amounts of \( \alpha Bgt \)-binding AChRs with \( \alpha 7 \) subunits, either as measured in cell extracts by RIA to assay total receptors or as measured in cell culture with intact cells to assay surface receptors selectively (Fig. 8). Similarly, cyclosporin A at 10 \( \mu M \) had no effect on the number of AChRs binding epibatidine in RIAs (127 \( \pm 4\% \), mean \( \pm SE \); \( n = 3 \) experiments) compared with untreated control cells. Higher concentrations of cyclosporin A (30 \( \mu M \)) were toxic and caused substantial cell loss. Blockade of new receptor expression by incubating cells with 0.5 \( \mu g/ml \) tunicamycin (which blocks N-linked glycosylation and prevents receptor maturation) showed that most of the \( \alpha Bgt \)-binding receptors are lost during a 24 hr period (Fig. 8). The tunicamycin-induced loss was apparent both in the total receptor population and in those confined to the cell surface, indicating an ongoing receptor turnover by the cells. If a cyclosporin A-sensitive cyclophilin were required in PC12 cells, as it is in \textit{Xenopus} oocytes for assembly of AChRs composed of \( \alpha 7 \) subunits, then the cyclosporin A treatment of PC12-C cells should have produced a large (90\%) reduction in the amount of \( \alpha Bgt \)-binding receptors tethered by anti-\( \alpha 7 \) mAbs in the RIA. It should also have substantially reduced the number of \( \alpha Bgt \)-binding receptors on the cell surface, given the ongoing turnover. As a result, it is unlikely that a lost or altered cyclophilin accounts for the posttranslational deficiency in receptor production by PC12-B cells.

**DISCUSSION**

This report provides the first demonstration, to our knowledge, of ACh-evoked currents in PC12 cells that can be blocked by \( \alpha Bgt \). It resolves a long-standing puzzle in the field and shows that the currents can vary dramatically among different PC12 populations. The currents correlate well with the presence of \( \alpha Bgt \)-binding AChRs on the cells, and the receptors contain \( \alpha 7 \) protein, but there is no correlation between receptor levels and the amount of \( \alpha 7 \) transcript in the cells. Instead, receptor expression is shown to depend on a posttranslational event that is absent in some PC12 populations. The posttranslational event seems to be most critical for expression of AChRs containing \( \alpha 7 \) subunits, although it may influence less severely the expression of other ligand-gated ion channels as well.

Like their counterparts on rat hippocampal neurons and chick ciliary ganglion neurons (Zorumski et al., 1992; Alkondon and Albuquerque, 1993; Alkondon et al., 1994; Zhang et al., 1994), \( \alpha Bgt \)-sensitive AChRs on PC12 cells seem to activate and desensitize rapidly. The quick desensitization of the receptors and their variable numbers among PC12 cell populations may explain why previous attempts failed to identify a current associated with the receptors. Another contributing factor is likely to have been the large \( \alpha Bgt \)-resistant ACh responses that may often obscure the \( \alpha Bgt \)-sensitive component. No antagonists have yet been identified that selectively block the \( \alpha Bgt \)-resistant receptors.

The deficiency of PC12-B cells in producing functional \( \alpha Bgt \)-binding AChRs cannot be ascribed to a defect in their \( \alpha 7 \) tran-
AChRs that bind αBgt were affinity-purified from PC12-B and -C cell extracts by adsorption to αBgt coupled to Actigel beads. The adsorbed material was eluted and analyzed by immunoblots probed with the anti-extracts by adsorption to αBgt-binding AChRs from an α7 subunit construct and visualized using a horseradish peroxidase-coupled secondary antibody followed by enhanced chemiluminescence. A single species of about 60 kDa was obtained from PC12-C samples (lane 1). Nicotine at 250 μM (lane 2) blocked adsorption of the component to the αBgt-Actigel, indicating the specificity of the affinity purification. No components were obtained specifically when PC12-B cell extracts were analyzed by similar methods (lanes 3 and 4). Molecular weight standards (Bio-Rad) are as follows: phosphorylase B, 97 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 31 kDa. Similar results were obtained in a second experiment using mAb A7–1 as probe and in two experiments using mAb 319 as probe. Total α7 protein from PC12-B and -C cell extracts was analyzed by immunoprecipitation using the α7-specific mAb 319 coupled to Actigel and probing immunoblots of the purified material with mAb A7–1. A species of 60 kDa component was obtained from both PC12-C (lane 1) and PC12-B (lane 2) cell extracts, although the latter contained much less of the component. Similar results were obtained in a second experiment. The band was absent when rat IgG-Actigel was substituted for the mAb 319-Actigel as a negative control (data not shown).

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The inability of PC12-A cells to produce αBgt-insensitive ACh responses deserves comment. Some epibatidine binding was detected in the cell extracts indicating the existence of AChRs. Possibly the receptors were sequestered in an intracellular pool and therefore unable to generate responses. The low levels of β4...
for $^{125}$I-subunit. Cell extracts were prepared 20–48 hr later and assayed by RIA for subunits ($a$ fmol/culture, respectively, for the experiments with $a$ subunits which have a large deficiency in the expression of AChRs with $a$ and $b$ subunits). Absolute levels of gene products to be assayed (receptors containing only endogenous gene components would not have been retained). Antibodies required that the receptors contain one or more transfected $a$ gene products, which recognizes the epitope-tagged $a$/5-HT$_3$ receptor protein and by RIA for $^{3}$H-epibatidine binding to receptors tethered with anti-$a$ mAb 318, which recognizes chick but not rat $a$ protein; or by anti-myc mAb 9E10, which recognizes the chick but not rat $a$ protein. The selectivities of the antibodies required that the receptors contain one or more transfected gene products to be assayed (receptors containing only endogenous gene components would not have been retained). $\alpha$ Ratio of receptor levels in the same experiment. Values represent the mean $\pm$ SEM of three or four experiments, containing either the $a$7 or the $a$7/5-HT$_3$, chimeric receptor subunit. Cell extracts were prepared 20–48 hr later and assayed by RIA for $^{125}$I-Abgt binding to receptors tethered either by anti-$a$ mAb 318, which recognizes chick but not rat $a$ protein, or by anti-myc mAb 9E10, which recognizes the epitope-tagged $a$7/5-HT$_3$ receptor protein and by RIA for $^{3}$H-epibatidine binding to receptors tethered with anti-$a$ mAb 289, which recognizes chick but not rat $a$ protein. The selectivities of the antibodies required that the receptors contain one or more transfected gene products to be assayed (receptors containing only endogenous gene components would not have been retained). $\alpha$ Ratio of receptor levels in the same experiment. Values represent the mean $\pm$ SEM of three or four experiments, containing either the $a$7 or the $a$7/5-HT$_3$, chimeric receptor subunit. Cell extracts were prepared 20–48 hr later and assayed by RIA for $^{125}$I-Abgt binding to receptors tethered either by anti-$a$ mAb 318, which recognizes chick but not rat $a$ protein, or by anti-myc mAb 9E10, which recognizes the epitope-tagged $a$7/5-HT$_3$ receptor protein and by RIA for $^{3}$H-epibatidine binding to receptors tethered with anti-$a$ mAb 289, which recognizes chick but not rat $a$ protein. The selectivities of the antibodies required that the receptors contain one or more transfected gene products to be assayed (receptors containing only endogenous gene components would not have been retained).

Figure 7. Expression of transfected receptor gene constructs in PC12 cells. PC12-B and -C cells were transfected with chicken AChR cDNA constructs encoding either the $a$7 or the $a$4 and $b$2 subunits or, in separate experiments, containing either the $a$7 or the $a$7/5-HT$_3$, chimeric receptor subunit. Cell extracts were prepared 20–48 hr later and assayed by RIA for $^{125}$I-Abgt binding to receptors tethered either by anti-$a$ mAb 318, which recognizes chick but not rat $a$ protein, or by anti-myc mAb 9E10, which recognizes the epitope-tagged $a$7/5-HT$_3$ receptor protein and by RIA for $^{3}$H-epibatidine binding to receptors tethered with anti-$a$ mAb 289, which recognizes chick but not rat $a$ protein. The selectivities of the antibodies required that the receptors contain one or more transfected gene products to be assayed (receptors containing only endogenous gene components would not have been retained). A. Ratio of receptor levels in PC12-C cell extracts divided by that in PC12-B cell extracts obtained from the same experiment. Values represent the mean $\pm$ SEM of three or four experiments. Absolute levels of Abgt binding to chick AChRs with $a$7 subunits in transfected PC12-B and PC12-C cells averaged 6 and 498 fmol/culture, respectively, for the experiments with $a$4$\beta$2 and 4 and 258 fmol/culture, respectively, for the experiments with $a$7/5-HT$_3$. Levels of epibatidine binding for PC12-B and PC12-C cells were 43 and 288 fmol/culture, respectively, in the $a$4$\beta$2 experiments; levels of Abgt binding to 5-HT$_3$ chimeras were 37 and 111 fmol/culture, respectively. PC12-B cells have a large deficiency in the expression of AChRs with $a$7 subunits compared with PC12-C cells. B, Relative number of Abgt-binding AChRs produced by transfected PC12-B (B) or PC12-C (C) cells with chick $a$7 subunits ($a$7-AChRs) divided by either the number of receptors with $a$4 and $b$2 subunits ($a$4$\beta$2-AChRs) or $a$7/5-HT$_3$, chimeric receptor subunits ($a$7/5-HT$_3$Rs). PC12-B cells are significantly more impaired at expressing Abgt-binding AChRs from the $a$7 construct than they are at expressing either AChRs from the $a$4 and $b$2 constructs ($p < 0.03$ by Student’s unpaired $t$ test) or receptors from the $a$7/5-HT$_3$ chimeric construct ($p < 0.002$).

Figure 8. Lack of cyclosporin A effects on levels of PC12 receptors binding Abgt. PC12-C cells in culture were treated either with vehicle (Control), tunicamycin (1 $\mu$g/ml), or cyclosporin A (10 $\mu$g/ml) for 24 hr and then assayed either for Abgt binding in RIAs with mAb 318 (which recognizes the chick but not rat $a$7 gene product) to immunotether receptors (Total Sites) or for Abgt-binding to intact cells in culture (Surface Sites). Values represent the mean $\pm$ SEM of three or four separate experiments and are expressed as percentages of those obtained from control cells. Tunicamycin treatment caused a large reduction in Abgt binding, indicating that receptors both on the cell surface and inside were normally turning over. Cyclosporin A treatment had little effect either on the total number of Abgt-binding receptors assayed or on those present on the cell surface.

transcript in PC12-A cells may have limited the ability of the cells to produce receptors targeted for the cell surface. It remains unclear, however, why the $a$3 and $b$2 gene products expressed by the cells were not alone sufficient to generate functional Abgt-resistant AChRs as they do in Xenopus oocytes (Boulter et al., 1987; Deneris et al., 1988; Gross et al., 1991).

PC12 cells passaged many times in culture under different conditions might well generate diverse variants. What is interesting about the present variations is the possibility that they reflect differences in the machinery cells use to assemble and maintain certain classes of membrane proteins. Ligand-gated ion channels are multimeric proteins containing both hydrophobic and hydrophilic transmembrane domains constituting the channel. They pose considerable challenges from a structural biological point of view in terms of proper assembly and insertion into the cell membrane. Neurons must efficiently produce large numbers of such channel proteins while avoiding potentially lethal errors such as creating spontaneously active channels or ones that permit excessive calcium to enter the cell. A gain-of-function mutation in a Caenorhabditis elegans gene homologous to $a$7 illustrates the potential danger of producing inappropriate receptors; the mutation causes cell death (Treinin and Chalfie, 1995). Quality control may come in the form of special chaperones that guide the assembly process and help prevent or discard errors.

REFERENCES


