A Desensitized Form of Neuronal Acetylcholine Receptor Detected by ^3H -Nicotine Binding on Bovine Adrenal Chromaffin Cells

Linda S. Higgins and Darwin K. Berg

Department of Biology, University of California, San Diego, La Jolla, California 92093

A nicotinic acetylcholine receptor (AChR) on bovine adrenal chromaffin cells in culture has previously been identified using the α -neurotoxin n-Bgt and the monoclonal antibody mAb 35. Here, we report that the cells have 2 classes of high-affinity binding sites for ^3H -nicotine, one being associated with the AChR and the other being associated with the α -bungarotoxin binding component that is distinct from the AChR. Scatchard analysis of ^3H -nicotine binding to the AChR site yields a K_D of 20 ± 3 nM and a B_{max} of 104 ± 12 fmol/mg protein. Nicotinic antagonists block ^3H -nicotine binding to the AChR site with the same rank order of potency and affinity with which they block nicotine-induced catecholamine release from the cells. About 80% of the AChRs are on the cell surface, as judged by the distribution of both ^3H -nicotine binding and ^{125}I -mAb 35 binding to the receptor, and the ratio of nicotine/mAb 35 binding to the AChR on the cell surface is approximately 1:1. Chronic treatment of the cells with mAb 35 results in receptor modulation such that all of AChR-related nicotine binding is lost from the cell surface, and all of the functional response to nicotine is lost as well. The results confirm that ^3H -nicotine binding is associated with AChRs on the cells.

The ^3H -nicotine binding observed to the AChR represents binding to a desensitized form of the receptor having increased affinity for agonists and unchanged affinity for antagonists. This conclusion derives from the following observations. The K_S for agonist competition of ^3H -nicotine binding indicate agonist affinities several orders of magnitude greater than do the K_D s measured for receptor activation. Exposing cultures to low concentrations of nicotine and substance P causes receptor desensitization, and the concentration dependence of the nicotine-induced desensitization displays an EC_{50} of 20 nM, in good agreement with the K_D obtained from equilibrium binding studies with ^3H -nicotine. In addition, the rate of ^3H -nicotine binding is increased both by substance P, which enhances the rate of agonist-induced desensitization on adrenal chromaffin cells, and by preincubation with nicotine. The increased rate of association, together with the dissociation rate, yields a kinetically derived K_D of 19 nM, again in good agreement with the K_D ob-

tained from equilibrium binding studies. These results demonstrate that the bovine adrenal chromaffin AChR is similar to AChRs from muscle and electric organ in undergoing an agonist-induced conversion to a desensitized state having increased affinity for agonists.

Nicotinic acetylcholine receptors (AChRs) trigger catecholamine release from bovine adrenal chromaffin cells when activated by synaptic transmission from the splanchnic nerve. The AChRs bind nicotinic ligands and display ion selectivities and single-channel properties generally similar to those previously described for AChRs from muscle, electric organ, and neurons (Brandt et al., 1976; Fenwick et al., 1982; Kidokoro et al., 1982). Unlike AChRs from muscle and electric organ, however, the receptors on bovine adrenal chromaffin cells are not blocked by neuronobungarotoxin (n-Bgt) (Trifaro and Lee, 1980; Kilpatrick et al., 1981; Higgins and Berg, 1987a); they are blocked instead by bungarotoxin 3.1 (Bgt 3.1) (Higgins and Berg, 1987a), previously referred to as bungarotoxin 3.1, toxin F or kappa-bungarotoxin (Loring and Zigmond, 1988). In this respect the receptors are similar to those on chick ciliary ganglion neurons (Ravdin and Berg, 1979) and rat sympathetic neurons (Sah et al., 1987). A resemblance between adrenal chromaffin AChRs and neuronal AChRs is to be expected since adrenal chromaffin cells share many properties with sympathetic neurons: both cell types derive from the neural crest, generate action potentials, synthesize and release catecholamines, and receive synaptic input from preganglionic neurons in the spinal cord.

Relatively little is known about adrenal chromaffin and vertebrate neuronal AChRs because until recently suitable probes were not available for studying the receptors. Two probes have now been demonstrated to distinguish the bovine adrenal chromaffin AChR. The first of these is a monoclonal antibody, mAb 35, to the "main immunogenic region" of muscle and electric organ AChR α subunit. mAb 35 cross-reacts with the AChR of chick ciliary ganglion neurons (Jacob et al., 1984; Smith et al., 1985, 1986; Halvorsen and Berg, 1987) and the AChR of bovine adrenal chromaffin cells (Higgins and Berg, 1987a). The second probe is n-Bgt, which blocks most of the nicotine-induced catecholamine release of bovine adrenal chromaffin cells and also modulates, or down-regulates, the number of AChRs on the cells available to bind mAb 35 (Higgins and Berg, 1987a). α -Bgt binds to a membrane component on bovine adrenal chromaffin cells, but the component appears to be distinct from the functional AChR (Higgins and Berg, 1987a, and unpublished observations), as has been shown for the α -Bgt binding component and functional AChRs on chick ciliary ganglion neurons (Jacob and Berg, 1983; Smith et al., 1983, 1985; Jacob et al., 1984; Halvorsen and Berg, 1986) and the rat pheochromocy-

Received June 18, 1987; revised Sept. 29, 1987; accepted Sept. 29, 1987.

We thank Dr. Palmer Taylor and Dr. Stanley Halvorsen for critical readings of the manuscript, and Dr. Jon Lindstrom for giving us the hybridoma cell line that produces mAb 35. Grant support was provided by the National Institutes of Health (NS 12601), the Muscular Dystrophy Association, and the American Heart Association. L.S.H. is a National Science Foundation Predoctoral Fellow.

Correspondence should be addressed to Darwin K. Berg, Department of Biology, B-022, University of California, San Diego, La Jolla, CA 92093.

Copyright © 1988 Society for Neuroscience 0270-6474/88/041436-11\$02.00/0

toma cell line PC12 (Patrick and Stallcup, 1977; Mitsuka and Hatanaka, 1983). The function of the α -Bgt binding component on these cells is unknown.

Labeled cholinergic ligands have been used to examine the regional distribution of AChRs in brain (Marks and Collins, 1982; Schwartz et al., 1982; Clarke et al., 1984, 1985a) and to characterize putative neuronal AChRs from brain tissue (Romano and Goldstein, 1980; Abood et al., 1983; Lippiello and Fernandes, 1986; Marks et al., 1986; Whiting and Lindstrom, 1986a, b, 1987a). A substantial discrepancy exists, however, between the measured affinities for labeled agonists binding to putative neuronal AChRs and the affinities inferred from physiological studies of AChR activation on neurons. The binding studies reveal agonist affinities that generally are several orders of magnitude higher than those suggested by the physiological responses of the receptors (Halvorsen and Berg, 1986; Margiotta et al., 1987). In contrast, the affinities of cholinergic antagonists measured in binding assays agree well with those obtained from physiological assays (Clarke et al., 1985b). A possible explanation is suggested by the binding properties of muscle and electric organ AChR whose agonists induce a slow conversion of the receptor to a desensitized state with dramatically increased affinity for agonists and little if any change in affinity for antagonists (Rang and Ritter, 1969, 1970a, b; Sine and Taylor, 1979; Weiland and Taylor, 1979; Heidmann et al., 1983). A similar phenomenon has been suggested for neuronal AChRs to account for the discrepancy in agonist affinities (Halvorsen and Berg, 1986; Whiting and Lindstrom, 1986b; Lippiello et al., 1987). No direct demonstration of a desensitized state with increased affinity for agonists has been provided for neuronal AChRs.

We show here that ^3H -nicotine binds with high affinity to a desensitized form of the AChR on bovine adrenal chromaffin cells. The receptor is the same as that previously identified by mAb 35 binding and can be modulated by exposure to mAb 35. Desensitization of the receptor is accompanied by an increase in agonist affinity with no apparent change in antagonist affinity. Some of these results have been described in a preliminary account (Higgins and Berg, 1987b).

Materials and Methods

Cell cultures. Bovine chromaffin cells were dissociated from adrenal medullae, purified on Percoll gradients, and maintained in culture as previously described (Higgins and Berg, 1987a). Cultures were prepared with 5×10^5 cells per 16 mm well (1 culture equivalent) or 1.5×10^6 cells/35 mm dish (3 culture equivalents) in culture medium consisting of Dulbecco's Modified Essential Medium (DMEM) containing 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin G, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were maintained at 35°C in 5% CO_2 /95% air and received fresh medium every 2 d. Cytosine arabinoside was included in the medium at a final concentration of 10^{-5} M after the first day. Cultures were taken for experiments 3–8 d after plating.

^3H -nicotine binding. To determine ^3H -nicotine binding to membrane fragments, cultures were scraped in 5 mM HEPES, pH 7.4, containing 137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 0.9 mM Na_2PO_4 , 0.4 mM KH_2PO_4 , 1.8 mM CaCl_2 , 5.6 mM glucose, and 2 mg/ml BSA (rinse buffer), homogenized in a glass tissue homogenizer, and centrifuged for 10 min in an Eppendorf microfuge at $15,600 \times g$. The pellet was resuspended in rinse buffer. Nicotine binding to the membrane fragments was carried out by incubating 3 culture equivalents of homogenate in 0.15 ml rinse buffer containing 20 nM ^3H -nicotine and 1 μM α -Bgt, unless otherwise indicated, for 70 min at room temperature (standard conditions). At the end of the reaction period, 0.05 ml aliquots of the reaction mixture were diluted to 5 ml, rapidly filtered over Whatman GF/B glass fiber filters that had been presoaked overnight in 0.01% polylysine to reduce nicotine binding to the filters, and washed 3 times with 5 ml of rinse

buffer at 37°C. The filter and rinse procedure was completed in a total of 15 sec. After the filters were allowed to dry, radioactivity retained on the filters was determined by liquid scintillation counting with a Beckman LS-100C counter using Aquasol-2 or Liquescent as solvent and fluor. Nonspecific binding was determined in the presence of 1 mM carbamylcholine (carbachol) unless otherwise indicated and has been subtracted.

The efficiency of receptor recovery after scraping and retention on the filters in the assay was determined in the following manner. Sister cultures labeled with ^{125}I -mAb 35 (see below) were either scraped in 0.2 ml of 0.6 N NaOH for measuring radioactivity in a Tracor analytic 1191 gamma counter directly or were scraped in rinse buffer, homogenized, centrifuged, resuspended, rapidly filtered as done for ^3H -nicotine binding assays and counted for radioactivity retained by the filters. A comparison of the 2 procedures indicated a mean value of $65 \pm 6\%$ ($\pm \text{SEM}$, $n=4$) for the overall efficiency of recovery in the filter assay. Data are presented as the mean $\pm$ SEM of triplicate determinations and have been corrected for assay efficiency.

Nicotine binding to intact cells was measured by replacing the media with media containing 20 nM ^3H -nicotine and 10^{-6} M α -Bgt, and incubating for 70 min at 37°C. Cultures were rinsed 4 times with 1 ml of rinse buffer, scraped in 0.1 ml 0.6 N NaOH, and assayed for radioactivity using liquid scintillation. Nonspecific binding was measured in the presence of 1 mM carbachol and has been subtracted.

Catecholamine release. Catecholamine release was determined as previously described (Higgins and Berg, 1987a). Briefly, cultures were preloaded with ^3H -norepinephrine (^3H -NE) for 2 hr at 37°C and then rinsed for 1 hr. Catecholamine release induced either by agonist at the indicated concentration or by 54 mM K^+ was measured by determining the radioactivity present in aliquots of the release media and of cell lysates obtained by scraping cultures in 0.6 N NaOH. ^3H -NE release is expressed as a percentage of the total radioactivity present in the cells prior to induced release. Single data points represent the mean $\pm$ SEM of triplicate determinations. Basal release, measured in the absence of secretagogue, has been subtracted.

mAb 35 binding. ^{125}I -mAb 35 binding to intact cells was measured as described by Higgins and Berg (1987a). Cultures were incubated with 5 nM ^{125}I -mAb 35 for 1 hr at 37°C, washed, scraped in 0.6 N NaOH, and analyzed for radioactivity. Nonspecific binding was determined in the presence of 5×10^{-7} M unlabeled mAb 35. Antibody binding to detergent extracts was determined using small DEAE cellulose columns as described by Stollberg and Berg (1987). Reaction mixtures contained 3 culture equivalents of extract and 2 nM ^{125}I -mAb 35, and bound ^{125}I -mAb 35 was determined in triplicate. Nonspecific binding was determined in the presence of excess unlabeled mAb 35. Data points represent the mean $\pm$ SEM of such triplicates and have been corrected for the efficiency of the assay ($70 \pm 6\%$, $n=5$), determined as described by Stollberg and Berg (1987).

α -Bgt binding. α -Bgt binding to intact cultures was determined as described by Smith et al. (1983). Briefly, culture media were replaced with media containing 10 nM ^{125}I α -Bgt. After incubating for 1 hr at 37°C, the cultures were washed, scraped in 0.6 N NaOH, and analyzed for radioactivity. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled α -Bgt.

Protein determination. Protein determinations were carried out by rinsing cultures 4 times with 1 ml aliquots of rinse solution lacking BSA, scraping the cells in 0.5 ml 0.6 N NaOH, diluting with an additional 0.5 ml of 0.4 N NaOH, and assaying for protein by the method of Lowry et al. (1951). BSA was used as a standard, and blanks were determined by assaying culture wells maintained with medium but lacking cells.

Materials. Bovine adrenal glands were obtained at Talone Packing Co., Escondido, CA. ^3H -(L)-nicotine (specific activity, 60–80 Ci/mmol) was purchased from New England Nuclear, Liquescent from National Diagnostics, and cholinergic ligands from Sigma with the exception of trimethaphan camsylate, which was the generous gift of Dr. W. E. Scott from Hoffman LaRoche, New Jersey. The hybridoma cell line secreting mAb 35 was generously provided by Jon Lindstrom of the Salk Institute. mAb 35 was purified and radioiodinated to specific activities of $2\text{--}3 \times 10^{18}$ cpm/mol as previously described (Smith et al., 1985). n-Bgt and α -Bgt were purified from *Bungarus multicinctus* venom (Miami Serpentarium) as previously described (Ravdin et al., 1981). ^{125}I - α -Bgt was prepared and isolated with initial specific activities of 250–300 Ci/mmol as previously described (Smith et al., 1985). Culture media components and other reagents were obtained as previously described (Higgins and Berg, 1987a).

Figure 1. Two classes of nicotine binding sites revealed by competition with α -Bgt. Binding of ^3H -nicotine was determined for membrane fragments prepared from cultures of bovine chromaffin cells in the presence of the indicated concentration of α -Bgt and is expressed as a percentage of the total binding that could be blocked by 1 mM carbachol. Values represent the mean $\pm$ SEM of 3 separate experiments, each done with triplicate cultures.

Figure 2. Time dependence of association and dissociation for ^3H -nicotine binding. Specific binding of ^3H -nicotine was determined for membrane fragments prepared from cultures of adrenal chromaffin cells under standard conditions, i.e., 20 nM ^3H -nicotine and 10^{-6} M α -Bgt for 70 min at 22°C followed by rapid filtration (15 sec), except for the indicated variations. *A*, Time of ^3H -nicotine binding was varied as indicated. *B*, At the end of the reaction period, aliquots of the reaction mixture were diluted 1000-fold with rinse buffer at 22°C, incubated, and washed within the total elapsed time indicated. The line in *B* represents the best least-squares fit of the data for a single-exponential decay process. Similar results were obtained in 2 additional experiments for *A* and 3 additional experiments for *B*. *Insets*, Pseudo-first-order (*A*) and first-order (*B*) plots of the data.

Figure 3. Concentration dependence of ^3H -nicotine binding. Membrane fragments were prepared from adrenal chromaffin cultures and incubated with the indicated concentration of ^3H -nicotine in the presence of 10^{-6} M α -Bgt using standard binding conditions. Scatchard analysis of the data (*inset*) indicated a K_D of 21 nM and a B_{max} of 16 fmol per culture (98 fmol/mg protein) for this experiment.

Results

Classes of nicotine binding sites

Previous studies suggested that bovine adrenal chromaffin cells are likely to have at least 2 classes of ^3H -nicotine binding sites since nicotine serves both as an agonist for the functional AChR and as a competitive ligand for the α -Bgt binding component (Wilson and Kirshner, 1977; Trifaro and Lee, 1980; Quick et al., 1986; Higgins and Berg, 1987a). As shown in Figure 1, ^3H -nicotine binding to adrenal chromaffin culture membranes can be divided pharmacologically into 2 classes. One class of binding sites, representing about 50% of the specific binding obtained with 20 nM ^3H -nicotine, is blocked by 10^{-7} M α -Bgt, while the remainder is not inhibited even by 10^{-5} M α -Bgt. Since α -Bgt does not prevent nicotine from activating AChRs on the cells (Trifaro and Lee, 1980; Higgins and Berg, 1987a), the α -Bgt-resistant ^3H -nicotine sites were chosen for additional study as candidates for functional AChRs. In all subsequent experiments, 10^{-6} M α -Bgt was routinely included in the binding reaction to block ^3H -nicotine binding to the α -Bgt binding component. This strategy facilitated characterization of ^3H -nicotine binding to the AChR described below.

Nicotine binding to a putative AChR

Specific binding of ^3H -nicotine to adrenal chromaffin membranes in the presence of 10^{-6} M α -Bgt reached equilibrium within 60 min at 22°C (Fig. 2*A*). Dissociation of the ligand occurred with a half-time under 2 min (Fig. 2*B*). Both association and dissociation curves can be described by single-exponential functions (*insets*, Figs. 2, *A*, *B*), as would be predicted for a single class of nicotine binding sites. Kinetic analysis of 4 such experiments yielded a value for the dissociation rate constant (k_{-1}) of $0.343 \pm 0.040 \text{ min}^{-1} \text{ mol}^{-1}$ (mean $\pm$ SEM). The association rate constant (k_1) was calculated to be $1.1 \pm 0.3 \times 10^6 \text{ min}^{-1}$ according to the method of Fields et al. (1978). The equilibrium dissociation constant (K_D) derived from k_1 and k_{-1} is 391 nM, 20-fold greater than the K_D derived from Scatchard

Figure 4. Inhibition of ³H-nicotine binding by cholinergic ligands. Binding of ³H-nicotine to membrane fragments prepared from chromaffin cultures was carried out in the presence of 10⁻⁶ M α -Bgt, 20 nM ³H-nicotine, and the indicated concentration of competing ligand. Membrane fragments were preincubated with α -Bgt and the competing ligand for 10 min prior to addition of ³H-nicotine. **A**, Competition by agonists: (-)nicotine (filled circles), ACh with 10⁻⁵ M eserine (open circles), carbachol (filled inverted triangles). **B**, Competition by antagonists: mecamylamine (filled circles), d-tubocurarine (filled triangles), trimethaphan (open circles), hexamethonium (open squares), decamethonium (open triangles), atropine (filled inverted triangles). Results are expressed as a percentage of the maximum specific binding, defined as that which was not blocked by 1 mM carbachol. Values represent the mean $\pm$ SEM of 3 experiments in **A** and of 4 experiments in **B**, each done in triplicate cultures.

analysis (see below). This discrepancy indicates that the observed ³H-nicotine binding cannot be described as a simple, reversible bimolecular reaction. Instead, the binding is complicated by an apparently rate-limiting, agonist-induced conversion of receptor sites to a form having high affinity for nicotine (see below). In subsequent experiments, binding reactions were normally carried out for 70 min and were terminated by rapid filtration and washing in a total of 15 sec.

Near saturation of binding was achieved with 45 nM ³H-nicotine (Fig. 3). Scatchard analysis revealed a single class of high-affinity binding sites with an apparent dissociation constant, K_D , of 20 ± 2 nM (mean $\pm$ SEM, $n=4$). This value falls within the wide range of affinities (2–30 nM) reported for high-affinity nicotine binding to rat and chicken brain membrane components (Romano and Goldstein, 1980; Clarke et al., 1984; Lippiello and Fernandes, 1986; Marks et al., 1986; Whiting and Lindstrom, 1986b). The B_{max} for binding from Scatchard analysis was 17 ± 2 fmol/culture equivalent or 104 ± 12 fmol/mg protein. Binding was linear with the amount of membrane ma-

Table 1. Binding parameters for cholinergic ligands at ³H-nicotine sites not blocked by α -Bgt

Competing ligand	n_H	IC ₅₀	K_i
Agonists			
ACh	1.1	1.4×10^{-7}	7.0×10^{-8}
Carbachol	1.0	6.6×10^{-7}	3.3×10^{-7}
Nicotine	1.1	3.4×10^{-8}	1.7×10^{-8}
Antagonists			
Atropine	0.93	7.6×10^{-5}	3.8×10^{-5}
Decamethonium	1.1	2.1×10^{-6}	1.0×10^{-6}
Hexamethonium	1.0	3.6×10^{-7}	1.8×10^{-7}
Mecamylamine	0.99	6.6×10^{-8}	3.3×10^{-8}
Trimethaphan	1.0	5.4×10^{-7}	2.7×10^{-7}
d-Tubocurarine	0.93	3.8×10^{-7}	1.9×10^{-7}

Inhibitory binding constants (K_i) were calculated as described by Cheng and Prusoff (1973) using data from the competition binding studies with ³H-nicotine described in Figure 4. In the case of ACh, 10⁻⁵ M eserine was included in the binding reactions to prevent hydrolysis of the agonist. Hill coefficients (n_H) were determined by transforming the data to Hill plots. Lines were fit by least-squares analysis with correlation coefficients ≥ 0.96 .

terial for the range tested (0–0.5 mg protein/assay, data not shown).

Pharmacology of nicotine binding

The pharmacology of the high-affinity nicotine binding sites was examined in competition binding studies using 20 nM ³H-nicotine and a variety of cholinergic ligands over a range of concentrations. Again 10⁻⁶ M α -Bgt was included throughout to prevent the ³H-nicotine from binding to the α -Bgt binding component. In each case the competing ligand was added 10 min prior to the ³H-nicotine, and binding was then carried out for the standard 70 min at 22°C followed by 15 sec of filtration and washing. All of the specific ³H-nicotine binding, defined by inhibition with 1 mM carbachol, was blocked as well by a number of nicotinic agonists (Fig. 4A) and antagonists (Fig. 4B). The inhibition constants (K_i) for various cholinergic ligands, calculated from data such as those shown in Figure 4, are listed in Table 1. In all cases the Hill coefficients (n_H) were near unity (Table 1).

n-Bgt blocks nicotine-induced catecholamine release from bovine adrenal chromaffin cells in culture (Higgins and Berg, 1987a) and so was also examined for blockade of ³H-nicotine binding to membrane fragments prepared from the cultures. The toxin was preincubated with the membranes for 40 min at 22°C to allow binding, and then ³H-nicotine was added to initiate the standard competition reaction. n-Bgt at 10⁻⁵ M blocked about three-quarters of the binding (Fig. 5); it was not practical to test higher concentrations. The breadth of the concentration dependence suggests heterogeneity in the ³H-nicotine sites with respect to n-Bgt binding.

Pharmacology of the functional AChR

The pharmacology of functional nicotinic receptors on bovine adrenal chromaffin cells was examined for comparison with the pharmacology of the ³H-nicotine binding sites described above. In these experiments, the cells were first loaded with ³H-NE, washed, and then tested for release of radioactivity induced by a cholinergic ligand within a 3 min test period. Previous studies demonstrated that the assay was specific for AChR-mediated catecholamine release and that it was dependent on extracellular

Figure 5. Inhibition of ^3H -nicotine binding by n-Bgt. Cultures were incubated with 10^{-6} M α -Bgt and the indicated concentrations of n-Bgt for 40 min prior to determination of ^3H -nicotine binding under standard conditions in the continued presence of the toxins. Values are expressed as a percentage of the specific binding (that not blocked by 1 mM carbachol) obtained in the absence of n-Bgt and represent the mean $\pm$ SEM of 7 experiments, each carried out in triplicate.

Ca^{2+} as expected for synaptic release (Trifaro and Lee, 1980; Kilpatrick et al., 1981; Higgins and Berg, 1987a). The maximal release elicited in the present experiments with each of the 3 agonists tested, namely, ACh, carbachol, and nicotine, was about 14% of the radioactivity associated with the cultures at the beginning of the test period (Fig. 6). Basal release, measured in the absence of secretagogue, has been subtracted. The submaximal levels of release observed with high concentrations of agonist (Fig. 6) suggest that AChRs on the cells desensitize (see below). Bovine adrenal chromaffin cells elaborate functional muscarinic as well as nicotinic receptors. Only nicotinic receptors, however, are thought to mediate catecholamine release from these cells (for review, see Livett, 1984). The 2 muscarinic agonists tested, oxotremorine and pilocarpine, did not elicit ^3H -NE release at 10^{-5} M (data not shown). Nevertheless, to exclude the possibility that catecholamine release in the present experiments with ACh and carbachol included a contribution from muscarinic receptors, the muscarinic antagonist atropine was included at 10^{-6} M in the release assay when testing these 2 agonists. Atropine at 10^{-6} M completely blocks activation of muscarinic receptors on bovine adrenal chromaffin cells by ACh (Fischer et al., 1981). In addition, eserine was included at 10^{-5} M in the release assay when ACh was tested to prevent hydrolysis

Figure 6. Catecholamine release induced by cholinergic ligands. The release of preloaded ^3H -NE from adrenal chromaffin cells in culture was measured in the presence of the indicated concentrations of ligand for a 3 min test period. Results are expressed as a percentage of the total radioactivity associated with the culture at the beginning of the test period and represent the mean $\pm$ SEM of 5 experiments, each carried out in triplicate. Basal release, measured in the absence of secretagogue was usually about 3%, and has been subtracted. Nicotine (filled circles), ACh with 10^{-5} M eserine and 10^{-6} M atropine (open circles), carbachol with 10^{-6} M atropine (filled triangles). No catecholamine release was induced by 10^{-5} M pilocarpine, oxotremorine, decamethonium, or hexamethonium in each of 3 experiments (data not shown).

of the agonist. No release was elicited by decamethonium (data not shown), a partial agonist at the muscle nicotinic AChR (Sine and Taylor, 1979). The concentrations eliciting half-maximal release ($\text{EC}_{50\text{app}}$) and the corresponding apparent Hill coefficients were calculated from data such as those shown in Figure 6 and are listed in Table 2. True $\text{EC}_{50\text{s}}$ and n_{Hs} could not be calculated since desensitization prevented accurate measurement of maximal responses. However, electrophysiological measurements have indicated EC_{50} values of $1\text{--}1.5 \times 10^{-5}$ M for ACh on rat adrenal chromaffin cells (Kidokoro and Ritchie, 1980; Kidokoro et al., 1982), which agree well with the value of 1.5×10^{-5} M calculated here and suggest that the $\text{EC}_{50\text{app}}$ may be good estimates of the true $\text{EC}_{50\text{s}}$.

The ability of cholinergic antagonists to block the catecholamine release elicited by nicotine was investigated by assaying for ^3H -NE release in the presence of 10^{-6} M nicotine and a variety of competing ligands over a range of concentrations (Fig. 7). Cultures were incubated with the antagonists for 60 min prior to the 3 min test period to allow binding to reach equilibrium. α -Bgt did not significantly block nicotine-induced catecholamine release over the concentration range tested, as expected since the α -Bgt binding component on the cells is thought to be distinct from the functional AChR (Trifaro and Lee, 1980; Kilpatrick et al., 1981; Higgins and Berg, 1987a). The apparent inhibition constants and Hill coefficients for each of the antagonists were calculated from data such as those shown in Figure 7 and are listed in Table 3.

Both the rank order potency and the K_i values for antagonists in the binding assay are in good agreement with those obtained for the antagonists in the functional assay. The results are consistent with the high-affinity binding site detected by ^3H -nicotine as being the same site at which nicotine elicits AChR-mediated catecholamine release from the cells. In contrast, there is a large discrepancy in affinities of agonists detected by the binding assay compared with those obtained from the functional release assay. Values derived from the former are several orders of magnitude

Table 2. Pharmacological parameters for activation of agonist-induced catecholamine release

Activating ligand	n_{Happ}	r	n	$\text{EC}_{50\text{app}}$
ACh	1.3	0.99	6	1.5×10^{-5}
Carbachol	1.1	0.99	5	2.2×10^{-4}
Nicotine	1.7	0.95	4	1.4×10^{-6}

Concentrations required to reach half-maximal activation ($\text{EC}_{50\text{app}}$) and apparent Hill coefficients (n_{Happ}) were determined by transforming the data in Figure 6 to Hill plots. True $\text{EC}_{50\text{s}}$ and n_{Hs} cannot be determined due to desensitization of the response, especially at higher doses of agonist. r , correlation coefficient for least-squares analyses of data for Hill plots; n , number of separate experiments.

Figure 7. Inhibition of nicotine-induced catecholamine release by cholinergic ligands. The release of preloaded ^3H -NE from adrenal chromaffin cells in culture was measured in the presence of 10^{-6} M nicotine and cholinergic ligand at the indicated concentrations for a 3 min test period. Cultures were preincubated with the competing ligand for 60 min prior to the test period in order to allow binding to reach equilibrium. Results are expressed as a percentage of the total radioactivity associated with the culture at the beginning of the test period and represent the mean $\pm$ SEM of 4–5 experiments, each carried out in triplicate. Basal release, measured in the absence of secretagogue, has been subtracted. Mecamylamine (filled circles), *d*-tubocurarine (filled triangles), trimethaphan (open circles), hexamethonium (open squares), decamethonium (open triangles), atropine (filled inverted triangles), α -Bgt (filled squares).

higher than those derived from the latter. A similar phenomenon has been described for muscle (Rang and Ritter, 1969, 1970a, b; Sine and Taylor, 1979) and electric organ AChRs (Weiland and Taylor, 1979; Heidmann et al., 1983), where cholinergic agonists have been shown to induce a slow transition of the AChR to a desensitized state with much increased affinity for agonists. In general, antagonists seem neither to induce the conversion nor to differentiate between the receptor states with respect to binding affinity. It is known that adrenal chromaffin AChRs desensitize (Clapham and Neher, 1984). The long incubation times used to achieve equilibrium in the ^3H -nicotine binding experiments may be necessary to allow desensitization of the AChR so that a high-affinity nicotine binding state will result that is detectable in the binding assay. To test this possibility, experiments were carried out to identify desensitizing conditions for the adrenal chromaffin AChR and to examine the effects of these conditions on ^3H -nicotine binding.

Desensitization of the AChR response

The effects of a 1 hr pre-exposure to agonist were tested on AChR desensitization since binding experiments were usually carried out for 1 hr. Cells were first loaded with ^3H -NE, incubated with low concentrations of nicotine for 60 min at 37°C , and then tested for ^3H -NE release in response to various concentrations of nicotine during a 3 min test period. (The nicotine concentrations chosen for the pre-exposure were inadequate to induce measurable ^3H -NE release.) To increase the rate of possible receptor desensitization by nicotine, 10^{-5} M substance P was included in the initial 1 hr incubation. Previous electro-

Table 3. Pharmacological parameters for inhibition of nicotine-induced catecholamine release

Competing ligand	n_H	IC_{50}	K_{app}
Atropine	1.0	5.6×10^{-5}	3.3×10^{-5}
Decamethonium	1.0	3.3×10^{-6}	1.9×10^{-6}
Hexamethonium	0.94	7.8×10^{-7}	4.6×10^{-7}
Mecamylamine	0.99	9.8×10^{-8}	5.7×10^{-8}
Trimethaphan	0.97	2.4×10^{-7}	1.4×10^{-7}
<i>d</i> -Tubocurarine	1.1	2.3×10^{-7}	1.3×10^{-7}

Concentrations required to inhibit half of the nicotine-stimulated ^3H -NE release (IC_{50}) and Hill coefficients (n_H) were calculated from indirect Hill plots using the data shown in Figure 7. K_{app} was calculated as described by Cheng and Prusoff (1973) using the $\text{EC}_{50,app}$ for nicotine shown in Table 2. Correlation coefficients of least-squares fit of data in modified Hill plots were ≥ 0.98 .

physiological (Clapham and Neher, 1984) and catecholamine release studies (Mizobe et al., 1979; Role et al., 1981; Livett and Boska, 1984) have demonstrated that substance P enhances the rate but not the extent of AChR desensitization on adrenal chromaffin cells. The pretreatment with nicotine and substance P dramatically reduced the maximal response of the cells without significantly changing the $\text{EC}_{50,app}$ of the response for nicotine (Fig. 8), as expected for treatments promoting receptor desensitization. Preincubation with substance P alone had no effect on nicotine-induced catecholamine release. The effects of nicotine and substance P were specific in that the treatment had no effect on basal release or on release induced by 54 mM K^+ (data not shown). The effects were reversible since a 1 hr wash restored nicotine-induced ^3H -NE release to control levels (data not shown).

The concentration dependence of receptor desensitization by nicotine was determined by incubating cultures with a range of nicotine concentrations for 1 hr in the presence of 10^{-5} M substance P and then assaying the release of ^3H -NE induced by 10^{-6} M nicotine in the standard 3 min test period (Fig. 9). Using Eadie-Hofstee analysis, a value of 20.2 ± 4.6 nM (mean $\pm$ SEM; $n = 6$) was calculated for the EC_{50} for nicotine desensitizing the functional response under these conditions. (Receptor desensitization was also obtained in several experiments in which substance P was omitted from the preincubation (data not shown).) The EC_{50} value is in excellent agreement with the K_D for ^3H -nicotine binding obtained by Scatchard analysis, supporting the hypothesis that the high-affinity site detected with ^3H -nicotine and the site at which nicotine desensitizes the functional cholinergic response are the same.

Effects of desensitizing conditions on ^3H -nicotine binding

The hypothesis that the high-affinity binding site detected by ^3H -nicotine represents the desensitized AChR on adrenal chromaffin cells was tested further by examining the effects of desensitizing conditions on the rate of association of ^3H -nicotine with its binding site. Inclusion of 10^{-5} M substance P in the reaction increased the rate of ^3H -nicotine binding but had no effect on the equilibrium levels of binding (Fig. 10). These results are consistent with substance P increasing the rate, but not the extent, of conversion for the nicotine binding site to a high-affinity state, as was found for the effects of substance P on AChR desensitization. The association rate of ^3H -nicotine was also measured after a 70 min exposure of membrane fragments to 20 nM unlabeled nicotine in the absence of substance P (Fig. 10). Equilibrium binding of ^3H -nicotine was reached by 6 min

Figure 8. Effect of prior exposure to substance P and nicotine on nicotine-induced catecholamine release. Cultures were preloaded with ^3H -NE and then incubated with 10^{-5} M substance P together with either 20 nM (open circles) or 100 nM (filled triangles) nicotine for 60 min at 37°C . The incubation media were removed and the cultures assayed for nicotine-induced catecholamine release in the presence of the indicated concentration of nicotine for a 3 min test period. Basal release, which was unaffected by the pretreatments, has been subtracted. Catecholamine release by cultures preincubated with substance P alone was indistinguishable from that of control cultures (filled circles). Values represent the mean $\pm$ SEM of 3 experiments with triplicate cultures per determination. EC_{50} values of 1.4, 1.8, and 2.7×10^{-6} M were obtained for the conditions of 0, 20, and 100 nM nicotine, respectively, in the preincubation.

rather than by 60 min as required in the absence of prior exposure of the membranes to agonist. An association rate constant was calculated from 4 experiments according to the method of Fields et al. (1978) and yielded a value of $1.8 \pm 0.2 \times 10^7 \text{ min}^{-1} \text{ mol}^{-1}$ (mean $\pm$ SEM). When this association rate constant is used together with the dissociation rate constant to calculate the K_D for ^3H -nicotine binding, a value of 19 nM is obtained. This is in good agreement with the K_D obtained from Scatchard analysis for ^3H -nicotine binding. The results indicate

Figure 9. Concentration dependence of the nicotine-induced desensitization of agonist-induced catecholamine release. Cultures were preloaded with ^3H -NE and then incubated with 10^{-5} M substance P and the indicated concentration of nicotine for 60 min. The incubation media were then removed and the cultures assayed for catecholamine release in the presence of 10^{-6} M nicotine during a 3 min test period. Basal release was unaffected by the pretreatment and has been subtracted. *Inset*, Eadie-Hofstee analysis reveals an EC_{50} of 20 nM. Values represent the mean $\pm$ SEM of 6 experiments in triplicate.

Figure 10. Rates of ^3H -nicotine binding. Membrane fragments prepared from adrenal chromaffin cultures were tested for specific binding of ^3H -nicotine in the presence (open circles) or absence (filled circles) of 10^{-5} M substance P under standard binding conditions. Alternatively, the membrane fragments were preincubated with 20 nM unlabeled nicotine (and 10^{-6} M α -Bgt) for 70 min at a concentration of 3 culture equivalents/0.050 ml and then assayed for ^3H -nicotine binding under standard conditions by adding 0.10 ml of ^3H -nicotine (and 10^{-6} M α -Bgt) (filled triangles). In all cases, binding reactions were carried out for the indicated times, and nonspecific binding was determined in the presence of 1 mM *d*-tubocurarine. Values represent the mean $\pm$ SEM of 3–4 experiments in triplicate.

that low concentrations of nicotine can induce a receptor state that is functionally desensitized. Moreover, the same conditions that produce desensitization also induce the high-affinity binding state for nicotine and strongly suggest that the ^3H -nicotine binding observed to adrenal chromaffin cell membranes represents binding to a desensitized form of the functional AChR.

Surface and internal pools of receptor

The ^3H -nicotine binding experiments described above were carried out with membrane fragments prepared from adrenal chromaffin cultures because the system presented a favorable signal to noise ratio for examining ^3H -nicotine binding. Binding to membrane fragments would be expected to include contributions from AChRs both in the plasma membrane and in internal membranes associated with intracellular organelles. There is precedence for large intracellular pools of AChRs both in neurons (Jacob et al., 1986; Stollberg and Berg, 1987) and in muscle (Devreotes et al., 1978; Merlie and Lindstrom, 1983; Pestronk, 1985). Accordingly, ^3H -nicotine binding experiments were also carried out with intact cells in adrenal chromaffin cultures to determine the proportion of high-affinity nicotine binding sites present on the surface of the cells. Background ^3H -nicotine binding was substantial in the cultures, but it was possible to estimate the proportion of surface sites as constituting approximately 80% of the total specific sites associated with the cells (Table 4). Scatchard analysis of ^3H -nicotine binding to intact cells indicated a single population of sites (in the presence of 10^{-6} M Bgt) with a K_D of about 20 nM (data not shown), as observed for binding to membrane fragments described above.

mAb 35 has previously been shown to cross-react with AChRs on adrenal chromaffin cells (Higgins and Berg, 1987a). The total number of specific mAb 35 binding sites associated with membrane fragments was measured and compared with the number

Figure 11. mAb 35 modulation of nicotine binding sites. Cultures were exposed to 7 nM mAb 35 for 24 hr and then assayed for ^3H -nicotine binding to AChRs (in the presence of 10^{-6} M α -Bgt) either to membrane fragments (Total) or to intact cells (Surface). The relative number of Bgt binding sites (α -Bgt-R) was determined by measuring the amount of ^3H -nicotine binding that could be inhibited by α -Bgt on membrane fragments (total) or by measuring the number of ^{125}I - α -Bgt binding sites on intact cells (surface). Hatched bars, mAb 35-treated cultures; open bars, sister cultures not exposed to mAb 35. Values represent the mean $\pm$ SEM of 4–6 experiments with triplicate cultures.

of mAb 35 sites present on intact cells to determine the proportions of surface and intracellular mAb 35 binding sites in the cultures. As in the case of nicotine binding sites, about 80% of the total mAb 35 binding in the cultures was associated with the cell surface. The ratio of mAb 35 binding sites to nicotine binding sites was about 1 both for surface sites and for total sites (Table 4). The ratio for internal sites could not be meaningfully calculated from the data because of the relatively large SEMs associated with the values.

mAb 35 modulation of nicotine binding sites

Extended exposure of adrenal chromaffin cells to mAb 35 has previously been shown to eliminate nicotine-induced catecholamine release, suggesting that the antibody can down-regulate the receptor through antigenic modulation (Higgins and Berg, 1987a). mAb 35 does not compete directly with ^3H -nicotine for binding (data not shown), a result consistent with the failure of mAb 35 to block AChR function (Lindstrom et al., 1983; Smith et al., 1986; Higgins and Berg, 1987a). It is known that antibodies to the "main immunogenic region" of muscle and electric organ AChR α subunit can induce the clustering, internalization, and degradation of muscle AChRs (Heinemann et al., 1977; Merlie et al., 1979). The possibility of antigenic modulation by mAb 35 to remove AChRs from the cell surface was tested here by measuring the number of ^3H -nicotine binding sites remaining on the cells and present in membrane fragments from cultures after incubation with mAb 35 for 24 hr. The treatment completely removed the surface sites and reduced the number of sites in membrane fragments by about 80% (Fig. 11). The remaining sites in the membrane fragments apparently represent the preexisting intracellular pool of AChRs. The numbers of total and surface α -Bgt binding sites were unaffected by the antibody (Fig. 11).

Table 4. Surface and internal AChRs

Ligand	Total sites (fmol/culture)	Surface sites (fmol/culture)	Calculated internal sites (fmol/culture)
^{125}I -mAb 35	10.2 ± 0.7 (6)	8.6 ± 0.4 (6)	1.6 ± 0.8
^3H -nicotine	11.4 ± 1.5 (12)	8.9 ± 1.6 (9)	2.5 ± 1.9
Ratio mAb 35/nic	0.9	1.0	

Total ^{125}I -mAb 35 binding sites were measured for extracts prepared from adrenal chromaffin cultures using small DEAE cellulose columns. Total ^3H -nicotine sites were assayed using rapid filtration under standard conditions. In both cases, values were corrected for assay efficiencies (70 and 65%, respectively), determined as described in Materials and Methods. Surface sites were assayed on intact cells, and the number of internal sites was calculated by subtracting the number of surface sites from the number of total sites. In all cases, values have been corrected for fractional occupancy (ρ) by the ligand according to the equation: $\rho = K_D / ([\text{ligand}] + K_D)$ using $K_D = 2$ nM for mAb 35 and $K_D = 20$ nM for nicotine. Values represent mean $\pm$ SEM for the number of separate experiments indicated in parentheses. The large relative SEMs associated with the calculated numbers of internal sites prevented a meaningful calculation for the ratio of mAb 35/nicotine binding in this case.

Discussion

The major findings reported here are that (1) the AChRs responsible for mediating catecholamine release from bovine adrenal chromaffin cells can be detected by high-affinity ^3H -nicotine binding, and (2) the rate-limiting step in obtaining the observed binding is the formation of a desensitized receptor state having increased affinity for agonists. The first conclusion derives from the following. Scatchard analysis indicates a single class of high-affinity ^3H -nicotine binding sites on the cells (when 10^{-6} M α -Bgt is used to prevent nicotine binding to α -Bgt binding sites). Binding to this site is inhibited by classical cholinergic antagonists with the same affinities that the antagonists inhibit AChR function, and the binding of the antagonists display Hill coefficients close to 1. The stoichiometry of mAb 35 and ^3H -nicotine binding on the cells is approximately 1, and mAb 35 has previously been shown to be a specific probe for AChRs on the cells (Higgins and Berg, 1987a). Antigenic modulation of AChRs on the cells by mAb 35 results in the specific and complete loss of AChR function and high-affinity ^3H -nicotine binding from the cells in a coordinate way.

The second conclusion derives from the observation that agonist-induced desensitization of the receptor accelerates the subsequent rate of ^3H -nicotine binding to the cells. The rates of association and dissociation under these conditions yield a kinetically derived K_D for nicotine binding that is equivalent in value to that obtained from equilibrium binding studies. Moreover, the apparent K_D obtained for receptor desensitization by nicotine is equivalent to the K_D obtained for ^3H -nicotine from equilibrium binding studies. Agonist-induced conversion of the receptor to a desensitized form having increased agonist affinity accounts for the differences between the apparent EC_{50} 's obtained for receptor activation by agonists in the short catecholamine release assay and the K_i 's measured for inhibition of ^3H -nicotine binding by the agonists in the longer binding assay.

These findings are consistent with the model proposed by Katz and Thesleff (1957) for desensitization of muscle AChR in which agonist association and dissociation are rapid and agonist-induced conversion of the receptor to the desensitized state is slow. The higher affinity of agonists for the desensitized state drives the binding reaction. Antagonists, with the excep-

tion of metaphilic antagonists, have the same affinity for the 2 forms and therefore do not induce the desensitized form. The model has been supported by a number of studies showing an agonist-induced conversion of muscle and electric organ AChRs to a desensitized form having increased affinity for agonist and no change in affinity for antagonists (Rang and Ritter, 1969, 1970a, b; Sine and Taylor, 1979; Weiland and Taylor, 1979; Heidmann et al., 1983). Similar explanations are likely to hold for differences observed in agonist affinity measured by receptor activation and that measured by competition with labeled receptor ligands for binding to AChRs both in the central and peripheral nervous systems (Halvorsen and Berg, 1986; Whiting and Lindstrom, 1986b).

Substance P was used in the present studies to enhance the rate of agonist-induced AChR desensitization and provided evidence that desensitization is the rate-limiting step in ^3H -nicotine binding. Substance P only exerts its modulatory action in the presence of agonist since prior exposure to substance P alone has no effect on receptor function. Enhancement of agonist-induced AChR desensitization by substance P has previously been reported for bovine chromaffin cells (Clapham and Neher, 1984) and also for the rat pheochromocytoma cell line PC12 (Stallcup and Patrick, 1980). In contrast, Livett and coworkers reported that, although substance P inhibits agonist-induced catecholamine release from chromaffin cells (Mizobe et al., 1979; Livett and Boska, 1984), it protects against receptor desensitization produced by prior exposure to agonist. This differs from our results and may be a consequence of the relatively high concentrations of ACh ($>10^{-4}$ M) and nicotine ($>2.5 \times 10^{-6}$ M) used in the preincubations in their protocols compared with the low concentrations of nicotine ($<10^{-7}$ M) used here.

The stoichiometry of about 1:1 for mAb 35 and nicotine binding sites on bovine adrenal chromaffin cells suggests similarities between the adrenal chromaffin AChR and AChRs from muscle and electric organ. In the latter cases, the receptors are known to contain 2 α subunits, each having an agonist binding site and a mAb 35 binding site (McCarthy et al., 1986). Immunoaffinity-purified AChRs from chicken brain (Whiting and Lindstrom, 1986a) and from bovine adrenal medulla (S. Swope and D. Berg, unpublished observations) appear to have at least 2 mAb 35 binding sites per receptor, since immobilization of receptor by mAb 35 coupled to Sepharose still permits the detection of additional mAb 35 binding sites on the receptors. Chick ciliary ganglion AChRs display Hill coefficients of about 2 for activation by agonists (Brenner and Martin, 1976; Margiotta et al., 1987), as do muscle AChRs (Hartzell et al., 1975; Dionne et al., 1978). Subunit analyses of neuronal and adrenal medullary AChRs, however, suggest substantial differences from that of muscle AChRs (Whiting and Lindstrom, 1986a, 1987a, b; Halvorsen and Berg, 1987; S. Swope and D. Berg, unpublished observations). The mAb 35 and nicotine binding sites may even be associated with separate subunits in neuronal AChRs (Halvorsen and Berg, 1987; Whiting and Lindstrom, 1987a, b).

The ratio of mAb 35 and nicotine binding is approximately 1 both for surface sites and for total sites in adrenal chromaffin culture membranes. In principle, one could calculate the ratio for internal sites, as well, by subtracting surface sites from total sites to obtain intracellular AChRs sites for each ligand. In the present case, the relatively large errors associated with calculated values for numbers of internal sites prevents a ratio from being determined. About 20% of the total AChRs associated with the adrenal chromaffin cells are intracellular. This contrasts with the large proportion of intracellular AChRs present in embry-

onic chick brain (Swanson et al., 1983) and ciliary ganglia (Jacob et al., 1986; Stollberg and Berg, 1987), as well as in developing chick and rat muscle (Devreotes et al., 1978; Merlie and Lindstrom, 1983; Merlie, 1984; Pestronk, 1985). The reduced proportion of intracellular receptors in adrenal chromaffin cells may reflect the fact that the cells were obtained from adult tissue and have lost an embryonic feature. Embryonic rat brain contains a large intracellular pool of voltage-dependent sodium channel protein, which is much reduced in adult tissue (Schmidt et al., 1985), and the intracellular pool of AChR in muscle is reduced in adult rats (Pestronk, 1985). Precedence for an altered ratio of binding sites on intracellular receptor comes from studies on muscle AChR intracellular precursors, which acquire binding sites for *d*-tubocurarine and α -Bgt at different times during receptor maturation (Carlin et al., 1986).

No evidence for heterogeneity of adrenal chromaffin AChRs was obtained from the equilibrium binding studies, kinetic analyses, or physiological assays of classical agonist and antagonist interactions with the receptor. The complete removal of high-affinity nicotine binding sites from the surface of the cells by mAb 35 treatment also supports this interpretation. n-Bgt competition with ^3H -nicotine for binding, however, occurs over a broad range of toxin concentrations and suggests heterogeneity in the interactions of the toxin with the receptor. A similar broad concentration dependence was previously observed for blockade of receptor function by n-Bgt (Higgins and Berg, 1987a). The apparent heterogeneity does not reflect a contribution from α -Bgt binding sites on the cells. Though 2 separate classes of high-affinity ^3H -nicotine binding sites were detected, one representing the AChR and the other representing the α -Bgt binding component, high concentrations of α -Bgt were consistently used to block the latter. This approach was adopted because previous studies (Trifaro and Lee, 1980; Kilpatrick et al., 1981), as well as those described here with mAb-mediated modulation, indicated that the α -Bgt binding component is distinct from the AChR on the cells. The molecular basis for the apparent heterogeneity of n-Bgt binding to the AChR is unknown.

The demonstration that desensitized adrenal chromaffin AChRs bind agonist with greater affinity resolves discrepancies between agonist affinities inferred from equilibrium binding studies and those inferred from receptor activation. To the extent that adrenal chromaffin AChRs are representative of neuronal receptors, the findings provide further evidence for the similarity between neuronal and muscle AChRs with respect to allosteric properties (Changeux et al., 1984). Both types of receptors require at least 2 molecules of agonist for activation, both are desensitized by exposure to agonist, both desensitize more rapidly in the presence of substance P, and both display increased agonist affinity when desensitized. The allosteric similarities are impressive in view of the likely differences in structure between neuronal and muscle AChRs (Boulter et al., 1986; Whiting and Lindstrom, 1986a, 1987a, b).

References

- Aboud, L. G., W. Latham, and S. Grassi (1983) Isolation of a nicotine binding site from rat brain by affinity chromatography. *Proc. Natl. Acad. Sci. USA* 80: 3536-3539.
- Boulter, J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick (1986) Isolation of a cDNA clone coding for a possible neuronal nicotinic acetylcholine α -subunit. *Nature* 319: 368-374.
- Brandt, B. L., S. Hagiwara, Y. Kidokoro, and S. Miyazaki (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol. (Lond.)* 263: 417-439.
- Brenner, H. R., and A. R. Martin (1976) Reduction in acetylcholine

- sensitivity of axotomized ganglion cells. *J. Physiol. (Lond.)* 260: 159–175.
- Carlin, B. E., J. C. Lawrence, Jr., J. M. Lindstrom, and J. P. Merlie (1986) An acetylcholine receptor precursor α subunit that binds α bungarotoxin but not δ -tubocurarine. *Proc. Natl. Acad. Sci. USA* 83: 498–502.
- Changeux, J.-P., A. Devillers-Thiery, and P. Chemarilli (1984) Acetylcholine receptor: An allosteric protein. *Science* 225: 1335–1345.
- Cheng, Y.-C., and W. H. Prusoff (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 22: 3099–3108.
- Clapham, D. E., and E. Neher (1984) Substance P reduces acetylcholine-induced currents in isolated bovine chromaffin cells. *J. Physiol. (Lond.)* 347: 255–277.
- Clarke, P. B. S., C. B. Pert, and A. Pert (1984) Autoradiographic distribution of nicotine receptors in rat brain. *Brain Res.* 323: 390–395.
- Clarke, P. B. S., R. D. Schwartz, S. M. Paul, C. B. Pert, and A. Pert (1985a) Nicotine binding in rat brain: Autoradiographic comparison of [3 H] acetylcholine, [3 H] nicotine, and [125 I]- α -bungarotoxin. *J. Neurosci.* 5: 1307–1315.
- Clarke, P. B. S., D. W. Hommer, A. Pert, and L. R. Skirboll (1985b) Electrophysiological actions of nicotine on Substantia nigra single units. *Br. J. Pharmacol.* 85: 827–835.
- Devreotes, P. N., J. M. Gardner, and D. M. Fambrough (1978) Kinetics of biosynthesis of acetylcholine receptor and subsequent incorporation into plasma membrane of cultured chick skeletal muscle. *Cell* 10: 365–373.
- Dionne, V. E., J. H. Steinbach, and C. F. Stevens (1978) An analysis of the dose-response relationship at voltage clamped frog neuromuscular junction. *J. Physiol. (Lond.)* 281: 421–444.
- Fenwick, E. M., A. Marty, and E. Neher (1982) A patch clamp study of bovine chromaffin cells and their sensitivity to acetylcholine. *J. Physiol. (Lond.)* 331: 599–635.
- Fields, J. Z., W. R. Roeske, E. Markin, and H. I. Yamamura (1978) Cardiac muscarinic cholinergic receptors: Biochemical identification and characterization. *J. Biol. Chem.* 253: 3251–3258.
- Fischer, S. K., R. W. Holz, and B. W. Agranoff (1981) Muscarinic receptors in chromaffin cell cultures mediate enhanced phospholipid labeling but not catecholamine secretion. *J. Neurochem.* 37: 491–497.
- Halvorsen, S. W., and D. K. Berg (1986) Identification of a nicotinic acetylcholine receptor on neurons using an α -neurotoxin that blocks receptor function. *J. Neurosci.* 6: 3405–3412.
- Halvorsen, S. W., and D. K. Berg (1987) Affinity labeling of neuronal acetylcholine receptors with an α -neurotoxin that blocks receptor function. *J. Neurosci.* 7: 2547–2555.
- Hartzell, H. C., S. W. Kuffler, and D. Yoshikami (1975) Post synaptic potentiation: Interaction between quanta of acetylcholine at the skeletal neuro muscular synapse. *J. Physiol. (Lond.)* 251: 427–463.
- Heidmann, T., J. Bernhardt, E. Neumann, and J.-P. Changeux (1983) Rapid kinetics of agonist binding and permeability response analyzed in parallel on acetylcholine receptor rich membranes from *Torpedo marmota*. *Biochemistry* 22: 5452–5459.
- Heinemann, S., S. Bevan, R. Kullberg, J. Lindstrom, and J. Rice (1977) Modulation of acetylcholine receptor by antibody against the receptor. *Proc. Natl. Acad. Sci. USA* 74: 3090–3094.
- Higgins, L. S., and D. K. Berg (1987a) Immunological identification of a nicotinic acetylcholine receptor on bovine chromaffin cells. *J. Neurosci.* 7: 1792–1798.
- Higgins, L. S., and D. K. Berg (1987b) A desensitized form of neuronal acetylcholine receptor detected by 3 H-nicotine binding on bovine adrenal chromaffin cells. *Soc. Neurosci. Abstr.* 17: 796.
- Jacob, M. H., and D. K. Berg (1983) The ultrastructural localization of α -bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. *J. Neurosci.* 3: 260–271.
- Jacob, M. H., D. K. Berg, and J. M. Lindstrom (1984) Shared antigenic determinant between the *Electrophorus* acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons. *Proc. Natl. Acad. Sci. USA* 81: 3223–3227.
- Jacob, M. H., J. M. Lindstrom, and D. K. Berg (1986) Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *J. Cell Biol.* 103: 205–214.
- Katz, B., and S. Thesleff (1957) A study of the “desensitization” produced by acetylcholine at the motor endplate. *J. Physiol. (Lond.)* 138: 63–80.
- Kidokoro, Y., and A. E. Ritchie (1980) Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol.* 307: 199–216.
- Kidokoro, Y., S. Miyazaki, and S. Osawa (1982) Acetylcholine-induced membrane depolarization and potential fluctuations in the rat adrenal chromaffin cell. *J. Physiol. (Lond.)* 324: 203–220.
- Kilpatrick, D. L., R. Slepetis, and N. Kirshner (1981) Inhibition of catecholamine secretion from adrenal medulla cells by neurotoxins and cholinergic antagonists. *J. Neurochem.* 37: 125–131.
- Lindstrom, M., S. Tzartos, W. Gullick, S. Hochswender, L. Swanson, P. Sargent, M. Jacob, and M. Montal (1983) Use of monoclonal antibodies to study acetylcholine receptors from electric organs, muscle, and brain, and the autoimmune response to receptor in myasthenia gravis. *Cold Spring Harbor Symp. Quant. Biol.* 48: 89–99.
- Lippiello, P. M., and K. G. Fernandes (1986) The binding of L-[3 H]nicotine to a single class of high affinity sites in rat brain membranes. *Mol. Pharmacol.* 29: 488–454.
- Lippiello, P. M., S. B. Sears, and K. G. Fernandes (1987) Kinetics and mechanism of L-[3 H]nicotine binding to putative high affinity receptor sites in rat brain. *Mol. Pharmacol.* 31: 392–400.
- Livett, B. G. (1984) Adrenal medullary chromaffin cells *in vitro*. *Physiol. Rev.* 64: 1103–1161.
- Livett, B. G., and P. Boska (1984) Receptors and receptor modulation in cultured chromaffin cells. *Can. J. Physiol. Pharmacol.* 62: 467–476.
- Loring, R. H., and R. E. Zigmond (1988) Characterization of neuronal nicotinic receptors by snake venom neurotoxins. *TINS* (in press).
- Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
- Margiotta, J. F., D. K. Berg, and V. E. Dionne (1987) The properties and regulation of functional acetylcholine receptors on chick ciliary ganglion neurons. *J. Neurosci.* 7: 3612–3622.
- Marks, M. J., and A. C. Collins (1982) Characterization of nicotine binding in mouse brain and comparison with binding of α bungarotoxin and quinuclidinyl benzolate. *Mol. Pharmacol.* 22: 554–564.
- Marks, M. J., J. A. Stitzel, E. Romm, J. M. Wehner, and A. C. Collins (1986) Nicotinic binding sites in rat and mouse brain: Comparison of acetylcholine, nicotine, and α -bungarotoxin. *Mol. Pharmacol.* 30: 427–436.
- Merlie, J. P. (1984) Biogenesis of the acetylcholine receptor, a multisubunit integral membrane protein. *Cell* 36: 573–575.
- Merlie, J. P., and J. Lindstrom (1983) Assembly *in vivo* of mouse muscle acetylcholine receptor: Identification of an alpha subunit species that may be an assembly intermediate. *Cell* 34: 747–757.
- Merlie, J. P., S. Heinemann, and J. M. Lindstrom (1979) Acetylcholine receptor degradation in adult rat diaphragms in organ culture and the effect of anti-acetylcholine receptor antibodies. *J. Biol. Chem.* 254: 6320–6327.
- Mitsuka, M., and H. Hatanaka (1983) Selective loss of acetylcholine sensitivity in a nerve cell line cultured in hormone-supplemented serum-free medium. *J. Neurosci.* 3: 1785–1790.
- Mizobe, F., V. Kozusek, D. M. Dean, and B. G. Livett (1979) Pharmacological characterization of adrenal paraneurons: Substance P and somatostatin as inhibitory modulators of the nicotinic response. *Brain Res.* 178: 555–566.
- Patrick, J., and W. B. Stallcup (1977) Immunological distinction between acetylcholine receptor and the α -bungarotoxin binding component on sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 74: 4689–4692.
- Pestronk, A. (1985) Intracellular acetylcholine receptors in skeletal muscles of the adult rat. *J. Neurosci.* 5: 1111–1117.
- Quick, M., S. Fournier, and J. M. Trifaro (1986) Modulation of the nicotinic α -bungarotoxin site in chromaffin cells in culture by a factor(s) endogenous to neuronal tissue. *Brain Res.* 372: 11–20.
- Rang, H. P., and J. M. Ritter (1969) A new kind of drug antagonism: Evidence that agonists cause a molecular change in acetylcholine receptors. *Mol. Pharmacol.* 5: 394–411.
- Rang, H. P., and J. M. Ritter (1970a) On the mechanism of desensitization at cholinergic receptors. *Mol. Pharmacol.* 6: 357–382.
- Rang, H. P., and J. M. Ritter (1970b) The relationship between desensitization and the metaphilic effect at cholinergic receptors. *Mol. Pharmacol.* 6: 383–390.
- Ravdin, P. M., and D. K. Berg (1979) Inhibition of neuronal acetylcholine sensitivity by α -toxins from *Bungarus multicinctus* venom. *Proc. Natl. Acad. Sci. USA* 76: 2072–2076.
- Ravdin, P. M., R. M. Nitkin, and D. K. Berg (1981) Internalization

- of α -bungarotoxin on neurons induced by a neurotoxin that blocks neuronal acetylcholine sensitivity. *J. Neurosci.* 1: 849–861.
- Role, L. W., S. E. Leeman, and R. L. Perlman (1981) Somatostatin and substance P inhibit catecholamine secretion from isolated cells of guinea pig adrenal medulla. *Neuroscience* 6: 1813–1821.
- Romano, C., and A. Goldstein (1980) Stereospecific nicotinic receptors in rat brain membranes. *Science* 210: 647–650.
- Sah, D. W. Y., R. H. Loring, and R. E. Zigmond (1987) Long-term blockade by toxin F of nicotinic synaptic potentials in cultured sympathetic neurons. *Neuroscience* 20: 867–874.
- Schmidt, J. W., S. Rossie, and W. A. Catterall (1985) A large intracellular pool of inactive sodium channel α subunits in developing rat brain. *Proc. Natl. Acad. Sci. USA* 82: 4847–4851.
- Schwartz, R. D., R. McGee, and K. J. Kellar (1982) Nicotinic cholinergic receptors labeled by [3 H]-acetylcholine in rat brain. *Mol. Pharmacol.* 22: 55–62.
- Sine, S., and P. Taylor (1979) Functional consequences of agonist-mediated state transitions in the cholinergic receptor. *J. Biol. Chem.* 254: 3315–3325.
- Smith, M. A., J. F. Margiotta, and D. K. Berg (1983) Differential regulation of acetylcholine sensitivity and α -bungarotoxin-binding sites on ciliary ganglion neurons in cell culture. *J. Neurosci.* 3: 2395–2402.
- Smith, M. A., J. Stollberg, J. M. Lindstrom, and D. K. Berg (1985) Characterization of a component in chick ciliary ganglia that cross-reacts with monoclonal antibodies to muscle and electric organ acetylcholine receptors. *J. Neurosci.* 5: 2726–2731.
- Smith, M. A., J. F. Margiotta, A. Franco, Jr., J. M. Lindstrom, and D. K. Berg (1986) Cholinergic modulation of an acetylcholine receptor-like antigen on the surface of chick ciliary ganglion neurons in cell culture. *J. Neurosci.* 6: 946–953.
- Stallcup, W. B., and J. Patrick (1980) Substance P enhances cholinergic receptor desensitization in a clonal nerve cell line. *Proc. Natl. Acad. Sci. USA* 77: 634–638.
- Stollberg, J., and D. K. Berg (1987) Neuronal acetylcholine receptors: Fate of surface and internal pools in cell culture. *J. Neurosci.* 7: 1809–1815.
- Swanson, L. W., J. Lindstrom, S. Tzartos, L. C. Schmued, D. D. O'Leary, and W. M. Cowan (1983) Immunohistochemical localization of monoclonal antibodies to the nicotinic acetylcholine receptor in chick midbrain. *Proc. Natl. Acad. Sci. USA* 80: 4532–4536.
- Trifaro, J. M., and R. W. H. Lee (1980) Morphological characteristics and stimulus-secretion coupling in bovine adrenal chromaffin cell cultures. *Neuroscience* 5: 1533–1546.
- Weiland, G., and P. Taylor (1979) Ligand specificity of state transitions in the cholinergic receptor: Behavior of agonists and antagonists. *Mol. Pharmacol.* 15: 197–212.
- Whiting, P., and J. Lindstrom (1986a) Purification and characterization of an acetylcholine receptor from chick brain. *Biochemistry* 25: 2082–2093.
- Whiting, P., and J. Lindstrom (1986b) Pharmacological properties of immuno-isolated neuronal nicotinic receptors. *J. Neurosci.* 6: 3061–3069.
- Whiting, P., and J. Lindstrom (1987a) Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc. Natl. Acad. Sci. USA* 84: 595–599.
- Whiting, P., and J. Lindstrom (1987b) Affinity labelling of neuronal acetylcholine receptors localizes acetylcholine-binding sites to their β -subunits. *FEBS* 213: 55–60.
- Wilson, S. P., and N. Kirshner (1977) The acetylcholine receptor of the adrenal medulla. *J. Neurochem.* 28: 687–695.