Metabolic Stability and Antigenic Modulation of Nicotinic Acetylcholine Receptors on Bovine Adrenal Chromaffin Cells

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Abstract. Bovine adrenal chromaffin cells have nicotinic acetylcholine receptors (AChRs) that are activated by the splanchnic nerve, resulting in release of catecholamines from the cells. Examination of the AChRs can provide information about the regulation and turnover of synaptic components on neurons and endocrine cells. Previous studies have shown that mAb 35 recognizes the AChR on the cells. Here we show that mAb 35 can remove AChRs from the surface of the cells by antigenic modulation, and that the modulation can be used together with other methods to examine the stability and turnover of the receptors in the plasma membrane. Unexpectedly, the results indicate a disparity between the rate at which AChRs reappear on the cells and the rate at which the ACh response recovers after preexisting AChRs have been removed.

Exposure of bovine adrenal chromaffin cultures to mAb 35 results in a parallel decrease in the magnitude of the nicotinic response and the number of AChRs on the cells. The decrease depends on the concentration and divalence of mAb 35, and on the time and temperature of the incubation. The antibody induces receptor aggregation in the plasma membrane under conditions where receptor loss subsequently occurs. After binding to receptor, mAb 35 appears to be internalized, degraded, and released from the cells through a temperature sensitive pathway that requires lysosomal function. These features are characteristic of antigenic modulation.

Appearance of new AChRs on the cells either after antigenic modulation or after blockade of existing AChRs with monovalent antibody fragments occurs at a rate equivalent to 3% of the receptors present on control cells per hour. The rate of receptor loss from the cells was measured in the presence of either tunicamycin or puromycin to block appearance of new receptors. Both conditions indicated a receptor half-life of ~24 h and a rate of loss of ~3%/h. The finding that the rate of receptor loss equaled the rate of receptor appearance was consistent with the observation that the total number of AChRs on untreated cells did not increase with time. In the presence of tunicamycin, loss of receptor-mediated response to nicotine also occurred with a half-time of 24 h. Paradoxically, the rate of recovery of the nicotinic response, determined using two procedures, was more than twice as great as the rate at which new AChRs appeared on the cells. The disparity was not generated by the existence of "spare" receptors or by differences in the time course or concentration dependence of the response to agonist. Instead the results suggest differences in the activity of new and old AChR populations in the plasma membrane.

MUCH of what is presently known about the regulation and turnover of synaptic components comes from studies of the vertebrate neuromuscular junction, where the best characterized component by far is the nicotinic acetylcholine receptor (AChR)[1]. With the recent advent of probes for neuronal AChRs, it has become possible to carry out comparative studies to determine the extent to which regulatory features of the muscle AChR can be extended to neurotransmitter receptors at other synapses. Initial results suggest certain differences between neuronal and muscle AChRs with respect to receptor structure (Boulter et al., 1986, 1987; Whiting and Lindstrom, 1986, 1987) and regulation (Baccaglini and Cooper, 1982; Brenner and Martin, 1976; Dunn and Marshall, 1985; Jacob and Berg, 1987, 1988; Tuttle, 1983). The differences may extend to the manner in which cell–cell interactions influence the function of AChRs in the plasma membrane (Margiotta et al., 1987*a*, *b*).

A promising system for gaining more information about neuronal AChRs is provided by adrenal chromaffin cells in culture. AChRs on adrenal chromaffin cells are likely to be

1. *Abbreviations used in this paper*: AChR, nicotinic acetylcholine receptor; BrACh, bromoacetylcholine; DTNB, 5,5-dithio-bis(2-nitrobenzoate); fAb, fragment of mAb retaining the antigen binding site; ^{3}H-NE, ^{3}H-norepinephrine.

of the neuronal type since the cells derive from the neural crest as do sympathetic neurons, and, like sympathetic neurons, the cells receive synaptic input from spinal cord neurons, generate action potentials, and synthesize and secrete catecholamines and neuropeptides (see Livett, 1984 for review). The pharmacology (Higgins and Berg, 1987, 1988*a*) and single channel properties (Fenwick et al., 1982) of bovine adrenal chromaffin AChRs suggest similarities to neuronal AChRs. We have previously demonstrated that several probes can be used to examine AChRs on bovine adrenal chromaffin cells (Higgins and Berg, 1987, 1988*a*). One of these is mAb 35, a mAb raised against AChR from *Electrophorus* electric organ that recognizes the main immunogenic region of muscle and electric organ AChRs (Tzartos et al., 1981) and cross reacts with chick ciliary ganglion AChRs (Halvorsen and Berg, 1987). A second is neuronal bungarotoxin, a protein toxin from the venom of *Bungarus multicinctus* that reversibly blocks neuronal AChRs (Ravdin and Berg, 1979) and is distinct from α-bungarotoxin. Neuronal bungarotoxin has previously been referred to as bungarotoxin 3.1, toxin F, and κ-bungarotoxin (Loring and Zigmond, 1988). A third probe for AChRs on bovine adrenal chromaffin cells is the agonist nicotine which binds with high affinity to a desensitized form of the receptor (Higgins and Berg, 1988*a*).

Here we show that chronic exposure of the cells to mAb 35 causes loss of the surface AChRs through antigenic modulation. We then take advantage of the modulation and other manipulations to demonstrate that the normal metabolic stability of AChRs in the plasma membrane is similar to that previously described for AChRs on skeletal muscle (Devreotes and Fambrough, 1975; Devreotes et al., 1977) and chick ciliary ganglion neurons (Stollberg and Berg, 1987) in culture. Unexpectedly, we find a substantial discrepancy between the rate at which receptors reappear in the plasma membrane after antigenic modulation and the rate at which recovery of the nicotinic response is obtained. The results suggest a difference in the functional properties of new and old AChRs in the plasma membrane. This hypothesis is explored further in the accompanying paper where a cAMP-dependent process is shown to enhance the functional response of old AChRs but not new AChRs in the plasma membrane (Higgins and Berg, 1988*b*).

Materials and Methods

Cell Cultures

Bovine adrenal chromaffin cell cultures were prepared as previously described (Higgins and Berg, 1987). Briefly, bovine adrenal glands were obtained from a local slaughter house, and medullary cells were isolated mechanically and enzymatically, using 0.2% (wt/vol) collagenase and 0.015% (wt/vol) DNase. Chromaffin cells were purified on a Percoll gradient and plated at a density of 5×10^5 cells/16-mm well in DME containing 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin. Cultures were maintained at 35°C in 5% CO_2/95% air and received fresh medium every 2 d, with 10^{-5} M cytosine arabinoside being included after the first 48 h.

mAb 35 Binding

^{125}I-mAb 35 binding was determined as previously described (Higgins and Berg, 1987; Higgins and Berg, 1988*a*). Binding to intact cells was measured by incubating cultures with 5 nM ^{125}I-mAb 35 for 60 min at 37°C, washing 4 times in a 2-min period, scraping in 0.6 N NaOH, and analyzing for radioactivity. Binding to AChRs in detergent extracts was determined using DEAE cellulose columns. In both assays nonsaturable binding, determined by including 5×10^{-7} M unlabeled mAb 35 in the binding reaction, constituted 10–20% of the total binding. Fractional occupancy was 70% at the concentration of ^{125}I-mAb 35 used.

fAb 35 Preparation

mAb 35 fragment (fAb 35) containing the antigen binding site was prepared as described (Wan and Lindstrom, 1985). mAb 35 (5 mg) in 0.50 ml of 10 mM $NaPO_4$, pH 7.4, with 100 mM NaCl and 10 nM sodium azide (PBS) was dialyzed using 3,500 MW cutoff spectrapor dialysis tubing (Spectrum Medical, Los Angeles, CA) for 18 h at 4°C against PBS, pH 8.0, containing 1 mM EDTA and 25 mM β-mercaptoethanol. Sufficient papain was then added to achieve an ~1:1 molar ratio with the mAb 35, and, after 4 h at 4°C, the digestion was terminated by adding 15 μl of 1 M iodoacetamide in PBS. After an additional 15 min, the reaction mixture was dialyzed against 10 mM $NaPO_4$, pH 7.5, overnight at 4°C. fAb 35 was purified by ion exchange chromatography using a 5-ml DEAE column eluted with a 0–0.5 M gradient of NaCl in 10 mM $NaPO_4$, pH 7.5. fAb 35 is not retained by DEAE at low ionic strength, while mAb 35 is eluted with intermediate concentrations of salt. Column fractions were assayed for protein and ability to compete for ^{125}I-mAb 35 binding, and were analyzed for the presence of undigested heavy chain using SDS-PAGE according to the method of Laemmli (1970).

Antigenic Modulation

Antigenic modulation of AChRs was achieved by incubating adrenal chromaffin cell cultures with 7 nM mAb 35 for 24 h, unless otherwise indicated.

Sucrose Gradient Centrifugation

Cultures were scraped at 4°C in a solution containing 137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.3 mM $NaPO_4$, 1.8 mM glucose, 2 mg/ml BSA, and 5 mM Hepes, pH 7.4, and centrifuged for 2 min at 4°C in an Eppendorf microfuge at 15,600 *g*. The pellet was detergent extracted with 180 μl of 10 mM $NaPO_4$, pH 7.4, containing 50 mM NaCl and 0.5% Triton X-100 (homogenate buffer). Aliquots (0.15 ml) of extract were layered onto 4.25-ml linear gradients of 5–20% sucrose in homogenate buffer. Centrifugation was carried out at 60,000 rpm for 60 min at 4°C rotor (model Vti 65; Beckman Instruments, Inc., Palo Alto, CA). Fractions of 0.2 ml were collected and either analyzed for radioactivity in the case of samples from cultures that had been prelabeled with ^{125}I-mAb 35 or ^{125}I-fAb 35, or assayed for mAb 35 binding using the DEAE column assay. The sedimentation markers catalase (11.3 S) and bacterial alkaline phosphatase (6.1 S) were included in the same tube as the experimental sample.

Catecholamine Release

Catecholamine release was determined as previously described (Higgins and Berg, 1987). Cells were preloaded with ^{3}H-norepinephrine (^{3}H-NE) for 2–3 h at 37°C and rinsed for 1 h. Release of the ^{3}H-NE was induced either by 10^{-6} M nicotine or by 54 mM K^+ in a 3-min test period. Radioactivity in aliquots of the media and of cell lysates obtained by scraping the cultures in 0.6 N NaOH was analyzed by liquid scintillation counting. ^{3}H-NE release is expressed as a percent of the radioactivity present in the cells before induced release. Basal release has been subtracted. The concentration of nicotine used was near the EC_{50} for ^{3}H-NE release (1.4×10^{-6} M; Higgins and Berg, 1988*a*). Using this protocol, nicotine-induced ^{3}H-NE release appears to be a linear function of the number of receptors occupied by agonist; antagonists compete for ^{3}H-nicotine binding and block nicotine-induced ^{3}H-NE release with the same concentration dependence (Higgins and Berg, 1988*a*). Release of preloaded catecholamines has been shown to be an accurate index of release of endogenous catecholamines under the conditions used here (Mizobe et al., 1979; Kilpatrick et al., 1980; Trifaro and Lee, 1980; Trifaro and Bourne, 1981; Role and Perlman, 1983).

Affinity Alkylation

Bovine adrenal chromaffin AChRs were affinity alkylated as described by Leprince (1983). Briefly, cultures were exposed to 1 mM dithiothreitol (DTT) for 15 min to reduce disulfide groups on the cells, washed, exposed to 1 mM bromoacetylcholine (BrACh) for 15 min to affinity label reactive sulfhydryl moieties near the AChR agonist binding site, washed, oxidized

by exposure to 1 mM 5,5-dithiobis (2-nitrobenzoate) (DTNB) for 15 min and washed a final time. This protocol resulted in an 88 ± 2% (n = 3) loss of nicotine-induced ^{3}H-NE release, while no more than 2 ± 9% (n = 3) loss was observed with control protocols in which the BrACh was either omitted or applied only after the reoxidation by DTNB. BrACh is an agonist for the bovine adrenal chromaffin AChR (data not shown). The blockade of AChR function after covalent attachment of the BrACh to the receptor may be caused by agonist-induced desensitization of the receptor or by the attached BrACh preventing reoxidation of a critical disulfide group on the receptor.

Intracellular Recording

The nicotine-induced membrane conductance (g_{nic}) of bovine adrenal chromaffin cells in culture was measured using single electrode intracellular recording techniques as described in the accompanying paper (Higgins and Berg, 1988*b*).

Materials

Bovine adrenal glands were obtained from Talone Packing Co. (Escondido, CA). mAb 35 was purified and radioiodinated to specific activities of 2–3 × 10^{18} cpm/mol as previously described (Smith et al., 1985). fAb 35 was radioiodinated to a specific activity of 10^{18} cpm/mol by the same method. Neuronal bungarotoxin and α-bungarotoxin were purified from *Bungarus multicinctus* venom (Miami Serpentarium, Salt Lake City, UT) as previously described (Ravdin et al., 1981). Tunicamycin, puromycin, BrACh, DTNB, and DTT were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-25 columns were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Culture components and other reagents were obtained as previously described (Higgins and Berg, 1987, 1988*a*).

Results

mAb 35-induced Loss of AChRs

Previous studies have shown that chronic exposure of bovine adrenal chromaffin cells to mAb 35 causes a loss of the nicotinic response and a loss of AChR-related ^{3}H nicotine binding sites from the surface of the cells (Higgins and Berg, 1987, 1988*a*). Acute exposure to antibody fails to reveal competition between mAb 35 and nicotine for binding. These results suggest that mAb 35 may slowly remove AChRs from the cell surface by antigenic modulation as previously described for muscle AChRs (Heinemann et al., 1977; Merlie et al., 1979*a*, *b*) and other surface proteins (Bourguinon and Singer, 1977; Taylor et al., 1971; Stall and Knopf, 1978).

To explore the possibility of antigenic modulation, the effects of mAb 35 on AChR number and function were examined further. Incubation of the cells in 7 nM mAb 35 at 37°C induced a time-dependent loss of AChR function, as indicated by a decrease in nicotine-induced ^{3}H-NE release from the cells (Fig. 1 *A*). In six experiments the loss occurred with a mean half-time of 5.5 ± 1.1 h. The effect was specific since there was no decrement in the secretory response induced by 54 mM K^+, and no decrement in basal release of ^{3}H-NE or in ^{3}H-NE uptake after a 24-h exposure to mAb 35 (data not shown).

The concentration dependence of the mAb 35 effect was examined by incubating cultures with antibody for 1 h to allow binding to reach equilibrium. The cultures were then washed to remove unbound antibody and, after 18 h at 37°C, were tested for a nicotinic response by measuring nicotine-induced ^{3}H-NE release. In parallel experiments, the number of AChRs on the cells was measured at the end of the incubation by quantitating the number of ^{125}I-mAb binding sites. This strategy was feasible because previously bound unlabeled mAb 35 should have dissociated during the 18-h incubation period: ^{125}I-mAb 35 dissociates from AChRs on the cells with a half-time of ~2.4 h (see below). The results indicate that loss of nicotinic response from the cells as reflected by nicotine-induced ^{3}H-NE release and loss of AChR number as reflected by ^{125}I-mAb 35 binding display similar dependences on the concentration of mAb 35 in the original binding incubation (Fig. 1 *B*). The parallel decline is consistent with the loss of function resulting from removal of AChRs from the cell surface. No loss of functional response was observed immediately after the initial 1-h incubation with mAb 35, demonstrating that events subsequent to antibody binding are required for the effect.

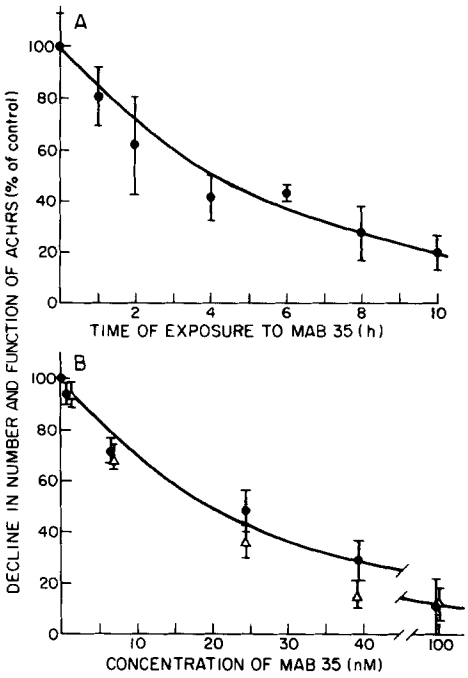

Figure 1. Time and concentration dependence of antigenic modulation. Cultures were incubated at 37°C either in 7 nM mAb 35 for the indicated times and then rinsed and assayed (*A*) or in the indicated concentrations of mAb 35 for 1 h at 37°C and then rinsed and incubated an additional 18 h without antibody before assay (*B*). In each case nicotine-induced ^{3}H-NE release was measured and compared with that in untreated control cultures to calculate the percent loss of this response (*solid circles*) caused by the treatment. In *B*, ^{125}I-mAb 35 binding was also measured since little if any unlabeled mAb 35 from the original incubation would have remained bound after the 18-h period. Again, the levels of ^{125}I-mAb 35 binding were compared to that obtained in untreated control cultures to calculate the percent loss (*open triangles*) induced by the treatment. Data represent the mean ± SEM of six experiments in *A* and four in *B*, each done with triplicate sets of cultures. Lines represent the best linear regression fit of data for nicotine-induced ^{3}H-NE release.

The loss of nicotinic response caused by mAb 35 depends both on temperature and the divalence of the antibody. Incubation with mAb 35 for 6 h at 37°C reduced the nicotinic response by more than half but had much less effect at 22°C and very little effect at 4°C (Fig. 2 *A*). Conditions that caused the complete loss of nicotinic response when mAb 35 was used as the antibody had no effect when the monovalent fAb 35 was used instead unless the fAb 35 was followed by goat anti-rat IgG (Fig. 2 *B*). A concentration of fAb 35 was used

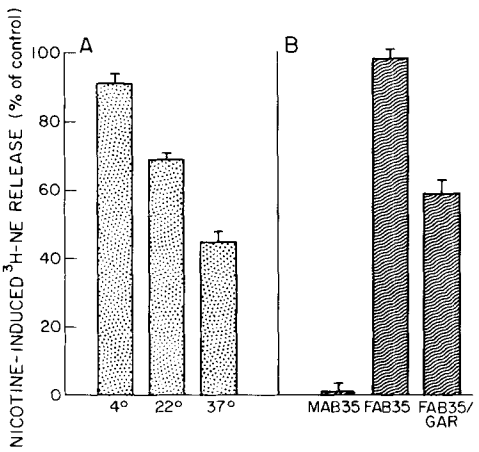

Figure 2. Dependence of antigenic modulation on temperature and divalence of antibody. (*A*) Cultures were incubated with 7 nM mAb 35 for 6 h at the indicated temperature, rinsed and transferred to 37°C for 15 min, and then assayed for nicotine-induced ^{3}H-NE release. (*B*) Cultures were incubated for 24 h at 37°C with 7 nM mAb 35, 20 nM fAb 35, or 20 nM fAb followed by a 1:500 dilution of goat anti-rat antiserum (*GAR*), and then assayed for nicotine-induced ^{3}H-NE release. The concentration of fAb used achieved a fractional site occupancy equivalent to that obtained with 7 nM mAb 35 as determined by competition binding with ^{125}I-mAb 35. Results are presented as the percent of release obtained in untreated control cultures. Data represent the mean ± SEM of five experiments, each done with triplicate sets of cultures.

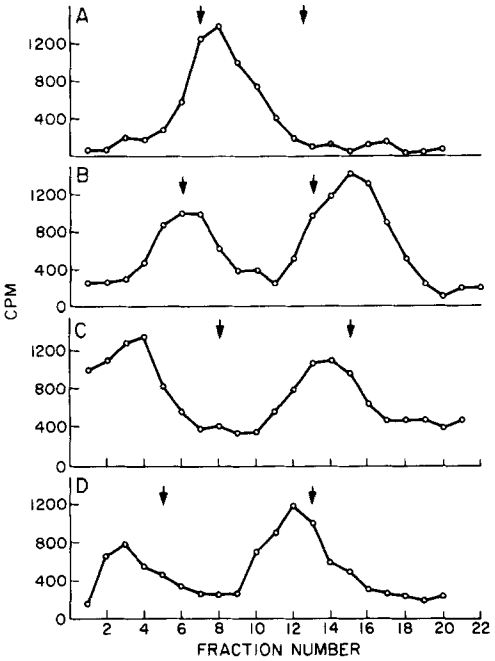

Figure 3. Sucrose gradient sedimentation of AChRs detergent solubilized before and after exposure to antibody. Cells were either solubilized in detergent (*A*) or were first incubated with 20 nM ^{125}I-fAb 35 for 2 h at 37°C (*B*), or with 5 nM ^{125}I-mAb 35 for 2 h at either 37 (*C*) or 4°C (*D*) and then solubilized in detergent. The detergent extracts were fractionated by sucrose gradient sedimentation, and fractions were collected and assayed for mAb 35 binding sites in the standard binding assay (*A*) or analyzed directly for radioactivity (*B*, *C*, and *D*). The major fast sedimenting species had S values of 10.3, 11.1, 14.6, and 12.6 in A, B, C, and D, respectively. The large amount of slowly sedimenting radioactivity, 4.6 S species in *B* and 6.8 S species in *C* and *D*, corresponds to free antibody in each case. Note that sedimentation was not allowed to proceed as far in *C* as in the other cases (separate rotor runs) to avoid centrifuging the large species to the bottom of the tube. *Left arrows*, catalase (11.3 S); *right arrows*, bacterial alkaline phosphatase (6.1 S). Similar results were obtained in two to five additional experiments for the various conditions.

for these experiments that gave the same fractional occupancy as mAb 35 under the test conditions as determined by competition binding experiments. These results are consistent with AChRs being removed through antigenic modulation and suggest that aggregation of receptors in the plasma membrane may be necessary as a first step.

The ability of mAb 35 to induce aggregation of AChRs in the plasma membrane was examined by determining the size of the receptor-antibody complex after detergent solubilization. This approach was taken both because it offered the promise of a direct demonstration of antibody-induced receptor aggregation and because the density of AChRs on the cell surface was sufficiently low as to discourage immunofluorescent studies of receptor distribution in the plasma membrane (Higgins and Berg, 1987). Analysis of solubilized AChRs by sucrose gradient centrifugation in the absence of antibody indicated a major species having a sedimentation coefficient of 10.6 ± 0.1 S (n = 4; Fig. 3 *A*), similar to the ~10 S value reported for AChRs from chick ciliary ganglion neurons (Smith et al., 1985). When ^{125}I-fAb 35 was used to label AChRs in the plasma membrane of intact cells, the ^{125}I-fAb-AChR complex obtained by detergent solubilization had a sedimentation coefficient of 11.2 ± 0.1 S (n = 3; Fig. 3 *B*). The slight increase presumably reflects the binding of fAb 35 molecules to the receptor. Some free ^{125}I-fAb 35 sedimenting at 3.9 S was also observed and is likely to represent antibody that had dissociated from receptor during the solubilization and centrifugation. In this and subsequent experiments an excess of unlabeled fAb was included during the cell homogenization to prevent binding of labeled antibody or aggregation of receptors by antibody after solubilization. Labeling surface AChRs with ^{125}I-mAb 35 instead of ^{125}I-fAb 35 resulted in antibody-receptor complexes with a different sedimentation profile when solubilized. A mixture of large components was usually observed; the major species had a sedimentation coefficient ranging from 12.6 to >17.0 S among experiments (Fig. 3 *C*), with a mean value of 14.8 ± 0.5 S (n = 8). In addition some free ^{125}I-mAb 35 was present, sedimenting at 6.8 S, again presumably due to dissociation of the antibody-receptor complex. The large antibody-receptor species must have formed in the plasma membrane because the excess of unlabeled fAb 35 present during the cell homogenization would have prevented subsequent ^{125}I-mAb 35 binding and cross-linking of AChRs. Since mAb 35 is divalent and the bovine chromaffin AChR may have more than 1 mAb 35 binding site, the large species could represent either cross-linked receptors or single receptors with multiple antibodies bound. To distinguish between these possibilities, cells were labeled with ^{125}I-mAb 35 at 4°C where membrane fluidity may be sufficiently reduced as to prevent aggregation of receptors from occurring. These conditions have been shown to permit binding of ^{125}I-mAb

35 while preventing the antibody from reducing nicotine-induced ^{3}H-NE release (Higgins and Berg, 1987), presumably because antigenic modulation is blocked. After binding an amount of labeled mAb 35 at 4°C equivalent to that achieved at 37°C, the antibody–receptor complex was solubilized and shown by sucrose gradient centrifugation to have a sedimentation coefficient of 12.7 ± 0.4 S (n = 8) (Fig. 3 *D*). This value is lower than that obtained for antibody–receptor complex formed at 37°C and indicates that the latter is likely to represent AChR aggregates induced by divalent antibodies. Apparently mAb 35 aggregates AChRs in the membrane at 37°C, while fAb 35 at 37°C and mAb 35 at 4°C are not able to do so. The conditions required for receptor cross-linking are, therefore, the same as those required for antibody-induced loss of surface AChRs from the cells.

The Fate of Bound mAb 35

If mAb 35 drives the internalization of surface AChRs through antigenic modulation, bound antibody may be internalized with the receptor and subsequently degraded in lysosomes. This possibility was examined by following the fate of ^{125}I-mAb 35 bound to surface AChRs on the cells. Sampling the culture medium at various times after binding ^{125}I-mAb 35 to the cells indicated that radioactivity was released with biphasic kinetics. The rapidly dissociating component displayed a half-time of 2.4 ± 0.1 h (n = 3). Gel filtration of culture media using a Sephadex G-25 column indicated that the rapidly released radioactivity was associated with material appearing in the void volume, as expected for intact antibody that dissociated from receptor (data not shown). The slowly dissociating component was a candidate for internalized antibody that had been degraded and released as radiolabeled fragments. The relative proportion of the slow component depended on how long the cultures had been incubated originally in ^{125}I-mAb 35. Longer times produced a greater proportion of slow component, consistent with a time-dependent internalization of bound antibody. To study the slow component in isolation, cultures were incubated in 7 nM ^{125}I-mAb 35 for 6 h, rinsed, incubated another 18 h without antibody, and rinsed again to eliminate the rapidly dissociating component, and then examined for subsequent release of radioactivity (Fig. 4). At 37°C the loss was described by a single exponential function with a half-time of 6.8 h. This value is similar to that obtained for degradation of AChRs in the muscle cell line BC3H-1 in the presence of anti-AChR antibodies (Lindstrom and Einarson, 1979). At 4°C the component had a half-time of 36 h, demonstrating a strong temperature dependence. Chloroquine, an inhibitor of proteolysis in lysosomes, also greatly decreased the rate at which radioactivity was lost from the cells (Fig. 4), implying that lysosomal proteolysis may be essential for the process.

Gel filtration with Sephadex G-25 columns indicated that much of the slowly released radioactivity was associated with small molecular weight material. Cells were labeled with 5 nM ^{125}I-mAb 35 for 6 h, rinsed, incubated another 18 h without antibody, rinsed again, and then incubated for 8 h more at 37°C to collect released radioactivity in the culture medium. Most of the released radioactivity (74 ± 12%, n = 4) migrated in the included volume of the column. Control experiments indicated that the remainder represented dissociation of nonspecifically bound antibody. Radioactivity remaining with the cells at the end of the incubations was excluded by the column after solubilization. These results are consistent with antigenic modulation of surface AChRs by mAb 35 in which the antibody binds to surface AChRs, induces receptor aggregation and internalization, and then is degraded in the lysosomes to release small radiolabeled products into the medium.

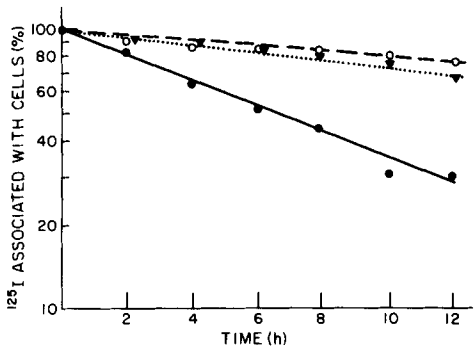

Figure 4. Loss of radioactivity from cells after binding ^{125}I-mAb 35. Cultures were incubated in 7 nM ^{125}I-mAb 35 for 6 h, rinsed, incubated for an additional 18 h without antibody, rinsed again, and then assayed for radioactivity retained by the cells as a function of time at 37°C (*solid circles*), 4°C (*open circles*), or 37°C in the presence of 100 μM chloroquine (*solid inverted triangles*). Data represent the mean of three experiments done with triplicate sets of cultures and are shown as a percent of the radioactivity present at the outset. SEMs were ≤9% of the values shown.

Rate of AChR Appearance

The rate of AChR appearance in the plasma membrane was determined in two ways. In the first case mAb 35 was used to remove surface AChRs by antigenic modulation. Reappearance of mAb 35 binding sites was then taken to represent accumulation of new AChRs on the cell surface. The initial rate of reappearance after antigenic modulation was equivalent to 2.8% of the number of receptors on cells in control cultures per hour (Fig. 5). No initial lag in the recovery was observed, consistent with the finding that antigenic modulation does not deplete the internal pool of AChRs, which presumably includes AChRs in transit to the surface (Higgins and Berg, 1988*a*). When puromycin was added to the cultures 4 h before initiation of the recovery period, no AChRs appeared (data not shown), demonstrating a requirement for protein synthesis in the recovery process.

The second approach involved blocking surface receptors with fAb 35, which does not induce antigenic modulation, and then measuring the appearance of new AChRs with ^{125}I-mAb 35. To correct for a contribution from preexisting surface AChRs from which the fAb might dissociate during the experiment, sister cultures were maintained in puromycin to block protein synthesis or in tunicamycin to block glycosylation of receptors. The drug treatments were begun 3.5 h before adding fAb 35 so that insertion of new receptors would be completely blocked during the period of interest (Stollberg and Berg, 1987). The appearance of ^{125}I-mAb 35 binding sites under these conditions was taken to represent sites exposed by fAb dissociation. Subtracting such sites from the total sites measured in the absence of drug treatment yielded a value for the initial rate of AChR reappearance as

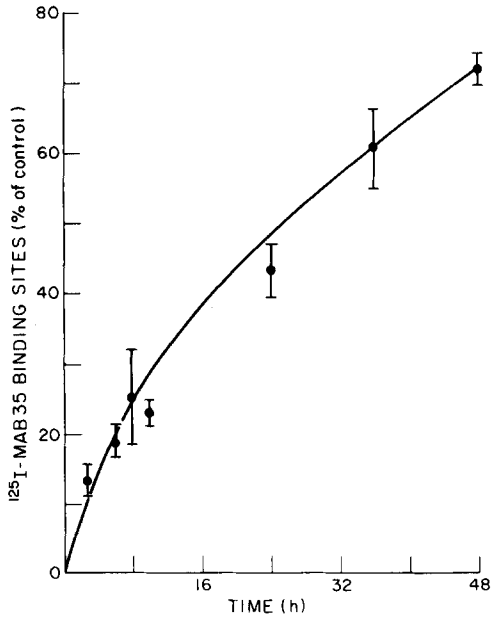

Figure 5. Appearance of AChRs on the cell surface after antigenic modulation. Surface AChRs were removed from cells by incubating cultures for 24 h with 7 nM mAb 35. After rinsing, the cultures were assayed for reappearance of mAb 35 binding sites using ^{125}I-mAb 35. The data were corrected for competition by residual unlabeled mAb 35 left over from the original incubation. Values are expressed as a percent of the number of sites present in untreated control cultures (100% = 6.0 ± 0.3 fmol/culture) and represent the mean ± SEM of five experiments done with triplicate sets of cultures.

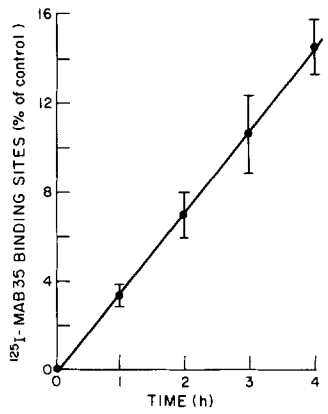

Figure 6. Appearance of new AChRs on the cell surface after blockade of existing AChR sites by fAb 35. Cultures were incubated with a near saturating concentration of fAb 35 (100 nM) for 90 min to block existing receptors, and then were rinsed and assayed for the appearance of new ^{125}I-mAb 35 binding sites at the indicated times. Corrections were made for fAb dissociation during the time course of the experiment which would have exposed sites on preexisting receptors. This was evaluated by carrying out parallel determinations on sister cultures in which the appearance of new sites was blocked by treating the cells either with 20 μg/ml puromycin or with 1 μg/ml tunicamycin beginning 3.5 h before addition of fAb 35. Appearance of ^{125}I-mAb 35 binding sites in such cultures ranged from 20 to 80% of the total sites obtained over the 4-h time period in the absence of puromycin and tunicamycin, and were subtracted to yield the values shown. The high rate of fAb dissociation prevented longer times from being examined. The results are expressed as a percent of the sites present on untreated cells and represent the mean ± SEM of five experiments done with triplicate sets of cultures.

being 3.6% of the number of receptors present on cells in control cultures per hour (Fig. 6).

Rate of AChR Degradation

The rate of AChR loss from the surface of the cells in the absence of antigenic modulation was determined by blocking the appearance of new AChRs with either tunicamycin or puromycin, and then measuring the number of remaining AChRs as a function of time. The concentrations of tunicamycin and puromycin used blocked >98% of the incorporation of ^{3}H-mannose or ^{3}H-leucine, respectively, into TCA precipitable material. AChRs, as measured by ^{125}I-mAb 35 binding sites, were lost from the surface of the cells by a first order process with a half-time of 23.7 ± 0.3 h (n = 3) in puromycin (Fig. 7 *A*), and 23.4 ± 0.8 h (n = 3) in tunicamycin (Fig. 7 *B*). In puromycin a lag of 3.2 ± 0.6 h was observed preceding the loss of surface AChRs; in tunicamycin the lag was 2.8 ± 0.3 h. The 3-h lag is likely to represent the transit time for newly synthesized AChRs to reach and be inserted into the plasma membrane. In the case of tunicamycin treatment, the decline in nitotinic response was also determined, using nicotine-induced ^{3}H-NE release as an assay. After a lag of 3.0 ± 0.3 h (n = 3), the response declined with a half-time of 25.4 ± 2 h (Fig. 7 *B*), in good agreement with the loss of surface receptors measured by ^{125}I-mAb 35 binding. The cells appeared to be viable after the tunicamycin treatment since the amount of ^{3}H-NE uptake, basal ^{3}H-NE release, and K^{+}-induced ^{3}H-NE release were similar to the levels measured in untreated sister cultures. Comparable experiments in puromycin were not meaningful because chronic blockade of protein synthesis impaired the release process independent of effects on AChRs. The half-times of AChR loss measured either as a decline in ^{125}I-mAb 35 binding sites or as a decline in nicotinic response from the cells, corresponds to an initial rate of loss of 2.8% of the receptors per hour.

Since the number of AChRs on bovine adrenal chromaffin cells in culture does not change over the time period examined (3–10 d in culture; Higgins and Berg, 1987), the rate of receptor appearance must equal the rate of receptor loss from the surface. In agreement with this prediction, the experimentally observed rates both of AChR appearance and of AChR loss are ~3% per hour. This corroborates, in part, the separate procedures used to measure AChR appearance and degradation.

Reappearance of AChR Function

Recovery of nicotinic response would be expected to coincide with recovery of AChR number on the surface of adrenal chromaffin cells after antigenic modulation. To examine this, recovery of nicotine-induced ^{3}H-NE release was measured at various times after antigenic modulation had been used to remove the surface AChRs initially present. As shown in Fig. 8, recovery of nicotine-induced ^{3}H-NE release under these conditions can be described as a single exponential process with a half-time of 8.9 h and an initial recovery rate of 9%/h. This is much more rapid than the reappearance of AChRs on the surface of the cells which recovers to control levels with a half-time of ~24 h and an initial rate of ~3%/h.

To corroborate the rapid recovery of the functional response, a second method was devised. In this case AChRs on the surface of the cells were irreversibly blocked by affinity alkylation as previously described for AChRs on PC12

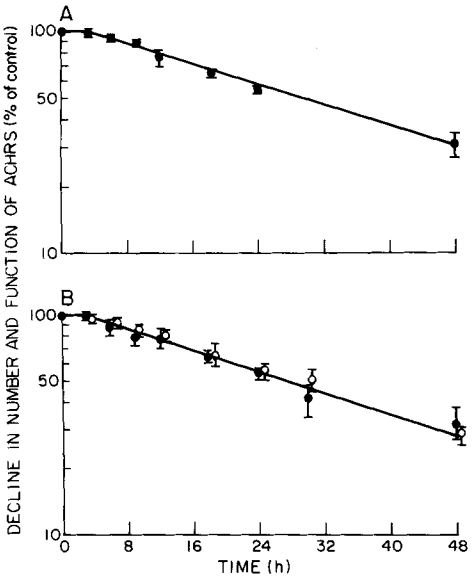

Figure 7. Loss of AChRs during blockade of either protein synthesis or glycosylation. Cultures were incubated in either 20 μg/ml puromycin (*A*) or 1 μg/ml tunicamycin (*B*) and assayed at the indicated times for ^{125}I-mAb 35 binding (*solid circles*) or for nicotine-induced ^{3}H-NE release (*open circles*). Values are expressed as a percent of the receptors present at the outset and represent the mean ± SEM of three experiments, each done with triplicate sets of cultures. After an initial lag, the number of AChRs on the cells declined with first order kinetics and a half-time of 23.7 h in *A* and 23.4 h in *B*. The lag period, determined by extrapolating the best fit of the exponential decay phase back to 100%, was found to be 3.2 h in *A* and 2.8 h in *B*. The lag period presumably reflects the time required for appearance of new receptors to be blocked; no increase is expected during the lag since the number of AChRs on the cells is normally at steady-state (Higgins and Berg, 1987). The loss of nicotine-induced ^{3}H-NE release in the presence of tunicamycin occurred with a half-time of 25.4 h after a lag of 3.0 h. ^{3}H-NE release in response to 54 mM K^+ was not affected by tunicamycin treatment over the time course examined (data not shown).

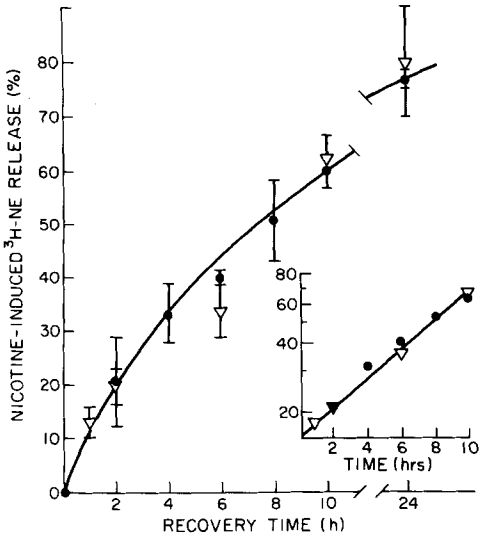

Figure 8. Recovery of nicotinic response after either antigenic modulation or blockade of receptors by affinity alkylation. Cultures were either incubated with 7 nM mAb 35 for 24 h to remove AChRs by antigenic modulation (*solid circles*) or were affinity alkylated with BrACh to block the activation of existing AChRs (*open inverted triangles*), and then were rinsed and assayed for nicotine-induced ^{3}H-NE release at the indicated times. Values are expressed as a percent of those obtained in untreated control cultures and represent the mean ± SEM of three to four experiments, each done with triplicate sets of cultures. ^{3}H-NE release induced by 54 mM K^+ was unaffected by the treatments (data not shown). (*Inset*) Semi-log plot of recovery rate indicates a first order process. Only values obtained during the first 2 h were used to calculate the initial rate of recovery; they were assumed to follow a zero order process.

cells (Leprince, 1983). The procedure involved using DTT to reduce disulfide moieties on the surface of the cells, then reacting the cells with BrACh which preferentially couples covalently to a sulfhydryl moiety near the active site of AChRs, and finally reoxidizing the cell surface with DTNB to reconstruct unreacted disulfides. Preexisting AChRs are blocked irreversibly under these conditions: the nicotinic response does not recover when protein synthesis is inhibited (data not shown). Accordingly, the reappearance of a nicotinic response must reflect new receptors being inserted into the plasma membrane. Applying the procedure to bovine adrenal chromaffin cells in culture (Fig. 8) produces a half-time of 8.1 ± 0.9 h (n = 4) for recovery of the nicotinic response with an initial recovery rate of 8.5%/h, in good agreement with recovery obtained after antigenic modulation.

The surprising finding that the nicotinic response returns to control levels more quickly than does the number of surface AChRs raises questions about the measurement of receptor function. Both the dependence on agonist concentration and the time course of nicotine-induced ^{3}H-NE release, however, are indistinguishable for AChRs on unmodulated cells and those on cells after half maximal recovery; i.e., 8 h after antigenic modulation (Fig. 9). In addition, the assays reveal no change in rates of agonist-induced receptor desensitization as reflected by declining ^{3}H-NE release in response to high concentrations of agonist (Fig. 9 *B*), though a change in a rapid component of desensitization would have gone undetected. Lastly, there is no evidence for "spare" receptors in the release assay: the loss of surface AChRs with time in tunicamycin closely matches the decline in nicotine-induced ^{3}H-NE release from the cells (Fig. 7 *B*). Similarly, no evidence for spare AChRs was obtained in experiments where the ability of neuronal bungarotoxin or classical antagonists to block the nicotinic response was compared with their ability to compete with ^{3}H-nicotine for binding to the cells (Higgins and Berg, 1988*a*). These results suggest that AChRs newly inserted into the plasma membrane are more active in some way than are older AChRs.

Intracellular recording corroborates the results of the ^{3}H-NE release assay, showing that new AChRs appear to be more functional than old AChRs. Cells with new AChRs were obtained by removing AChRs with antigenic modulation and then allowing the cells to recover for 6–8 h, during which time new AChRs appeared in the plasma membrane. Though such cells had only ~20% of the AChRs present on untreated control cells (Fig. 5), they had ~70% of the nicotinic response. The g_{nic} value was 26 ± 3 nS (n = 24) for treated cells compared with 37 ± 6 nS (n = 32) for control cells. In contrast, cells maintained in tunicamycin for 27 h had ~50% of the AChRs initially present (Fig. 7 *A*) and

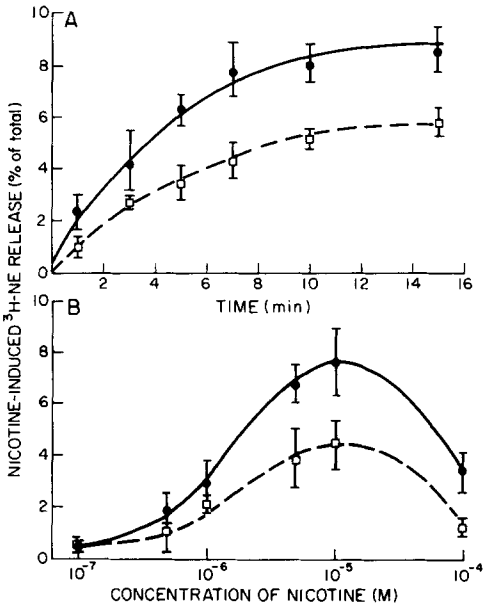

Figure 9. Agonist concentration and time dependence of nicotine-induced ^{3}H-NE release after recovery from antigenic modulation. Nicotine-induced ^{3}H-NE release was examined with untreated cells (*solid circles*) and with cells that had been antigenically modulated by incubation with 7 nM mAb 35 for 24 h and then allowed to recover for 8 h (*open squares*). The time of exposure to the standard concentration of 10^{-6} M nicotine was varied as indicated in *A* while the concentration of nicotine in the standard 3-min release period was varied as indicated in *B*. The only difference observed between the two kinds of cultures in the assays is that unmodulated cells had a larger response to agonist. Values are expressed as a percent of the ^{3}H-NE present in cells at the beginning of the test period and represent the mean ± SEM of three experiments, each done with triplicate sets of cultures.

~54% of the nicotinic response compared to untreated control cells. The g_{nic} value was 20 ± 3 nS (n = 31) for treated cells in this case. The results indicate a two-to-threefold difference in the relative nicotinic responses of new and old AChR populations defined in this way.

Discussion

The major findings reported here are that (*a*) AChRs can be removed from the surface of bovine adrenal chromaffin cells by antigenic modulation, (*b*) in the absence of modulation the AChRs have a half-life of ~24 h in the plasma membrane, and (*c*) newly inserted AChRs appear to be more active than older receptors in the plasma membrane.

Antigenic modulation has been widely documented for membrane proteins and can occur when divalent antibodies induce clustering of surface molecules in the plasma membrane. The clustered components are then internalized and degraded by the cell (Bourguinon and Singer, 1977). The evidence for antigenic modulation of AChRs on bovine adrenal chromaffin cells by mAb 35 is substantial. Previous studies indicated that chronic exposure to mAb 35 induces the loss of high affinity ^{3}H-nicotine binding sites from the surface of the cells, though mAb 35 does not compete with nicotine for binding (Higgins and Berg, 1988*a*). The present studies demonstrate that the agonist response as measured by nicotine-induced release of ^{3}H-NE and the number of AChRs on the surface as measured by ^{125}I-mAb 35 binding are removed in parallel after exposure to mAb 35. The removal is dependent on events that occur subsequent to antibody binding, and the process is dependent on time, temperature, and the concentration and divalence of mAb 35. mAb 35 induces aggregation of AChRs in the plasma membrane, and some of the bound antibody is apparently internalized, degraded to small molecular weight material, and released into the culture medium. This latter process is also temperature dependent and can be blocked by chloroquine, an inhibitor of lysosomal proteolysis. All of these characteristics are consistent with antigenic modulation of the AChR by mAb 35.

Anti-AChR antibodies dramatically increase the rate of degradation of muscle AChRs (Heinemann et al., 1977; Drachman et al., 1978; Merlie et al., 1979*a*, *b*) and, as a result, cause a reduction in miniature endplate potential amplitude and in sensitivity to ACh (Albuquerque et al., 1976). Antigenic modulation of muscle AChRs by anti-receptor antibodies has been advanced as a contributing factor in myasthenia gravis (Lindstrom and Einarson, 1979). Since mAb 35 recognizes the main immunogenic region of the AChR, as do most of the antibodies in the serum of patients with myasthenia gravis (Tzartos et al., 1982), it is possible that antigenic modulation of adrenal chromaffin AChRs may have relevance for symptoms of the disease as well. The ability of mAb 35 to bind but not antigenically modulate AChRs on chick ciliary ganglion neurons in the absence of secondary antibodies (Halvorsen and Berg, 1987) implies that ciliary ganglion AChRs differ in some way from AChRs on bovine adrenal chromaffin cells. Ciliary ganglion neurons are not refractory to internalization of membrane components per se in culture (Ravdin et al., 1981).

Antigenic modulation was used in the present study to clear the cell surface of AChRs so that appearance of new AChRs could be measured. This, together with several methods for examining receptor appearance and degradation, indicated a value of ~24 h for the half-life of AChRs in the plasma membrane of bovine adrenal chromaffin cells. Similar values have been obtained for AChRs of vertebrate skeletal muscle (Devreotes and Fambrough, 1975; Devreotes et al., 1977) and chick ciliary ganglion neurons in culture (Stollberg and Berg, 1987). Since the number of AChRs per cell does not change over the culture ages used in the present experiments (Higgins and Berg, 1987), the rate of appearance of new receptors in the plasma membrane must equal the rate of receptor degradation. Consistent with this prediction, the hourly rate of receptor appearance as determined by two independent methods was equivalent to ~3% of the number of receptors present on the cells at equilibrium, and the rate of degradation, measured separately, was also ~3%/h.

The 3-h lag that precedes loss of surface AChRs when the appearance of new receptors is blocked by tunicamycin or puromycin is likely to represent the period during which previously synthesized intracellular AChRs are transported to and inserted into the plasma membrane. Bovine adrenal chromaffin cells from mature animals have an intracellular pool of AChRs that comprise ~20% of the total AChRs (Higgins and Berg, 1988*a*). The measured lag time and rate of insertion predict that about half of the intracellular receptors are destined to reach the surface. Even larger pools of inter-

nal AChRs (Olsen et al., 1983; Pestronk, 1985; Stollberg and Berg, 1987) and Na channel α subunits (Schmidt et al., 1985) have been reported in other systems.

The most surprising finding was that the nicotinic response of the cells recovered more rapidly than the surface complement of AChRs. Four possible explanations are considered here: (*a*) the assay for nicotinic response may not have been proportional to the number of AChRs, (*b*) mAb 35 binding sites may have included a substantial non-AChR population with a slow rate of appearance, (*c*) recovery of the response may have included a contribution from receptors previously on the cell surface, or (*d*) new and old AChRs in the plasma membrane may differ with respect to function. Several lines of evidence argue against the first explanation. Antigenic modulation of surface AChRs by mAb 35 caused a parallel decline in the number of AChRs and in the nicotinic response of the cells. Tunicamycin treatment caused a similar parallel decrease. These observations indicate that few spare receptors exist on the cells, at least with respect to the ^{3}H-NE release assay. No changes were observed in either the time course of release or in the dependence on agonist concentration when the release assay was performed on cells recovering from antigenic modulation. Lastly, results obtained with intracellular recording techniques corroborated those obtained with the ^{3}H-NE release assay. The second explanation is equally unlikely. ^{125}I-mAb 35 binding studies reveal a single class of high affinity binding sites on the cells, and the binding component has the properties expected for the AChR (Higgins and Berg, 1987). Moreover, the number of ^{125}I-mAb 35 binding sites is in good agreement with the number of high affinity ^{3}H-nicotine binding sites representing AChRs on the cells (Higgins and Berg, 1988*a*). In addition, the rates of appearance and disappearance of mAb 35 sites followed zero and first order kinetics, respectively, as expected for a single population of sites. The third possibility is also improbable. Bound antibody is degraded, strongly suggesting internalization of receptors. In addition, AChR-associated ^{3}H-nicotine binding sites are completely removed from the cell surface after mAb 35 treatment (Higgins and Berg, 1988*a*). Receptor recycling after removal of bound antibody is unlikely since recovery of the nicotinic response does not occur in the presence of protein synthesis inhibitors, a treatment which does not block insertion of AChRs into the plasma membrane that have already been synthesized. These considerations point to the fourth explanation and suggest that newly inserted AChRs are under a unique regulatory control or are different from older AChRs with respect to function. The accompanying paper supports this interpretation and demonstrates that not only can a cAMP-dependent process enhance the nicotinic response of the cells, but that it can discriminate between new and old AChRs in the plasma membrane when doing so (Higgins and Berg, 1988*b*).

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