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Agonist-induced Alteration in the Membrane Form of Muscarinic Cholinergic Receptors*

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Incubation of 1321N1 human astrocytoma cells with carbachol resulted in a rapid loss of binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) to muscarinic cholinergic receptors measured at 4 °C on intact cells; loss of muscarinic receptors in lysates from the same cells measured with [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) at 37 °C occurred at a slower rate. Upon removal of agonist from the medium, the lost [^{3}H]NMS binding sites measured on intact cells recovered with a $t_{½}$ of approximately 20 min, but only to the level to which [^{3}H]QNB binding sites had been lost; no recovery of "lost" [^{3}H]QNB binding sites occurred over the same period. Based on these data and the arguments of Galper *et al.* (Galper, J. B., Dziekan, L. C., O'Hara, D. S., and Smith, T. W. (1982) *J. Biol. Chem.* 257, 10344–10356) regarding the relative hydrophilicity of [^{3}H]NMS *versus* [^{3}H]QNB, it is proposed that carbachol induces a rapid sequestration of muscarinic receptors that is followed by a loss of these receptors from the cell. These carbachol-induced changes are accompanied by a change in the membrane form of the muscarinic receptor. Although essentially all of the muscarinic receptors from control cells co-purified with the plasma membrane fraction on sucrose density gradients, 20–35% of the muscarinic receptors from cells treated for 30 min with 100 μM carbachol migrated to a much lower sucrose density. This conversion of muscarinic receptors to a "light vesicle" form occurred with a $t_{½} \approx 10$ min, and reversed with a $t_{½} \approx 20$ min. In contrast to previous results in this cell line regarding β-adrenergic receptors (Harden, T. K., Cotton, C. U., Waldo, G. L., Lutton, J. K., and Perkins, J. P. (1980) *Science* 210, 441–443), agonist binding to muscarinic receptors in the light vesicle fraction obtained from carbachol-treated cells was still regulated by GTP. One interpretation of these data is that agonists induce an internalization of muscarinic receptors with the retention of their functional interaction with a guanine nucleotide regulatory protein.

Many peptide hormone receptors undergo a process of agonist-induced receptor clustering and internalization (1). In the case of transferrin (2), asialoglycoprotein (3), and low-density lipoprotein (4) receptors, receptor-mediated endocytosis is thought to be involved in the transport of ligand to intracellular sites. The receptor is conserved by recycling back to the cell surface. Although delivery of ligands to intracellular sites and receptor recycling clearly are components of the ligand-induced internalization process in the insulin and epidermal growth factor receptor systems, receptor degradation also is observed (5, 6).

A number of investigators have suggested that catecholamine-induced desensitization of adenylate cyclase also is accompanied by ligand-induced internalization of the β-adrenergic receptor (7–9). Although current technology has not permitted a morphological analysis of this process, the occurrence of β-adrenergic receptors in a membrane form that is not associated with the plasma membrane (7–9) and that is relatively inaccessible to hydrophilic ligands (10, 11) is consistent with this idea. As with insulin and epidermal growth factor receptors, internalization of β-adrenergic receptors may be a necessary first step for the eventual degradation of receptors that occurs during long-term exposure of cells to catecholamines (12).

The 1321N1 human astrocytoma cell line has proven very useful for studies of catecholamine-induced desensitization of β-adrenergic receptors (12, 13). In addition, these cells express a muscarinic cholinergic receptor that when activated results in the breakdown of phosphoinositides (14), the mobilization of intracellular Ca^{2+} (14), and the activation of a Ca^{2+}/calmodulin-regulated phosphodiesterase (15, 16). We previously have reported (17) that short-term exposure of 1321N1 cells to carbachol results in a reduced capacity of cholinergic agonists for stimulation of phosphodiesterase; extended exposure to agonist results in a loss of muscarinic receptors. The potential role of receptor internalization in these processes has not been examined. Indeed, little is known about the importance of this process in the muscarinic receptor system and for other hormone receptors that produce their physiological effects through a breakdown of phosphoinositides. In light of the fact that catecholamine-induced alteration in the membrane form of β-adrenergic receptors has been studied extensively in 1321N1 cells (8, 11, 18), we have applied similar methodology to examine the fate of muscarinic receptors during exposure of these cells to cholinergic agonists.

MATERIALS AND METHODS

Cell Culture—Human astrocytoma cells (1321N1) were grown on 150-mm plastic culture dishes or six-well plates in Dulbecco's modification of Eagle's medium containing 5% fetal calf serum, penicillin (25 unit/ml), and streptomycin (25 μg/ml). Cells were subcultured at a density of 10,000–15,000 cells/cm^2 and maintained in a 37 °C humidified incubator in an atmosphere of 92% air and 8% CO_2. Growth medium was replenished every 3 days, and experiments were conducted on postconfluent cultures 6–8 days after subculture.

Sucrose Density Gradient Experiments—Growth medium was replaced with 10 ml of Eagle's minimal essential medium buffered with

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20 mM Hepes[1] (Hepes-Eagle's) at the start of each experiment. For most experiments, cells were incubated with vehicle or a muscarinic receptor agonist for 30 min at 37 °C.

After the completion of experimental treatments, the medium was replaced with 4 °C Hepes-Eagle's containing 0.25 mg/ml of concanavalin A (19, 20), and the dishes were placed on ice for 20 min. Cells were hypotonically lysed by rinsing the dishes once with 10 ml of cold lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.4) followed by a 20-min incubation on ice in lysis buffer and subsequent scraping of the swollen cells with a rubber policeman.

For most experiments, pooled lysates (3 ml) from three to four 150-mm dishes were layered on top of a 9-ml continuous 30–60% sucrose density gradient (all gradients were buffered with 10 mM Tris, pH 7.4). The gradients were centrifuged in a Beckman SW 40 rotor at 154,000 × *g* for 1 h. Twenty 0.6-ml fractions were collected from the top of each gradient at a flow rate of 1.5 ml/min.

Some experiments (carbachol competition curves) required larger amounts of tissue and, thus, larger gradients. Lysates from 25–35 150-mm dishes were pooled and layered on three 30-ml 30–60% continuous sucrose gradients and centrifuged in a Beckman SW 27 rotor at 114,000 × *g* for 1 h. Twenty 2-ml fractions were collected from the top of each gradient at a flow rate of 3.0 ml/min. Light vesicle fractions and plasma membrane fractions were identified by [^{3}H]QNB binding assays (see below), pooled, diluted with 10 mM Hepes, pH 7.5, 10 mM EDTA, and centrifuged for 30 min at 170,000 × *g*. The supernatant was discarded, and the pellet was resuspended in 10 mM Hepes, 10 mM EDTA using a hand-operated Teflon and glass homogenizer and centrifuged a second time at 170,000 × *g* for 30 min. The final pellet was resuspended in 10 mM Hepes, pH 7.5, 5 mM $MgCl_2$.

Broken Cell Receptor Assays—Muscarinic receptors were quantitated with [^{3}H]QNB as previously described (21). Briefly, samples were incubated at 37 °C for 60–90 min in 10 mM Hepes, pH 7.5, and 5 mM $MgCl_2$. The concentration of [^{3}H]QNB was 300 pM for assay of gradient fractions and 30 pM for carbachol competition binding experiments. The assay volume was 1 ml for assay of gradient fractions and 10 ml for saturation binding isotherms and competition binding assays. Assays were terminated by the addition of 10 ml of wash buffer (145 mM NaCl, 10 mM Tris, pH 7.5) to the reaction tubes and filtration over glass fiber filters. The filters were washed with an additional 10 ml of wash buffer and counted in a liquid scintillation counter at an efficiency of 40%. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 1 μM atropine.

Cyanopindolol was iodinated and the binding of ^{125}I-cyanopindolol to β-adrenergic receptors was measured as described elsewhere (22). Isoproterenol (100 μM) was used to define nonspecific binding.

Intact Cell Muscarinic Receptor Assay—Cells grown on six-well plates were rinsed twice with 1 ml of 37 °C Hepes-Eagle's to remove any previously used drugs. The plates then were placed on ice, and 1 ml of Hepes-Eagle's containing [^{3}H]NMS (usually 400 pM) was added to each well. The plates were incubated for 2.5 h on ice and subsequently washed twice with ice-cold Hepes-Eagle's. The cells were solubilized in 1 ml of 1 N NaOH. Aliquots were neutralized with 1 N HCl and radioactivity measured in a liquid scintillation counter. Nonspecific binding, defined as radioactivity retained in the presence of 1 μM atropine, was less than 10% of the total counts.

Galper *et al.* (23) have previously reported on the relative hydrophilicity of [^{3}H]NMS *versus* [^{3}H]QNB and have proposed that [^{3}H]NMS can be used to measure "cell-surface" muscarinic receptors whereas the lipophilic radioligand [^{3}H]QNB will detect muscarinic receptors in a "sequestered" as well as cell surface form. Although the data presented below are consistent with this conclusion, the assays utilized in the current study are somewhat different from those employed by Galper *et al.* (23). First, since the agonist-induced changes in muscarinic receptors in 1321N1 cells were rapidly reversible at 37 °C it was necessary to carry out intact cell binding assays at 4 °C (a temperature at which the observed agonist-induced changes were not reversible). Second, since binding of [^{3}H]QNB to muscarinic receptors reaches equilibrium very slowly even at elevated temperature, intact cell binding assays at 4 °C with [^{3}H]QNB were not feasible. In addition, even at elevated temperatures, there was a high level of nonspecific binding of [^{3}H]QNB in intact cell assays. Thus, the comparisons in the manuscript are made between intact cell assays using [^{3}H]NMS at 4 °C and [^{3}H]QNB binding assays with lysates from the same cells at 37 °C.

We have previously reported the details of the [^{3}H]QNB binding reaction in these cells (15, 17, 21). [^{3}H]NMS bound to muscarinic receptors on intact 1321N1 cells at 4 °C with a K_d of 181 ± 41 pM (n = 3 experiments). This affinity is 3–4-fold less than the K_d of [^{3}H]NMS obtained in the same cells at 37 °C (21). Although some variability was observed, the number of [^{3}H]NMS binding sites observed in intact 1321N1 cells at 4 °C was similar (70–100%) to the number observed with [^{3}H]QNB in 37 °C assays using lysates from the same cells. As we have previously reported (15), the density of muscarinic receptors (50–200 fmol/mg of protein) varied with the age and density of 1321N1 cell cultures. Equilibrium binding of [^{3}H]NMS was obtained within 2 h at a concentration of 200 pM.

Materials—The following compounds were purchased from Sigma: arecoline hydrobromide, atropine sulfate, carbamyl-β-methylcholine chloride (bethanechol), carbamylcholine chloride (carbachol), methacholine chloride, and oxotremorine sesquifumarate. Concanavalin A was purchased from Calbiochem-Behring and ultra-pure sucrose from Schwarz/Mann. [^{3}H]QNB (specific activity = 33.4 Ci/mmol) and [^{3}H]NMS (specific activity = 84.8 Ci/mmol) were from New England Nuclear. Na^{125}I was from Amersham. Cyanopindolol was a generous gift from Drs. Gunther Engel and Dan Hauser of Sandoz Pharmaceuticals (Basel, Switzerland).

RESULTS

Two types of radioligand binding assays were used for assessment of muscarinic receptors in this study. [^{3}H]QNB binding assays were carried out with homogenates at 37 °C and [^{3}H]NMS binding assays were carried out using intact cells at 4 °C. As discussed under "Materials and Methods" and by Galper *et al.* (23), there are several reasons to believe that the [^{3}H]QNB binding assay measures "total" receptors while the [^{3}H]NMS binding assay measures cell-surface or "nonsequestered" receptors. Although there was some variability among cultures, the number of muscarinic receptors detected on control cultures with each type of radioligand binding assay was similar (Fig. 1). Such was not the case subsequent to preincubation of 1321N1 cells with carbachol. Whereas the number of [^{3}H]NMS binding sites decreased by 30–40% during a 30-min exposure of cells to 100 μM carbachol, little decrease in the number of [^{3}H]QNB sites occurred (Fig. 1). In neither case was there a change in the apparent affinity of radioligand, suggesting that the wash conditions fully remove carbachol from the cultures.

The time course of loss of [^{3}H]NMS binding sites is presented in Fig. 2. Receptor density decreased rapidly ($t_{½} \approx 10$ min) to a new level from which further decreases occurred at a much slower rate. The $K_{0.5}$ value for carbachol for induction of the loss in binding of [^{3}H]NMS to intact cells after a 30-min incubation with agonist was approximately 10 μM (data not shown). The carbachol-induced decrease in the amount of binding of [^{3}H]NMS was rapidly reversible. That is, if 1321N1 cells were first incubated with 100 μM carbachol for 45 min, then washed free of agonist and the incubation continued in agonist-free medium at 37 °, [^{3}H]NMS binding sites reappeared with a $t_{½}$ of 15–20 min (Fig. 2).

We previously have reported (Ref. 17 and Fig. 1) that only a small decrease in the number of binding sites measured with [^{3}H]QNB in homogenates occurs during the first hour of incubation of 1321N1 cells with carbachol. Furthermore, in contrast to the rapid recovery of sites measured with [^{3}H]NMS in an intact cell binding assay (Fig. 2), recovery of lost [^{3}H]QNB binding sites measured in a homogenate required hours ($t_{½} \approx 12$ h) of incubation of carbachol-pretreated cells in agonist-free medium (17). This dichotomy was examined further in the experiment depicted in Fig. 3. Cells were incubated for the indicated times with 100 μM carbachol. The cells then were washed free of agonist and incubation continued in

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{3}H]QNB, L-[*benzilic*-4,4-^{3}H]quinuclidinyl benzilate; [^{3}H]NMS, [*N-methyl*-^{3}H]scopolamine chloride.

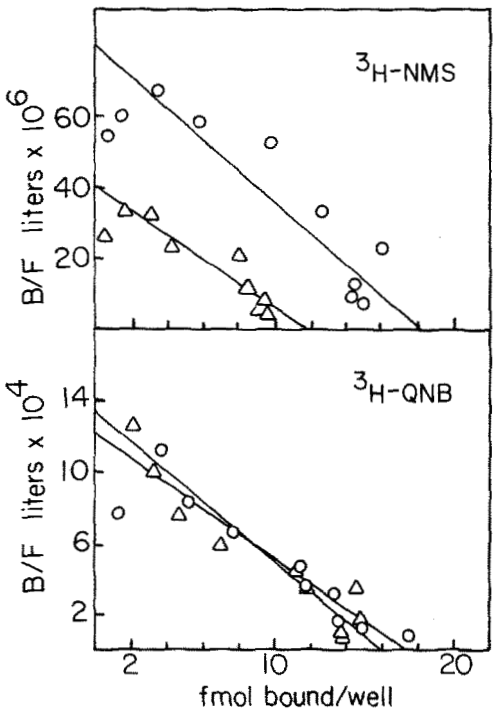

FIG. 1. **[^{3}H]NMS and [^{3}H]QNB binding to muscarinic receptors from carbachol-pretreated 1321N1 cells.** Cells were incubated in the absence (○) or presence (△) of 100 μM carbachol for 30 min. The cells then were washed free of agonist, and two types of muscarinic receptor binding assays carried out. In one half of the cultures, intact cell binding assays were carried out at 4 °C using [^{3}H]NMS as described under "Materials and Methods" (*top panel*). The other half of the cultures were lysed and [^{3}H]QNB binding assays carried out at 37 °C as described under "Materials and Methods" (*bottom panel*). Scatchard plots are presented with each data point representing the average of triplicate determinations. The K_d and B_{max} values for [^{3}H]NMS binding with control cultures were 230 pM and 18 fmol/well, respectively, and for carbachol-pretreated cultures were 290 pM and 11 fmol/well, respectively. The K_d and B_{max} values for [^{3}H]QNB binding with control cultures were 14 pM and 17 fmol/well, respectively, and for carbachol-pretreated cultures were 12 pM and 16 fmol/well, respectively. The results are representative of those from three experiments.

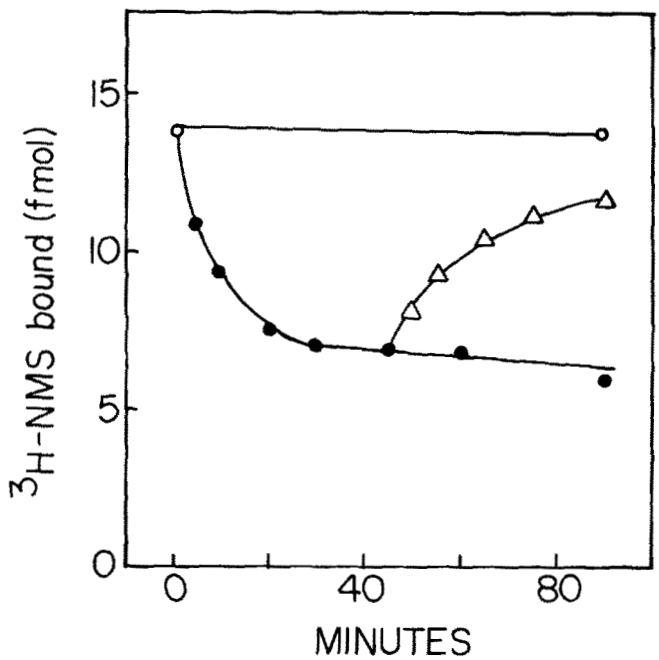

FIG. 2. **Carbachol-induced changes in [^{3}H]NMS binding to intact 1321N1 astrocytoma cells.** Cells were incubated at 37 °C without agonist (○), with 100 μM carbachol (●) for the indicated times, or with 100 μM carbachol for 45 min prior to washing the cultures free of agonist and continuing the incubation in agonist-free medium (△). At the indicated times, the cells were washed and intact cell [^{3}H]NMS binding assays were carried out at 4 °C as described under "Materials and Methods." Each point represents the mean value for triplicate plates of cells. The results are representative of those observed in four experiments.

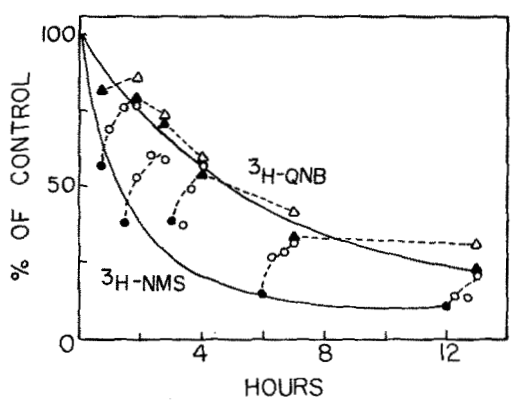

FIG. 3. **Reversal of carbachol-induced changes in [^{3}H]NMS and [^{3}H]QNB binding.** Cells were incubated with 100 μM carbachol for the indicated times. The cells then were washed free of agonist, and either [^{3}H]NMS binding (●) assays (intact cells at 4 °C; see "Materials and Methods") or [^{3}H]QNB binding (▲) assays (lysates at 37 °C; see "Materials and Methods") were carried out or incubation was continued in agonist-free medium prior to carrying out [^{3}H]NMS (○) or [^{3}H]QNB (△) binding assays. The data are presented as the per cent of the specific binding of each radioligand measured in cells not incubated with carbachol. The data are the average of two experiments carried out with triplicate dishes.

agonist-free medium. At the indicated times, receptor number was assessed in homogenates with [^{3}H]QNB and on intact cells using [^{3}H]NMS at 4 °C as described under "Materials and Methods." As was previously discussed, the loss of [^{3}H]NMS binding sites occurred at a faster rate than did loss of [^{3}H]QNB binding sites. In addition, the changes in receptor number measured with these two assays subsequent to removal of agonist from the incubation medium were very different. Whereas the number of [^{3}H]NMS binding sites recovered to approximately the level of [^{3}H]QNB binding sites, there was no increase in the number of [^{3}H]QNB sites (if anything, there was a tendency for a further small decrease in the number of [^{3}H]QNB sites). Thus, during incubation of 1321N1 cells with carbachol, muscarinic receptors measured in an intact cell assay with [^{3}H]NMS decrease and recover much faster than those measured with [^{3}H]QNB, and recovery of [^{3}H]NMS binding sites occurs approximately to the level of [^{3}H]QNB binding sites that remain after the precincubation with agonist.

We previously demonstrated that incubation of 1321N1 cells with catecholamine results in a change in the membrane form of β-adrenergic receptors (8, 11, 18). Using similar methodology, the possibility was examined that changes in [^{3}H]NMS binding to intact cells are accompanied by changes in the membrane form of muscarinic receptors. Cells were incubated with 100 μM carbachol for 30 min prior to lysis and centrifugation of lysates on sucrose density gradients. As we have reported previously (18), essentially all of the muscarinic receptors identified with [^{3}H]QNB co-purified with markers for the plasma membrane (Fig. 4). Subsequent to incubation of 1321N1 cells with carbachol for 30 min, there was a decrement in the number of receptors appearing in the plasma

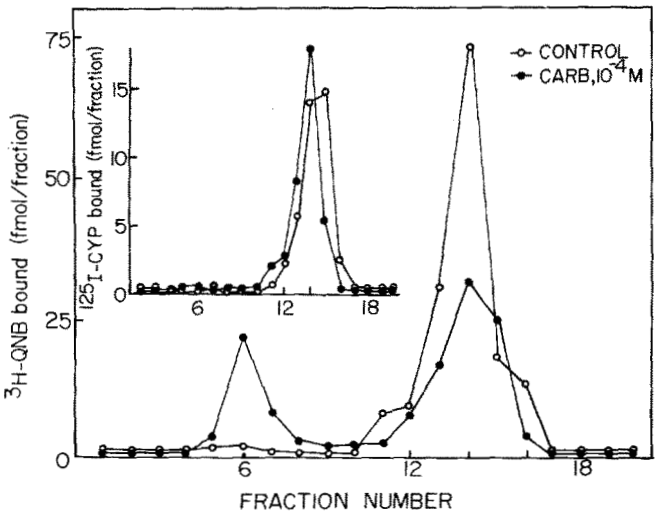

FIG. 4. **Carbachol-induced change in the membrane form of muscarinic cholinergic receptors.** 1321N1 cells were incubated with 100 μM carbachol (*CARB*) for 30 min. The cells then were washed free of agonist and hypotonically lysed, and sucrose density gradient centrifugation was performed as described under "Materials and Methods." [^{3}H]QNB binding assays were carried out on each fraction as described under "Materials and Methods." The data are presented as the amount of [^{3}H]QNB bound specifically per fraction. The data are representative of 15 similar experiments. *Inset*, β-adrenergic receptors were measured in each gradient fraction using ^{125}I-cyanopindolol (*^{125}I-CYP*). The results are representative of those obtained in two experiments.

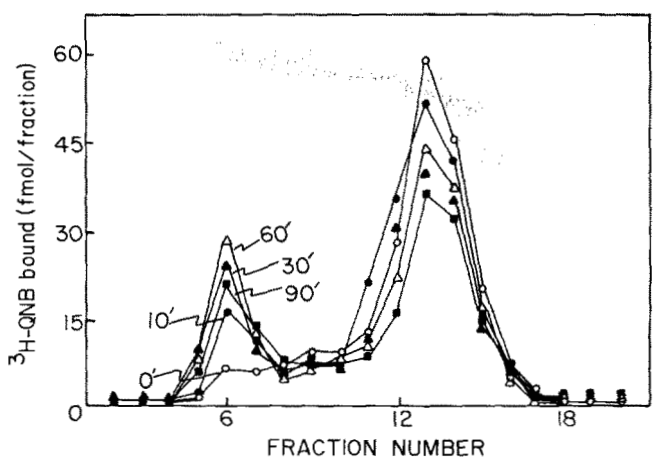

FIG. 5. **Time course of carbachol-induced change in the membrane form of muscarinic receptors.** Cells were incubated for the indicated times with 100 μM carbachol prior to hypotonic lysis and sucrose density gradient centrifugation as described under "Materials and Methods." The data are presented as the amount of specific [^{3}H]QNB binding/fraction and are representative of results from three experiments.

membrane fraction of the gradients and a corresponding increase in the number of receptors appearing at much lighter sucrose densities. This carbachol-induced appearance of muscarinic receptors in a "light vesicle" fraction was apparently receptor-selective since no carbachol-induced change in the distribution of β-adrenergic receptors (Fig. 4, *inset*) or H_1-histamine receptors measured with [^{3}H]pyrilamine (data not shown) occurred.

The time course of the agonist-induced change in muscarinic receptor distribution on sucrose density gradients also was measured (Fig. 5). The $t_{1/2}$ (10–15 min) for the agonist-induced change in membrane form of muscarinic receptors was similar to that for the loss of [^{3}H]NMS binding sites measured in intact cell binding assays (Fig. 2). The maximal change in receptor distribution occurred within 45–60 min in the presence of 100 μM carbachol. With extended incubation, *e.g.* 90 min in Fig. 5, a decrease in muscarinic receptor number occurred in the light vesicle as well as the plasma membrane fractions.

The reversibility of the change in membrane form also was examined. Cells were incubated for 30 min with 100 μM carbachol prior to washing the cultures free of agonist and continuing the incubation in agonist-free medium. The cells were lysed after various times of incubation in agonist-free medium and sucrose density gradient centrifugation carried out as for Figs. 4 and 5. Following removal of carbachol, there was a time-dependent decrease in the number of receptors occurring in the light vesicle form and a corresponding increase in the number of receptors appearing in the plasma membrane fraction (data not shown). The $t_{1/2}$ of this "recovery" was 15–20 min. The gradient distribution of muscarinic receptors from cultures that had been incubated for 30 min with carbachol and then incubated in agonist-free medium for 60 min was indistinguishable from that of control cultures (data not shown).

The concentration dependence for the carbachol-induced change in the membrane form of muscarinic receptors is presented in Fig. 6. The $K_{0.5}$ for carbachol for induction of this process was approximately 10 μM which is very similar to the $K_{0.5}$ of carbachol for stimulation of the breakdown of phosphoinositides (5–30 μM; Refs. 14 and 24) and Ca^{2+} mobilization (5–30 μM; Refs. 14 and 24). Atropine (1 μM) blocked the effects of carbachol on the gradient distribution of muscarinic receptors; no change in the distribution of muscarinic receptors occurred when incubation of 1321N1 cells was with atropine (1 μM) alone (data not shown).

A series of partial agonists (relative to carbachol) have been identified based on their capacity to stimulate the breakdown of phosphoinositides (14, 24). Cells were incubated with a saturating concentration (based on their capacity to stimulate phosphoinositide breakdown) of these agonists for 30 min. The number of receptors appearing in the light vesicle fraction was then quantitated (Table I). There was a very good corre-

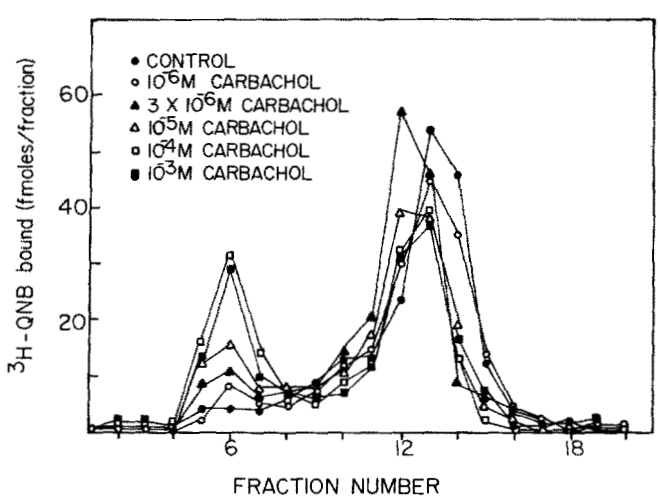

FIG. 6. **Concentration dependence of carbachol-induced change in the membrane form of muscarinic receptors.** Cells were incubated with the indicated concentrations of carbachol for 30 min. The cells then were hypotonically lysed, and sucrose density gradient centrifugation was carried out as described under "Materials and Methods." Muscarinic receptors in each gradient fraction were measured as described under "Materials and Methods." The data are presented as specific [^{3}H]QNB binding/fraction and are representative of results from three experiments.

TABLE I

Induction of light vesicle form of muscarinic receptors by different cholinergic agonists

1321N1 cells were incubated for 30 min with the indicated agonists at a maximally effective (100 μM with all agonists except bethanechol which was present at a concentration of 1000 μM) concentration regarding stimulation of phosphoinositide breakdown (Refs. 14, 24). The cells were then lysed, and sucrose density gradient centrifugation carried out as described under "Materials and Methods." The femtomoles of receptors appearing in the peak (fraction 6) fraction of the light vesicle population of receptors was quantitated with [^{3}H]QNB. The data are representative of results obtained in two experiments.

Agonist in incubation	Muscarinic receptors in light vesicle fraction
	fmol
None	12
Carbachol	52
Methacholine	44
Bethanechol	34
Arecoline	31
Oxotremorine	25

lation between the relative efficacy of agonists for stimulation of phosphoinositide breakdown and their effectiveness relative to carbachol for induction of the change in membrane form of muscarinic receptors. For example, oxotremorine, which is approximately 20% as efficacious as carbachol for stimulation of phosphoinositide breakdown (14, 24), was approximately 30% as effective as carbachol regarding conversion of muscarinic receptors to the light vesicle form (Table I).

One important observation made concerning the β-adrenergic receptor of 1321N1 cells was that β-receptors appearing in the light vesicle fraction on sucrose density gradients generated from catecholamine-preincubated cells no longer interacted with the stimulatory (regarding adenylate cyclase) guanine nucleotide regulatory protein, N_s (8). We have presented evidence that the muscarinic receptors of 1321N1 cells interact with a guanine nucleotide regulatory protein that is neither N_s or the inhibitory (regarding adenylate cyclase) regulatory protein, N_i (25). It was of interest to determine if the altered membrane form of the muscarinic receptor still interacted with this putative guanine nucleotide regulatory protein. Cells were preincubated with carbachol (100 μM) for 30 min prior to lysis and centrifugation of the lysates on sucrose density gradients. Both the light vesicle and the plasma membrane fractions were pooled from several gradients, and competition curves for carbachol were generated in the absence and the presence of GTP. The agonist competition curves with plasma membrane receptors (Fig. 7, *top panel*) were indistinguishable from those of washed membrane preparations from control cell lysates (data not shown and Ref. 25). That is, the addition of GTP markedly shifted the agonist competition curve to the right. In contrast to the results previously obtained in similar studies with the β-adrenergic receptors of these cells (8), the binding properties of muscarinic receptors in the light vesicle fraction from carbachol pretreated cells (Fig. 7, *bottom panel*) were the same as that of the plasma membrane fraction. That is, carbachol inhibited [^{3}H]QNB binding with high affinity in the absence of GTP, and the carbachol competition curve was markedly shifted to the right in assays carried out in the presence of 100 μM GTP.

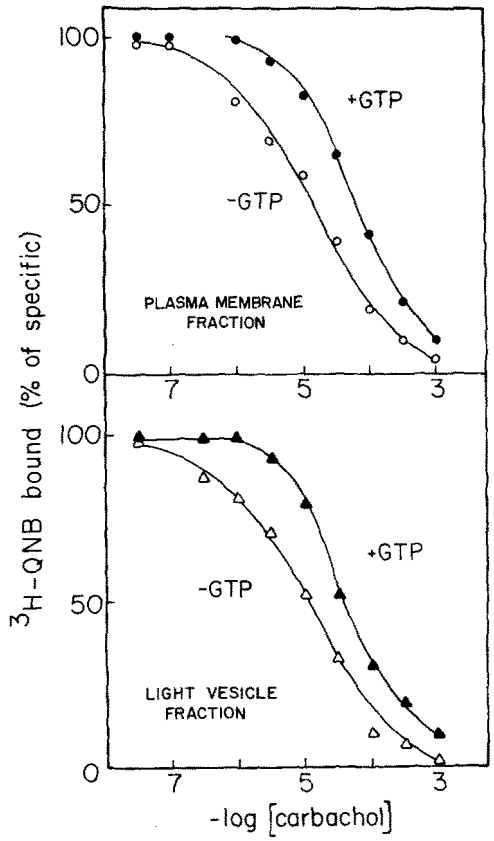

FIG. 7. **Guanine nucleotide regulation of agonist binding to muscarinic receptors of plasma membrane and light vesicle fraction.** Cells were incubated with 100 μM carbachol for 30 min. The cells then were hypotonically lysed and subjected to sucrose density gradient centrifugation as described under "Materials and Methods." The gradient fractions comprising the plasma membrane and light vesicle population of muscarinic receptors were both pooled and subjected to further centrifugation as described under "Materials and Methods." The washed gradient fractions were incubated with [^{3}H]QNB (30 pM) and the indicated concentrations of carbachol. Competition curves were generated in the absence (○, △) or in the presence (●, ▲) of 100 μM GTP. The data are the average of triplicate assays and are representative of results obtained in seven experiments.

DISCUSSION

Galper and co-workers (23) have presented evidence that, based on their relative hydrophilicities, [^{3}H]NMS can be utilized in intact cell binding assays to measure muscarinic receptors in a nonsequestered (cell-surface?) form, whereas [^{3}H]QNB can be utilized as a measure of total muscarinic receptors. Our preliminary data (see "Materials and Methods") as well as the data presented in Figs. 1–3 support this idea. However, unequivocal conclusions are compromised by the poor quality of the data obtained using [^{3}H]QNB in an intact cell binding assay, as well as by its very slow rate of association with the muscarinic receptor at temperatures that are sufficiently low to prevent reversal of agonist-induced changes in muscarinic receptors.

Assuming that our ideas regarding the [^{3}H]NMS and [^{3}H]QNB binding assays are correct, then the following sequence of events can be proposed. Incubation of 1321N1 cells with carbachol results in rapid conversion of muscarinic receptors to a form that is inaccessible to the hydrophilic radioligand [^{3}H]NMS in reduced temperature assays with intact cells. Return of these sequestered receptors to the cell surface is rapid upon removal of agonist from the culture medium. Loss of [^{3}H]QNB binding sites during extended exposure to cholinergic stimuli occurs at a slower rate than does the loss of [^{3}H]NMS binding sites measured on intact cells due to the fact that [^{3}H]QNB binding assays in lysed cells at 37 °C would

detect both cell surface receptors and sequestered receptors. It can be further proposed that only those receptors that have been modified in a long-lasting way are lost to detection by [^{3}H]QNB. The very slow recovery of muscarinic receptors measured with [^{3}H]QNB following transfer of cells to agonist-free medium (Ref. 17 and Fig. 3) supports the idea that resynthesis of receptors is necessary to recover lost [^{3}H]QNB binding sites. Although none of the data directly address this issue, it is attractive to consider the sequestered form of the receptor as representative of a step in the sequence of events that results in long-term down-regulation of muscarinic receptors. Thus, it can be hypothesized on the basis of the two types of radioligand binding assays that muscarinic receptors exist in at least a cell surface form, a sequestered form, and a "lost" form after several hours of incubation of 1321N1 cells with carbachol.

These proposals concerning regulation of muscarinic receptors of 1321N1 cells are consistent with those initially made by Galper and co-workers (23). Based on intact cell binding studies with cultured chick embryo heart cells, these workers proposed that [^{3}H]NMS and [^{3}H]QNB recognize different forms of the muscarinic receptor during agonist-induced down-regulation and that one of these forms represented receptors in a sequestered, *i.e.* internalized, state. These conclusions have been supported subsequently by studies of muscarinic receptors in several other cell types (26, 27). A major difference between the results obtained with 1321N1 cells and those previously reported for chick heart cells should be mentioned. That is, whereas lost [^{3}H]NMS binding sites rapidly recovered to the level of [^{3}H]QNB binding sites upon transfer of 1321N1 cells to agonist-free medium, the large difference in the number of [^{3}H]NMS *versus* [^{3}H]QNB binding sites in carbachol-pretreated chick heart cells (23) remained even after incubation in agonist-free medium for 60 min at 37 °C. The reasons for this difference are not clear.

The interpretation of [^{3}H]NMS and [^{3}H]QNB binding data obtained with carbachol-pretreated cells is supported by results obtained using a very different approach. That is, it was hypothesized that if carbachol induces conversion of muscarinic receptors to a sequestered form and if this form at least in part represents internalized receptors, then these receptors should no longer co-purify with plasma membrane fractions on sucrose density gradients. This, indeed, turned out to be the case when lysates from carbachol-treated cells were subjected to sucrose density gradient centrifugation. Although there was little or no change in total muscarinic receptors measured with [^{3}H]QNB, a portion of these receptors no longer co-purified with plasma membrane fractions after incubation of 1321N1 cells with carbachol. The fact that the time courses of formation of and recovery from this altered membrane form were essentially the same as for the agonist-induced changes in [^{3}H]NMS binding to intact cells strongly argues that these two very different experimental approaches at least in part measure the same phenomenon. However, it should be pointed out that the amounts of altered receptors measured by these two means were not the same. That is, whereas a 30-min incubation of 1321N1 cells with carbachol resulted in a 35–50% decrease in [^{3}H]NMS binding to intact cells, the amount of receptor converted to a light vesicle form measured on sucrose density gradients under the same conditions was 20–35%. Perhaps this difference simply is due to reversal of the altered membrane form during the long period of time necessary to process samples in the sucrose density gradient experiments. Alternatively, incubation with agonists may induce a form of the muscarinic receptor that is no longer accessible to a hydrophilic radioligand on the cell surface, but that, nonetheless, is still associated with the plasma membrane. Such a form of the β-adrenergic receptor has been proposed to be induced by incubation of this cell line (11) or other tissues (28) with catecholamines.

Agonist-occupied muscarinic receptors of 1321N1 cells interact with a guanine nucleotide regulatory protein (24, 25). This protein is apparently not N_i based on the insensitivity to pertussis toxin of GTP-sensitive high affinity binding of agonists (25) and the lack of effect of pertussis toxin on the capacity of muscarinic receptor agonists to stimulate phosphoinositide breakdown (29), Ca^{2+} mobilization (29), or phosphodiesterase activity (30). Several lines of evidence from 1321N1 cells (24) and other tissues (31, 32) suggest that this yet to be identified guanine nucleotide regulatory protein is an important component of the coupling mechanism involved in receptor-mediated stimulation of phosphoinositide breakdown. We previously have shown that the uncoupling mechanism involved in catecholamine-induced desensitization of the β-adrenergic receptor/adenylate cyclase system of 1321N1 cells involves a loss of capacity of agonist-occupied β-receptors to interact with N_s (12, 13). Also, β-adrenergic receptors that occur in a light vesicle fraction subsequent to incubation of 1321N1 (8) or other (9) cells with catecholamine no longer interact with N_s. Considering these results, it was surprising to observe that the agonist-induced light vesicle form of the muscarinic receptor retained capacity to interact with its guanine nucleotide regulatory protein. It is possible that muscarinic receptors in an agonist-induced vesicular form could fuse during the isolation process with other structures that contain the guanine nucleotide regulatory protein. However, this possibility seems highly unlikely. Thus, incubation of 1321N1 cells with carbachol may result in a co-alteration of the membrane form of both muscarinic receptors and their associated guanine nucleotide regulatory protein. Unfortunately, this putative protein only can be quantified in a very indirect way (GTP-sensitive agonist binding), and direct examination of this question must await development of methodology for identification of the protein.

Activation of the Ca^{2+}/calmodulin-sensitive phosphodiesterase that occurs in response to cholinergic stimuli rapidly desensitizes during incubation of 1321N1 cells with carbachol (17). Based on the work of Masters *et al.* (33), desensitization may not occur at the level of the muscarinic receptor. They have demonstrated that carbachol-stimulated accumulation of inositol-1-PO_4 occurs linearly during a 60-min incubation of 1321N1 cells with agonist. We do not know at this time what percentage of the inositol-1-PO_4 comes from the second messenger, inositol 1,4,5-trisphosphate, and what percentage comes from the recently discovered isomer of unknown activity regarding Ca^{2+} mobilization, inositol 1,3,4-trisphosphate (34). Irrespective of this point, the fact remains that phosphoinositide breakdown is occurring linearly under conditions where up to one half of the receptors are apparently in a sequestered form. One explanation of these results is that the altered vesicular form of the muscarinic receptor is in an environment where both phospholipase C and polyphosphoinositide substrates are available for the formation of inositol trisphosphates. Thus, assuming carbachol has sufficient accessibility to this environment, phosphoinositide breakdown would continue to occur in a cellular location of receptors that is not classically considered in neurotransmitter action. Clearly, much more work is needed addressing all aspects of this question to determine its true significance.

In summary, we have presented evidence that the muscarinic receptor that regulates phosphoinositide breakdown in 1321N1 cells undergoes a change in its membrane form upon

exposure of these cells to cholinergic agonists. The properties of this change in muscarinic receptors in 1321N1 cells are remarkably similar to the catecholamine-induced changes that occur with the adenylate cyclase-linked β-receptor of the same cell. The one notable exception to this generalization is the observation that the light vesicle form of the muscarinic receptor still interacts with a guanine nucleotide regulatory protein. This work together with that of Masters *et al.* (33) suggests that phosphoinositide breakdown in response to agonists may occur in a state of the muscarinic receptor that is not equivalent to its normal cell surface localization.

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