Agonist and Guanine Nucleotide Modulation of Muscarinic Cholinergic Receptors in Cultured Heart Cells*

(Received for publication, January 29, 1980, and in revised form, May 12, 1980)

Jonas B. Galper† and Thomas W. Smith
From the Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Massachusetts

Agonist modulation of muscarinic cholinergic receptor properties was studied in cultured chick embryo heart cells. Exposure of cultured heart cells to muscarinic agonists caused a biphasic decrease in the number of muscarinic receptors as measured by binding of the potent muscarinic antagonist [3H]quinuclidinyl benzilate ([3H]QNB) to homogenates of these cultures. A rapid loss of 28% of receptors occurred during the first minute of exposure to agonist, followed by a gradual loss of another 44% of receptors by 3 h. Changes in the extent of [3H]QNB binding after a 3-h exposure to agonist were not the result of agonist-induced changes in affinity or rate of [3H]QNB binding to the receptor since [3H]QNB bound with a k_d of 0.21 × 10^{-5} M and a half-time to equilibrium of 13.0 min to homogenates of control cells compared to a k_d of 0.19 × 10^{-5} M and half-time to equilibrium of 13.9 min in homogenates of agonist-treated cells. However, studies of the rapid phase of agonist-induced receptor loss indicated that: 1) this phase was accompanied by a shift in the affinity of the remaining receptors for agonist from an IC_{50} for carbamylcholine inhibition of [3H]QNB binding of 1.5 × 10^{-2} M to 9.2 × 10^{-2} M; 2) exposure of cell homogenates to 10^{-4} M guanosine 5'-[(β,γ-imino)triphosphate caused a similar shift in affinity unaccompanied by alteration in receptor number; 3) incubation with guanine nucleotides caused the reappearance of [3H]QNB-binding sites lost during the first minute of agonist exposure; and 4) incubation with colchicine had no effect on the rapid phase of receptor loss. Studies of the slow phase of agonist-induced receptor loss indicated that: 1) inhibitors of microtubule function including colchicine and vinblastine inhibited up to two-thirds of agonist-induced receptor loss with half-maximal effects at 1.4 × 10^{-7} M and 8.0 × 10^{-7} M, respectively; 2) recovery of receptors following washout of agonist was preceded by a 3-h lag period after which receptor number returned to 95% of control levels over 9 h; and 3) the protein synthesis inhibitor cycloheximide (2 μg/ml) prevented recovery. These data indicate a complex set of interactions of muscarinic agonists, guanine nucleotides, and cytokinetic events in the modulation of muscarinic receptor activity.

The role of agonists in modulating the number and function of cell surface receptors and the relationship of this modulation to the dynamic nature of the cell membrane has been described for several systems (1). Incubation of cultured human lymphocytes with insulin has been shown to cause up to a 70% decrease in the binding of [125I]labeled insulin to the cells, while the affinity of the remaining receptors for insulin was unchanged (2). The number of sites for binding of [125I]-labeled human epidermal growth factor in human fibroblasts has been shown to decrease after treatment of cells with epidermal growth factor. The data suggest that endocytosis of the receptor-growth factor complex, with subsequent lysosomal degradation of both the hormone and receptor within the cell, may be responsible for this process (3). The activity of β-adrenergic receptors in avian and frog erythrocytes (4-6) and astrocytoma cells (7, 8) is modulated by β-agonists as well as guanine nucleotides. In astrocytoma cells, short term incubation with β-agonists is followed by uncoupling of hormone binding from the adenylate cyclase response, while more prolonged incubation is followed by an actual decrease in receptor number (5). Studies utilizing radiolabeled β-adrenergic agonists suggest that persistent binding of agonist and agonist-induced modification of the receptor might be responsible for these effects (9, 10).

Our previous studies of the effects of carbamylcholine in modulating the responsiveness of the explanted embryonic chicken heart to this muscarinic agonist demonstrated a rapid but transient decrease in the frequency (11) and force¹ of contraction with return to base-line within 3 min. Earlier studies have been interpreted as suggesting that muscarinic cholinergic effects are mediated by increased intracellular cGMP levels and that rapid desensitization occurs because of the transient nature of the cGMP elevation (12). Other possible mechanisms can now be probed because of the availability of the potent radiolabeled muscarinic antagonist [3H]quinuclidinyl benzilate, allowing more direct studies of the interaction of agonists and antagonists with the muscarinic receptor. Studies utilizing [3H]QNB² (13) have defined the properties of [3H]QNB binding to muscarinic receptors in embryonic chicken heart (11), rabbit, rat, and guinea pig heart (14), and in monolayer cultures of embryonic chicken heart cells (15).

Using embryonic chicken heart cell cultures, we have demonstrated that prolonged exposure to muscarinic agonists (up to 3 h) was accompanied by marked decreases (65 to 70%) in both the number of muscarinic receptors and in the physiological responsiveness of these cells to muscarinic agonists (15). The present investigation deals with the effect of both short and long term exposure of muscarinic receptors of

1 J. B. Galper and T. W. Smith, unpublished observation.
2 The abbreviations used are: QNB, quinuclidinyl benzilate; Gpp(NH)p, guanosine 5'-[(β,γ-imino)triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* This study was supported by research grants from the National Heart, Lung and Blood Institute, National Institutes of Health Grants HL-22775 and HL-19003; American Heart Association Grant 79875, and an award from the William P. Miller Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Recipient of Young Investigator Award, National Heart, Lung and Blood Institute, National Institutes of Health.
cultured heart cells to muscarinic agonists. The time course of changes in receptor number and receptor properties was studied, together with the effect of guanine nucleotides and inhibitors of endocytosis on these agonist-mediated events.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were obtained from the following sources: carbamylcholine chloride, vinblastine sulfate, 8 Br-adenosine 3'-cyclic monophosphoric acid, N7,O7'-dibutylryl guanosine 3'5'-cyclic monophosphoric acid, cycloheximide, guanosine 3'-5' cyclic monophosphoric acid, GMP, and GTP from Sigma; podophyllotoxin, cytochalasin B, colchicine, and oxotremorine from Aldrich Chemical Co., guanosine 5'-β,γ-imino)triphosphate tetrasodium salt from ICN Pharmaceuticals; medium M-199 from Microbiological Associates; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer from Calbiochem; [3H]QNB from New England Nuclear (specific activity 28 Ci/mmol); embryonated Leghorn chicken eggs (flock MR58) from Sphats, Inc., Norwich, CT.

**Media**—Cells were grown in a modification of medium M-199 consisting of 21% (v/v) M-199 and 79% (v/v) Earle's salt solution supplemented with 4% fetal calf serum and 2% horse serum, both heat inactivated (30 min, 57°C). Ca2+ was adjusted to 1.8 mM with CaCl2. Medium used for all [3H]QNB-binding studies was Hepes-buffered M-199 consisting of 21% (v/v) M-199 and 79% (v/v) of a buffered salt solution containing 117 mM NaCl, 4.4 mM KCl, 0.8 mM MgSO4, 25 mM Heps (adjusted to pH 7.4 with NaOH), 1 mM NaH2PO4, 5 mM glucose, 0.001% (v/v) phenol red, and 1.8 mM CaCl2.

**Heart Cell Cultures**—Heart cell cultures were prepared by a modification of the method of DeHaan (16) as described (17) except that Ca2+/Mg2+-free Hanks' balanced salt solution was used in place of Flock's saline G. Hearts were removed, minced, and incubated with 0.025% (v/v) trypsin in Ca2+/Mg2+-free Hanks' balanced salt solution at 37°C for 8 min. The trypsinization solution was removed and diluted into medium M-199 containing 50% heat-inactivated horse serum at room temperature. After successive trypsinizations, suspensions of trypsinized cells were sedimented at 1000 rpm in a desk-top centrifuge, resuspended in medium, and incubated in a 100-mm Petri dish (Falcon) for 45 min at 37°C in a humidified atmosphere of 5% CO2/95% air. During this incubation, nearly 95% of the fibroblasts in the suspension adhered to the dish. The heart cells then were plated at a density of 2.0 x 106 cells per cm2 on collagen-coated 100-mm Petri dishes. On the third day of incubation, the medium was changed. Cells were harvested on culture day 4 unless otherwise indicated.

**Measurements of [3H]QNB Binding to Homogenates**—The assay procedure was a method of the modification of Yamamura and Snyder (11, 13). After three rinses with ice-cold M-199, the cells were harvested in a small volume of M-199/Hepes. After freezing and thawing twice at -70°C, the cells were homogenized in a glass homogenizer and allowed to thaw at 22°C for 30 min. The homogenate protein with trichloroacetic acid. Preparations of LUMICOLCHICINE—A solution of colchicine in 95% ethanol gave two peaks, λmax 350 nm (ε 16,700) and 244 nm (ε 30,000) (19). For conversion of choline to lumicolchicine, a 33 μM solution of choline in 95% ethanol, which gave an optical density reading of 1.0 at 244 nm, was exposed to a 366-nm source in a Chromata-Vu black box, Blacklight Eastern Corporation, Port Washington, NY, in order to avoid extraneous light. Conversion to lumicolchicine was followed by comparison of the ultraviolet absorption spectrum of an aliquot determined on a Cary recording spectrophotometer at various times after initiation of UV exposure. The conversion to β and γ lumicolchicines was nearly complete after 90 min as compared to known spectra (19).

**RESULTS**

**Effects of Agonist Exposure on Binding of [3H]QNB to Muscarinic Receptors**—We have demonstrated previously that exposure of intact cultured chick heart cells for 3 h to 10-7 M carbamylcholine caused a 65 to 70% decrease in muscarinic receptor number as measured by the binding of the potent muscarinic antagonist [3H]QNB to homogenates of these cells (15). Such an agonist-induced decrease in [3H]QNB binding might reflect a decrease in the number of receptors available for binding. Alternatively, either a decreased affinity of the receptor for [3H]QNB or a substantial decrease in the rate of approach of [3H]QNB binding to equilibrium could explain an apparent decrease in [3H]QNB binding.

In order to distinguish among these mechanisms, an experiment comparing the binding of [3H]QNB to homogenates of intact cells which had been exposed to agonists and to homogenates of control cells is illustrated in Fig. 1. The specific binding was saturable. Scatchard analysis of the data gave two nearly parallel straight lines for carbamylcholine-treated and control cells corresponding to KD values of 0.18 ± 0.03 (S.E.) and 0.21 ± 0.04 (n = 3) nm, respectively, intersecting the x axis at 78 and 194 fmol/mg of protein (Fig. 1). Hence the data are consistent with a 60% carbamylcholine-mediated decrease in [3H]QNB binding with no significant alteration in affinity for [3H]QNB.

In order to determine whether carbamylcholine treatment affected the time course of [3H]QNB binding, a kinetic analysis of QNB binding to homogenates of control cells and homogenates of intact cells treated with carbamylcholine was performed. The rate of binding of [3H]QNB at a concentration of 1 nm is illustrated in Fig. 2. In homogenates of both control and carbamylcholine-treated cells, binding proceeded without a lag and reached 50% saturation at 13.2 ± 0.7 (S.E.) and 12.0 ± 0.8 min (n = 3), respectively, with saturation levels

![Scatchard plot of binding of [3H]QNB to chick heart cell homogenates at various [3H]QNB concentrations.](image-url)
referring to an IC, for the agonist which is specific for the experimental
interaction between ['HIQNB and the competing ligand, the Hill
coefficients for agonists are less than one, and a simple competitive
inhibition of ['HIQNB binding by agonists cannot be assumed. Hence
we refer to an IC, for the agonist which is specific for the experimental
conditions described.

reflecting a 63% decrease in receptor number after agonist exposure. Previous studies have demonstrated that the binding of ['HIQNB to whole heart homogenates and homogenates of cultured heart cells is a biphasic process (11, 15). When the data in Fig. 2, left panel, were plotted according to the equation for a simple biomolecular process, superimposable biphasic curves were obtained (Fig. 2, right panel) for homog-

an effect of nucleotides on agonist modulation of muscarinic receptor number after prolonged agonist exposure, we exposed cultured heart cells to 10^{-3} M carbachol for 3 h, washed, homogenized, and measured ['HIQNB binding in the presence of 10^{-5} M guanine or adenine nucleotides including GTP, ATP, Gpp(NH)p, GDP, GMP, cAMP, and cGMP. No significant restoration of ['HIQNB-binding sites toward control levels was detected following this 3-h exposure of cells to agonist. ['HIQNB binding remained decreased 70 ± 3% (n = 3) below control levels. In a similar experiment homogenates of intact cells exposed for 3 h to 10^{-3} M carbachol were incubated with 10^{-4} M guanine and adenine nucleotides prior to addition of ['HIQNB. ['HIQNB binding remained decreased 68 ± 4% (n = 3) below control levels.

It was not possible to determine whether high intracellular levels of GTP or Gpp(NH)p might enhance recovery of recep-
tors in intact cells after prolonged agonist exposure, since these agents have limited ability to enter intact cells. No role
of cyclic nucleotides in modulation of agonist-induced changes in receptor number was apparent in experiments in which intact cells were incubated with 10^{-3} M carbachol for 3 h in the presence of 10^{-4} M concentrations of 8-Br-guanosine 3',5'-cyclic monophosphoric acid or dibutyryl cyclic AMP, cyclic nucleotide derivatives thought to be capable of entering the cell. When these cells were washed and homogenized, ['HIQNB-binding sites remained decreased to 70 ± 4% (n = 3) below control levels.

Effect of Prior Exposure to Agonist on the Properties of
inhibition of ['HIQNB binding to embryonic chick heart cell homogenates were 1.0 × 10^{-7} M and 0.9 × 10^{-5} M, respectively, with Hill coefficients of 0.58 and 0.55 (15). The marked similarities among the data for the relative ability of these agonists to compete with ['HIQNB for receptor binding, their relative potencies in inducing decreases in receptor number (Fig. 3), and their relative pharmacologic potencies indicate that ago-

Effect of Nucleotides on Agonist-mediated Decreases in
Antagonist Binding to Muscarinic Receptors—It has been demonstrated in a number of systems including the β-adre-
nergic receptors of frog erythrocyte membranes that following an agonist-mediated decrease in receptor-binding sites, the number of receptors can be restored to near-control levels by incubation with GTP or with Gpp(NH)p, a nonhydrolyzable GTP analogue (21). To determine the effects of nucleotides on agonist modulation of muscarinic receptor number after prolonged agonist exposure, we exposed cultured heart cells to 10^{-3} M carbachol for 3 h, washed, homogenized, and measured ['HIQNB binding in the presence of 10^{-4} M concen-	rakines of guanine or adenine nucleotides including GTP, ATP, Gpp(NH)p, GDP, GMP, cAMP, and cGMP. No significant restoration of ['HIQNB-binding sites toward control levels was detected following this 3-h exposure of cells to agonist. ['HIQNB binding remained decreased 70 ± 3% (n = 3) below control levels. In a similar experiment homogenates of intact cells exposed for 3 h to 10^{-3} M carbachol were incubated with 10^{-4} M guanine and adenine nucleotides prior to addition of ['HIQNB. ['HIQNB binding remained decreased 68 ± 4% (n = 3) below control levels.

It was not possible to determine whether high intracellular levels of GTP or Gpp(NH)p might enhance recovery of recep-
tors in intact cells after prolonged agonist exposure, since these agents have limited ability to enter intact cells. No role
of cyclic nucleotides in modulation of agonist-induced changes in receptor number was apparent in experiments in which intact cells were incubated with 10^{-3} M carbachol for 3 h in the presence of 10^{-4} M concentrations of 8-Br-guanosine 3',5'-cyclic monophosphoric acid or dibutyryl cyclic AMP, cyclic nucleotide derivatives thought to be capable of entering the cell. When these cells were washed and homogenized, ['HIQNB-binding sites remained decreased to 70 ± 4% (n = 3) below control levels.

Effect of Prior Exposure to Agonist on the Properties of

![FIG. 2. Kinetic analysis of ['HIQNB binding to homogenates of agonist-treated cells. Left panel, time course of ['HIQNB binding to cultured heart cells. Replicate culture dishes were divided into a control group and a group incubated for 3 h with 10^{-5} M carbachol; cell homogenates from each group were prepared and incubated in 1 nm ['HIQNB for the times indicated, at which time aliquots were removed and binding terminated by addition of 4 ml of ice-cold wash solution and filtration. ['HIQNB bound was determined as described under "Experimental Procedures." O, control; O, cells incubated with 10^{-3} M carbachol prior to harvest. Right panel, data plotted according to the equation in Bm = (kBm(Bm - B0)) / (kBm + k-0) t in which Bm and B0 are concentrations of bound ['HIQNB at equilibrium and at time t; k-0 is the second order association rate constant and k+0 is the first order rate constant for dissociation of the QNB-receptor complex. Free ['HIQNB is present in large excess and hence remains essentially constant (for discussion of this equation see Ref. 11).

![FIG. 3. Effect of prior exposure of cells to graded concentrations of agonist on ['HIQNB binding by cell homogenates. Left panel, media from three replicate cultures were replaced by fresh medium containing the indicated concentration of either oxotremo-

rline or carbachol, incubated for 3 h at 37°C in a humidified chamber in an atmosphere of 5% CO2/95% air, and washed three times with ice-cold Hepes-buffered M-199. Cells from all three dishes were then harvested, combined, and assayed for ['HIQNB binding in the presence of 1 nm ['HIQNB as described under "Experimental Procedures." Each point represents the mean of three determinations and is corrected for nonspecific binding. O, oxotremorine; O, carba-
mylecholine. Right panel, same data plotted on the coordinates of Brown and Hill (20); y is the fractional decrease in ['HIQNB binding.
Agonist Binding to Muscarinic Receptors—Although the affinity of the receptor for agonist and the kinetics of binding of agonist indicate that the unaltered by agonist exposure, the binding properties of agonists might reflect more subtle changes in the receptor than the less specific binding of antagonists. Therefore, the affinity of receptor for agonist in control cells and in cells exposed to $10^{-3}$ M carbamylcholine for 3 h was estimated by determining the ability of agonists to compete with $[^3H]QNB$ binding to homogenates of control and agonist-treated cells.

The experiment summarized in Fig. 4 demonstrated an increase in the IC$_50$ for agonist displacement of $[^3H]QNB$ binding in carbamylcholine-treated cells from 1.5 $\pm$ 0.3 (S.E.) $\times 10^{-5}$ M in homogenates of control cells to 9.5 $\pm$ 2.0 $\times 10^{-5}$ M (n = 6) in homogenates of cells exposed to carbamylcholine prior to homogenization. This 6-fold difference was highly reproducible. Oxotremorine exposure resulted in a similar shift in IC$_50$ from 1.0 $\times 10^{-7}$ M to 6.8 $\times 10^{-7}$ M. Analysis of these inhibition curves by the method of Brown and Hill revealed that the shift in IC$_50$ was not accompanied by a significant change in the Hill coefficient; values in this experiment were 0.65 and 0.63 for control and carbamylcholine-treated cells, respectively.

**Time Course of Agonist-induced Decrease in $[^3H]QNB$-binding Sites and Receptor Affinity for Agonist—**Since our prior studies (15) primarily involved agonist exposure times greater than 30 min, a more detailed analysis of the effects of short periods of exposure of cells to $10^{-3}$ M carbamylcholine was undertaken. As shown in Fig. 5, the time course of the effect of agonist on receptor number was biphasic. A 26% loss of $[^3H]QNB$-binding capacity during the first minute of exposure to agonist was followed by a short plateau period during which continued exposure to agonist had no apparent further effect on $[^3H]QNB$ binding. For periods in excess of 20 min of agonist exposure a gradual loss of an additional 43% of $[^3H]QNB$-binding capacity occurred over the next 2 h. The half-time of this second phase of loss of $[^3H]QNB$-binding capacity was about 30 min.

One possible explanation for the loss of $[^3H]QNB$-binding sites is persistent binding of agonist to a fraction of receptors with subsequent interference with $[^3H]QNB$ binding to that fraction of receptors. In order to study the reversibility of the early effects of agonist exposure on $[^3H]QNB$ binding, cells were treated with agonist for 15 min, washed free of unbound carbamylcholine, and incubated in fresh medium for up to 3 h.

Upon having shown (Fig. 4) that the apparent affinity of receptors for agonist was decreased 6-fold after a 3-h exposure of cells to agonist, we studied the time course over which agonist-induced changes in affinity took place. As summarized in Fig. 6, a 15-min exposure of cells to $10^{-3}$ M carbamylcholine resulted in a shift of the IC$_50$ for carbamylcholine displacement of $[^3H]QNB$ from 1.4 $\pm$ 0.3 $\times 10^{-5}$ M (n = 15) to 8.9 $\pm$ 0.9 $\times 10^{-5}$ M (n = 9). A similar change in IC$_50$ could be detected as early as 1 min after exposure of cells to $10^{-3}$ M carbamylcholine (data not shown). No change in KD for antagonist could be detected after such a brief exposure to agonist. This shift in affinity for agonist was not reversible after washout of agonist and incubation for 3 h in fresh medium (Fig. 6). Thus, both the loss of 26% of $[^3H]QNB$-binding sites and the decrease in apparent affinity of the remaining receptors occur during the first minute of agonist exposure and are not reversible with incubation in fresh medium without agonist for 3 h.

The Effect of Guanine Nucleotides on Binding of Agonists to Muscarinic Receptors—Guanine nucleotides have been shown to decrease the affinity of agonists for the $\beta$-adrenergic receptor and in homogenates to modulate the release of bound agonist from the receptor (10, 22). It has also been shown that GTP and Gpp(NH)p are capable of lowering the apparent affinity of carbamylcholine for the muscarinic receptor in homogenates of rat heart (23, 24). The IC$_50$ for carbamylcholine displacement of $[^3H]QNB$ binding in homogenates of heart cell cultures increased from 1.5 $\pm$ 0.2 $\times 10^{-7}$ M (n = 15) to 8.7 $\pm$ 1.5 $\times 10^{-5}$ M (n = 4) with the addition of 0.1 mM Gpp(NH)p (Fig. 6). This shift was similar to that seen in homogenates of intact cells exposed for 1 to 15 min to carbamylcholine (Fig. 6 and Table I). However, unlike the effect of exposure to carbamylcholine, no effect of Gpp(NH)p on total number of $[^3H]QNB$-binding sites could be demonstrated. Incubation with GTP and GDP also increased the concentration of carbamylcholine required to inhibit 50% of $[^3H]QNB$ binding while GMP and cGMP had no significant effect on carbamylcholine binding (Table I).

If the loss of 26% of $[^3H]QNB$-binding sites after brief exposure of cultured heart cells to muscarinic agonists represents persistent binding of agonist to the receptor, and if muscarinic receptors are subject to regulation by guanine nucleotides, treatment with guanine nucleotides of homoge-
Receptors. Replicate culture dishes were incubated with and without 10^{-5} M carbamylcholine for 15 min; cells were harvested and homogenized as described under "Experimental Procedures." Aliquots of homogenates of control and agonist-treated cells were incubated for 1 h in medium containing 1 nM [3H]QNB with or without 10^{-4} M Gpp(NH)p and the concentrations of agonist indicated and [3H]QNB binding determined. Several of the carbamylcholine-treated cultures were rinsed 3 times with fresh medium and incubated in fresh growth medium for 3 h. [3H]QNB binding to homogenates of these cells in the presence of the indicated concentrations of carbamylcholine was then determined. Each point represents the mean of 3 replicate determinations of [3H]QNB binding. Inhibition of [3H]QNB binding is plotted as fmol/mg. 100% inhibition corresponds to a decrease in [3H]QNB bound of 140 fmol/mg. , control; , control plus 10^{-4} M Gpp(NH)p; , carbamylcholine pretreatment for 15 min; , carbamylcholine pretreatment for 15 min followed by incubation in the presence of 10^{-4} M Gpp(NH)p; , carbamylcholine pretreatment for 15 min followed by 3-h recovery.

**Table I**

Effect of guanine nucleotides on apparent agonist affinity for muscarinic receptors

| Nucleotide | IC_{50} for carbamylcholine (nM) |
|------------|----------------------------------|
| None       | 1.5 ± 0.2 × 10^{-3} (n = 15)     |
| None*      | 9.5 ± 2.0 × 10^{-3} (n = 9)      |
| Gpp(NH)p   | 8.7 ± 1.5 × 10^{-3} (n = 5)      |
| GTP        | 7.1 ± 1.1 × 10^{-3} (n = 3)      |
| GDP        | 6.9 ± 1.0 × 10^{-3} (n = 3)      |
| GMP        | 1.1 ± 1.7 × 10^{-3} (n = 3)      |
| GMP        | 1.0 ± 1.5 × 10^{-3} (n = 3)      |

* Cells exposed to 10^{-3} M carbamylcholine for 15 min prior to harvesting for determination of IC_{50}.

* p < 0.001 by group comparison with IC_{50} for cells not treated with nucleotides.

Not significantly different from control.

Fig. 6. Effect of brief agonist exposure and treatment with guanine nucleotides on the binding of agonist to muscarinic receptors. Replicate culture dishes were incubated with and without 10^{-5} M carbamylcholine for 15 min; cells were harvested and homogenized as described under "Experimental Procedures." Aliquots of homogenates of control and agonist-treated cells were incubated for 1 h in medium containing 1 nM [3H]QNB with or without 10^{-4} M Gpp(NH)p and the concentrations of agonist indicated and [3H]QNB binding determined. Several of the carbamylcholine-treated cultures were rinsed 3 times with fresh medium and incubated in fresh growth medium for 3 h. [3H]QNB binding to homogenates of these cells in the presence of the indicated concentrations of carbamylcholine was then determined. Each point represents the mean of 3 replicate determinations of [3H]QNB binding. Inhibition of [3H]QNB binding is plotted as fmol/mg. 100% inhibition corresponds to a decrease in [3H]QNB bound of 140 fmol/mg. , control; , control plus 10^{-4} M Gpp(NH)p; , carbamylcholine pretreatment for 15 min; , carbamylcholine pretreatment for 15 min followed by incubation in the presence of 10^{-4} M Gpp(NH)p; , carbamylcholine pretreatment for 15 min followed by 3-h recovery.

Fig. 7. Time course of recovery of receptors after agonist pretreatment. Sets of replicate cultures were incubated for 3 h with 10^{-5} M carbamylcholine and then washed 3 times. Half the dishes were then fed with fresh medium and the other half with fresh medium containing 2 pg/ml of cycloheximide. At the times indicated, sets of three dishes from each group were harvested, homogenized, and assayed for [3H]QNB binding. Each point represents the mean of three replicate determinations. Control cultures not subjected to agonist pretreatment bound 145 fmol of [3H]QNB/mg of protein. , cells incubated in fresh medium; , cells incubated in fresh medium plus 2 pg/ml of cycloheximide.
with a time course indistinguishable from that shown in Fig. 5 in the presence of cycloheximide. In all of these recovery experiments, cells were used on the fourth day of culture at which time there is little cell division as judged by [3H]-thymidine incorporation (25) and repeated cell counts. We conclude that the rate of recovery of receptors is too rapid to be explained merely by cell division with appearance of new receptor-containing membrane.

In order to study the recovery of that subgroup of 26% of receptors lost rapidly during the early phase of agonist exposure, cells were incubated for 15 min in 10−6 M carbamylcholine, washed, and incubated in fresh medium. As shown in Fig. 6, these [3H]QNB-binding sites did not recover during a 3-h incubation in fresh medium. Unlike the recovery of receptors lost after a 3-h incubation with agonist, however, further incubation for up to 12 h demonstrated no significant recovery. Thus, recovery of this class of rapidly lost receptors was significantly slower than recovery of receptors lost after a 3-h incubation with agonist. However, data in Fig. 7 indicate that after a 3-h incubation with agonist, receptors recover to control levels after 12 h in fresh medium, suggesting that all classes of receptors recover during this period. One possible explanation of these findings is that a 3-h exposure to agonist alters the subgroup of receptors lost during the first 15 min of exposure to agonist, rendering these receptors unresponsive to guanine nucleotides and shortening their prolonged recovery period to approximate the recovery kinetics shown in Fig. 7.

**Effect of Inhibitors of Endocytosis on Agonist-induced Loss of Receptor Number**—The preceding data are consistent with loss of [3H]QNB-binding sites either due to irreversible alteration of the properties of the receptor by agonist exposure or actual agonist-induced removal of the receptor from the cell surface membrane. Both of these mechanisms are consistent with a requirement for synthesis of protein for recovery of receptors lost rapidly during the early phase of agonist exposure, however, further incubation for up to 12 h demonstrated no significant recovery. Thus, recovery of this class of rapidly lost receptors was significantly slower than recovery of receptors lost after a 3-h incubation with agonist. However, data in Fig. 7 indicate that after a 3-h incubation with agonist, receptors recover to control levels after 12 h in fresh medium, suggesting that all classes of receptors recover during this period. One possible explanation of these findings is that a 3-h exposure to agonist alters the subgroup of receptors lost during the first 15 min of exposure to agonist, rendering these receptors unresponsive to guanine nucleotides and shortening their prolonged recovery period to approximate the recovery kinetics shown in Fig. 7.

The effect of colchicine reached a maximum at a concentra-

| Concentration (M) | % decrease in [3H]QNB Bound |
|------------------|---------------------------|
| 0                | 20                        |
| 10−7             | 40                        |
| 10−6             | 60                        |
| 10−5             | 80                        |

**Fig. 8.** Effect of pretreatment of cells with colchicine on agonist-induced loss of receptors. Replicate cultures were incubated with either 10−5 M, 10−4 M, or 10−3 M colchicine for the times indicated (three plates for each time point); incubation was then continued for 3 h in fresh medium containing 10−6 M carbamylcholine plus the indicated concentration of colchicine. Homogenates of each set of three dishes were pooled and assayed for [3H]QNB binding. Each point is the mean of three replicate determinations. The zero time point represents cells treated for 3 h with the indicated concentration of colchicine plus 10−6 M carbamylcholine, without prior exposure to colchicine alone. *10−5 M colchicine; □, 10−4 M colchicine; △, 10−3 M colchicine.

| Concentration (M) | % decrease in [3H]QNB Bound |
|------------------|---------------------------|
| 0                | 20                        |
| 10−7             | 40                        |
| 10−6             | 60                        |
| 10−5             | 80                        |

**Fig. 9.** Effect of inhibitors of microtubule and microfilament function on agonist modulation of receptor number. Groups of three replicate cultures were preincubated with the indicated concentrations of agent for 2 h. Fresh medium containing 10−3 M carbamylcholine and the indicated concentration of each agent was then added. After further incubation for 3 h, cells were harvested and [3H]QNB binding to homogenates determined. Each point is the mean of three replicate determinations. ▲, cytochalasin B; △, lumicolchicine; ●, colchicine; □, podophyllotoxin; ○, vinblastine.
Control of Muscarinic Receptors in Cultured Heart Cells

The effect of colchicine on the loss of \(^{3}H\)QNB-binding sites

Replicate culture dishes were incubated with fresh medium or medium containing \(10^{-4}\) M colchicine for 2 h, after which fresh medium containing no additions (control), \(10^{-4}\) M colchicine, \(10^{-9}\) M carbamylcholine, or \(10^{-7}\) M carbamylcholine plus \(10^{-4}\) M colchicine was added and incubated for 15 min. Cells were then homogenized and \(^{3}H\)QNB binding determined.

| Treatment                  | \(^{3}H\)QNB Bound (\% of Control) |
|----------------------------|----------------------------------|
| Control                    | 100                              |
| Colchicine                 | 95 ± 5                           |
| Carbamylcholine            | 98 ± 4                           |
| Carbamylcholine + colchicine| 97 ± 3                           |

**TABLE II**

The affinity of receptors for \(^{3}H\)QNB and the kinetics of \(^{3}H\)QNB binding were the same in control and agonist-treated cells, the agonist-induced decrease in the number of \(^{3}H\)QNB-binding sites presumably reflects a decrease in the number of receptor sites available to interact with this potent antagonist. Although exposure of cells to muscarinic agonists did not affect affinity or kinetics of antagonist binding, a more subtle and potentially important agonist-induced effect was the significant decrease in the apparent affinity of the receptor for agonist (Fig. 6).

The regulation of receptor number and receptor affinity by agonist was separable temporally into two distinct phases, shown schematically in the upper portion of Fig. 10. As early as 1 min after addition of agonist, a 26% decrease in \(^{3}H\)QNB-binding sites occurred, together with a concomitant decrease in the apparent affinity of all remaining receptors for agonist. With more prolonged agonist exposure, another 44% of \(^{3}H\)QNB-binding sites were lost over 2 h. We also found that guanine nucleotides decreased the affinity of muscarinic receptors for agonists in homogeneous cell populations to the same extent as a brief exposure to agonist. Furthermore, guanine nucleotides were able to facilitate the recovery of the \(^{3}H\)QNB-binding sites lost during the early phase of exposure to agonists.

These findings suggest that muscarinic receptors in cultured heart cells may exist in three states: 1) a high affinity guanine nucleotide-sensitive state characterizing a subset of receptors (26% shown schematically in the upper portion of Fig. 10) capable of binding agonist in a quasi-reversible fashion, subsequently unavailable for \(^{3}H\)QNB binding; 2) a resting state that characterizes the majority of receptors (74%; lower portion of Fig. 10) prior to exposure of intact cells to agonist or prior to exposure of homogenates to elevated levels of guanine nucleotides, with an IC\(_{50}\) for carbamylcholine inhibition of \(^{3}H\)QNB binding of \(1.4 \times 10^{-7}\) M (Fig. 6); and 3) a low affinity state assumed by receptors either after treatment of homogenates with guanine nucleotides or immediately after exposure of cells to agonist, with an IC\(_{50}\) for carbamylcholine displacement of \(^{3}H\)QNB of \(8.9 \times 10^{-7}\) M (Fig. 6).

In this scheme, rapid quasi-reversible binding of agonist to a group of high affinity receptors would cause a loss of \(^{3}H\)QNB-binding sites and be accompanied by conversion of additional receptors in the resting state to the low affinity activated state. The concept of partial occupancy by agonist of a group of receptor sites with an associated decrease in the affinity for agonist of the remaining unbound sites is quite analogous to the concept of negative cooperativity, which is well demonstrated for muscarinic agonist binding (Fig. 3). We postulate that binding of guanine nucleotides to a nucleotide regulatory site associated with the high affinity receptor may convert these high affinity receptors to a low affinity form and, in addition, convert the remaining receptor population to the low affinity form. Any agonist persistently bound to the high affinity form would be released during this process with recovery of \(^{3}H\)QNB-binding sites. Alternatively, all receptors may be associated with a guanine nucleotide regulatory site and be converted independently to the low affinity state.

Under physiologic conditions, muscarinic cholinergic agonists and guanine nucleotides may act in concert at their respective sites to mediate rapid conversion of the receptor to the low affinity activated state. Whether or not the low affinity state of the receptor is actually the physiologically active form of the receptor cannot be determined from the data presented.

The model outlined in Fig. 10 depends on the presence of a high affinity receptor and persistent binding of agonist to this receptor associated with regulation of receptor affinity. Traditional analysis of hormone-receptor interactions has been...
based on the assumption that the hormone-receptor complex is readily reversible. However, studies of the $\beta$-adrenergic receptor (9) and the glucagon receptor (25) have demonstrated persistent binding of agonists and a role of guanine nucleotides in mediating the release of agonist from receptor in homogenates. Furthermore, exposure of astrocytoma cells (7) to either guanine nucleotides or $\beta$-adrenergic agonists has been reported to decrease the affinity of the receptor for agonist as well as to mediate a decrease in adenylate cyclase activity, suggesting that such agonist-induced affinity changes are also involved in regulation of $\beta$-adrenergic receptor activity. Although we have not added direct evidence in the present studies for the existence of the high affinity form of the receptor, Birdsall et al. have presented a computer analysis of data from studies of direct binding of tritiated muscarinic agonists to synaptosomal preparations from rat cerebral cortex suggesting that 25 to 30% of receptors may exist in a high affinity form (30).

Of the 60 to 70% of $[^3H]$QNB-binding sites lost during agonist exposure, 26% are lost during the first minute of exposure while 33% of sites are lost between 30 min and 3 h during a slow second phase (Fig. 5). Agents known to interfere with microtubule function (Fig. 9) inhibit 40 to 45% of the total agonist-induced receptor loss. Hence a subclass of receptors (25 to 30%) is subject to agonist control by a mechanism independent of microtubule function. The absence of an effect of colchicine on the rapid loss of receptor sites during brief exposure of cells to agonist (Table II) suggests that these sites may represent the 25 to 30% subset insensitive to colchicine. The total receptor population, then, may be divided into subgroups as shown schematically in Fig. 10.

Studies of recovery of receptors lost during brief exposure to agonist revealed that the receptor sites did not recover significantly over periods greater than 12 h after removal of agonist. However, if cells were incubated for 3 h with agonist, guanine nucleotides had no effect on recovery of receptors, and recovery to base-line levels occurred over a 12-h period and required protein synthesis (Fig. 7). One possible explanation of these data is that after a 3-h exposure to agonist, receptors in the 26% subgroup undergoing rapid loss are also subject to endocytosis. The role of microtubules in the process of agonist-induced loss of muscarinic receptors is further supported by the finding that the half-maximal inhibitory effect of colchicine on agonist-mediated receptor loss occurs at a concentration of 1.4 $\times 10^{-6}$ M (Fig. 9), comparable to the value of 2.3 $\times 10^{-6}$ M observed for half-maximal binding of colchicine to the tubulin of sea urchin eggs (31). However, it should be noted that colchicine exerts an inhibitory effect on nucleotide transport (28) which could also interfere with agonist modulation of receptors. However, lumicolchicine, which had no effect on agonist-induced changes in receptor number (Fig. 9), also has a potent inhibitory effect on nucleoside transport. Polymerization of tubulin involves GTP (27) which was found (Table I) to decrease apparent receptor affinity for carbamylcholine. Shifts in GTP concentrations during depolymerization of microtubules by colchicine could secondarily alter receptor affinities and perhaps also interfere with agonist-induced changes in receptor number. The most likely explanation is that disruption of microtubular structure interferes with the agonist-induced disappearance of $[^3H]$QNB-binding sites, presumably by inhibition of endocytosis. Agonist stimulation of endocytosis of cell surface receptors has been demonstrated following epidermal growth factor interaction with human fibroblasts (3), antigen interaction with antigen receptor sites on B lymphocytes (32, 33), and low density lipoprotein interaction with fibroblasts (26). Recently Siman and Klein have demonstrated

that agonist-mediated decreases in muscarinic receptors in neuroblastoma hybrids were inhibited by cytochalasin B, which interacts with microfilaments (34). The absence of an effect of cytochalasin B in our system is presumably related to differences in species and cell type.

Further studies to explore the presence of a high affinity subclass of receptors, the role of endocytosis of receptor-ligand complexes, and persistent binding of agonist to high affinity receptors after short agonist exposure will be necessary to elucidate more fully the mechanism of agonist control of muscarinic receptor activity and the interaction of agonists, guanine nucleotides, and the cell membrane in this process.

Acknowledgments—We thank Dr. Eva J. Neer for helpful discussions. We also thank Ms. Louise Dziekan and Ms. Lisa Morrow for excellent technical assistance and Ms. Deborah Kilion for typing the manuscript.

REFERENCES
1. Cett, K. J., Harwood, J. P., Aguillera, G., and Dufau, M. L. (1979) Nature 280, 109-116
2. Kastakos, F. C., and Roth, J. (1978) in Protein Turnover and Lysosome Function (Segal, H. L., and Doyle, D. J., eds) pp. 653-777, Academic Press, New York
3. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 71, 159-171
4. Mukherjee, C., Caron, M. G., and Lefkowitz, R. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1945-1949
5. Shear, M., Insel, P. A., Melmor, K. L., and Coffino, P. (1976) J. Biol. Chem. 251, 7572-7578
6. Mukherjee, C., and Lefkowitz, R. J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1494-1498
7. Su, Y.-F., Harden, T. K., and Perkins, J. P. (1979) J. Biol. Chem. 254, 38-41
8. Harden, T. K., Su, Y.-F., and Perkins, J. P. (1979) J. Cyclic Nucleotide Res. 5, 98-106
9. Wessel, M. R., Mullikin, D., and Lefkowitz, R. J. (1978) J. Biol. Chem. 253, 3371-3373
10. Limbird, L. E., and Lefkowitz, R. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 228-232
11. Galper, J. B., Klein, W., and Catterall, W. A. (1977) J. Biol. Chem. 252, 3371-3373
12. George, W. J., Polson, J. B., O'Toole, A. G., and Goldberg, N. D. (1970) Proc. Natl. Acad. Sci. U. S. A. 66, 398-403
13. Yamamura, H., and Snyder, S. H. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1729-1732
14. Fields, J. Z., Roeseke, W. R., Morkin, E., and Yamamura, H. (1978) J. Biol. Chem. 253, 3251-3258
15. Galper, J. B., and Smith, T. W. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5831-5835
16. DeHaan, R. L. (1967) Dev. Biol. 16, 216-249
17. Galper, J. B., and Catterall, W. A. (1978) Dev. Biol. 65, 216-227
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
19. Wilson, L., and Friedkin, M. (1966) Biochemistry 5, 2463-2468
20. Brown, W. E., and Hill, A. V. (1922) Proc. R. Soc. Lond. B. Biol. Sci. 94, 297-304
21. Mukherjee, C., and Lefkowitz, R. J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1494-1498
22. Lefkowitz, R. J., Mullikin, D., and Caron, M. G. (1976) J. Biol. Chem. 251, 4686-4692
23. Berrie, C. P., Birdsall, N. J. M., Burgen, A. S. U., and Hullme, E. C. (1979) Biochem. Biophys. Res. Commun. 87, 1000-1005
24. Rosenberger, L. B., Roeseke, W. R., and Yamamura, H. I. (1971) Eur. J. Pharmacol. 26, 179-180
25. Fischman, D. A., Doyle, C. M., and Zak, R. (1975) in Developmental and Physiological Correlations of Cardiac Muscle (Lieberman, M., and Sano, T., eds) pp. 67-79, Raven Press, New York
26. Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1979) Nature 278, 679-685
27. Weller, L., Hamborg, F. G., Meele, S. B., Grisham, L., and Creswell, K. M. (1974) Fed. Proc. 33, 158-166
28. Wessels, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T., and Yamada, K. M. (1971) Science 171, 135-142
29. Rodbell, M., Lin, M. C., and Salomon, Y. (1974) J. Biol. Chem. 249, 59-65
30. Birdsall, N. J. M., Burgen, A. S. U., and Hulme, E. C. (1978) Mol. Pharmacol. 14, 723-736
31. Yahara, I., and Edelman, G. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4141-4145
32. DePetrus, S., and Raff, M. (1972) Eur. J. Immunol. 2, 523-527
33. DePetrus, S., and Raff, M. (1973) Nature New Biol. 241, 257-259
34. Siman, R. G., and Klein, W. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4141-4145