The Biphasic Response of Muscarinic Cholinergic Receptors in Cultured Heart Cells to Agonists

**EFFECTS ON RECEPTOR NUMBER AND AFFINITY IN INTACT CELLS AND HOMOGENATES**

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A biphasic time course of the agonist-mediated loss of muscarinic cholinergic receptors has been demonstrated in cultured chick embryo heart cells by radioligand binding studies using the muscarinic antagonist [3H]quinuclidinyl benzilate ([3H]QNB). This agonist-mediated receptor loss was associated with decreased affinity of the receptor for agonist as judged by competitive binding of the agonist carbamylcholine with [3H]QNB to cell homogenates (Galper, J. B., and Smith, T. W. (1980) *J. Biol. Chem.* 255, 9571-9579). In the current studies the concentration dependence of agonist-mediated receptor loss was also found to be biphasic. The apparent shift of affinity following brief (15 min) agonist exposure coincided with the agonist-mediated loss of a subclass of high affinity receptors with an IC50 for carbamylcholine inhibition of [3H]QNB binding of 3.9 × 10^-7 M. Those receptors remaining constituted a subclass of low affinity receptors with IC50 = 8.2 × 10^-4 M. The data further suggest that an apparent decrease in agonist affinity after guanine nucleotide exposure represents conversion of high affinity receptors to a similar low affinity state, IC50 = 8.6 × 10^-5 M. The rapid loss of [3H]QNB binding sites in the presence of agonist did not require interaction of agonist with intact cells, but also occurred if cells were homogenized and then subjected to a brief (15 min) exposure to agonist. The slow loss over 3 h of [3H]QNB binding sites could only be demonstrated in intact cells incubated with agonist prior to homogenization.

To probe further the later phase of agonist-mediated receptor loss, we developed new assay methods for determining muscarinic antagonist binding to intact cells. In control cells, binding of the hydrophobic antagonist [3H]QNB was quite similar in extent to binding of [3H]MS, with KD values of 0.11 and 0.47 nM, respectively. Kinetic analysis of the binding of these two ligands was performed to determine whether they might distinguish between two states of the receptor. Both ligands bound to the receptor by a two step mechanism consistent with the formation of a low affinity complex followed by conversion to a high affinity complex. The ratio of reverse to forward rate constants of the second step of [3H]MS binding was roughly 100-fold greater than that for the more hydrophobic ligand [3H]QNB. Comparison of the time course of agonist-induced receptor loss as measured by binding of [3H]MS or [3H]QNB was consistent with muscaminic agonist mediation of a stepwise alteration in receptor configuration from a form that bound both [3H]MS and [3H]QNB to a form that bound only [3H]QNB and thence to a form that bound neither [3H]MS nor [3H]QNB. The relationship of such a sequential mechanism to agonist-induced changes in the relationship of the receptor to the cell membrane and agonist-induced endocytosis of the receptor is discussed.

The ability of hormones and neurotransmitters to regulate the number and/or affinity of cell surface receptors has been demonstrated in excitable tissues, endocrine glands, and other tissues subject to hormonal control. Guanine nucleotides have also been shown to modulate the binding of agonists to several classes of receptors including the β-adrenergic receptor, the glucagon receptor, and the muscarinic cholinergic receptor. Recently, experiments using the potent muscarinic antagonist [3H]QNB for the identification of muscarinic binding sites have demonstrated that prior exposure of neuroblastoma cells or cultures of embryonic chick heart cells to muscarinic cholinergic agonists decreased the number of muscarinic binding sites by greater than 70%. In cultured heart cells the time course of loss of binding sites during agonist exposure was biphasic with an early rapid loss (1 min) of 26% of binding sites followed by a 20-min lag phase and the gradual loss of another 45% of receptors over 3 h. The rapid loss of 26% of receptor sites was accompanied by a 6-fold decrease in apparent affinity of the remaining receptors for agonist. When homogenates of rat heart (8, 9) or chick embryo heart cell cultures (7) were exposed to guanine nucleotides, affinity of muscarinic receptors for agonist decreased while receptor number remained unchanged. When homogenates of chick embryo heart cell cultures which had been exposed briefly to agonist were homogenized and incubated with guanine nucleotides, affinity remained decreased while the apparent number of receptor sites recovered to control levels.

The slow loss of 43% of muscarinic receptors during a 3-h exposure to agonist was only reversible after a 12-h incubation in the absence of agonist, and required protein synthesis (7). Inhibitors of microtubule function inhibited 43% of total agonist-mediated receptor loss.

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4 The abbreviations used are: QNB, quinuclidinyl benzilate; Gpp(NH)p, guanosine 5'-[γ-guanosine] triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [3H]MS, [3H]methylscopolamine; RLow, high affinity receptor; RLow, low affinity receptor.
nast-induced receptor loss. These data suggested that the subclass of 43% of receptors that disappear slowly during agonist exposure of cultured heart cells might be subject to enzymatic or metabolic degradation.

Agonist-induced endocytosis of cell surface receptors has been demonstrated for several types of hormone binding sites (1). In one of the most extensively studied cases, epidermal growth factor interaction with the human fibroblast has been shown to involve endocytosis of the receptor-growth factor complex with probable subsequent lysosomal degradation of both the hormone and receptor within the cell (10).

The experiments described here address two major aspects of the interaction between muscarinic agonists and receptors in cultured heart cells. First, we extend previous studies of the early rapid phase of agonist binding. The findings presented support the hypothesis that the rapid loss of 26% of receptors after brief exposure to agonist is due to persistent binding of agonist to a class of high affinity receptor sites, and that guanine nucleotides mediate recovery of these sites by facilitating release of persistently bound agonist during guanine nucleotide-mediated conversion of the high affinity receptor to a low affinity form. A second set of experiments in which we developed an assay for the binding of muscarinic antagonists to intact heart cells explores further the mechanism by which prolonged agonist exposure mediates loss of receptors. These experiments are divided into two parts. First, we compared the binding of the muscarinic antagonists [³H]QNB and [³H]HMS to the intact cell. Kinetic studies demonstrated that although both antagonists bound to the receptor via a sequential two-step mechanism, the antagonist-high affinity receptor complex formed with the more hydrophobic [³H]QNB demonstrated marked kinetic differences from the [³H]HMS-high affinity receptor complex. In the second set of experiments we compared the binding of [³H]QNB and [³H]HMS to cells which had been exposed for various times to high concentrations of agonist prior to antagonist binding. Our findings support the view that properties of binding of these radioligands to intact cells are capable of differentiating between two states of the receptor: the control state (before agonist binding) which bound both [³H]HMS and [³H]QNB and a state present during the late phase of agonist exposure which bound only [³H]QNB and which may be an intermediate state in the process of receptor endocytosis. Thus, alterations in the muscarinic receptor subsequent to binding of agonist involve distinct and separable initial and late events that can be distinguished by analysis of specific radioligand binding data.

**EXPERIMENTAL PROCEDURES**

**Materials.—**Chemicals were obtained from the following sources: carbamylcholine chloride, cycloheximide, and GTP from Sigma; oxotremorine from Aldrich; guanosine 5′(β, γ-imino) triphosphate tetra- sodium salt (Gpp[NH]p) from ICN Pharmaceuticals (Cleveland, OH); medium M-199 from Microbiological Associates (Bethesda, MD); HEPES buffer from Calbiochem; [³H]QNB (specific activity 43 Ci/ mmol) and [³H]HMS (specific activity 53 Ci/mm) from New England Nuclear; embryonated Leghorn chicken eggs (flock MR62) from Spafas, Inc. (Norwich, CT).

**Heart Cultures.—**Heart cell cultures were prepared by a modification of the method of DeHaan (11). as described (7). Embryos were removed from embryonated Leghorn chicken eggs on day 10 in ovo. Hearts were removed, minced, and incubated with 0.25% (w/v) trypsin in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution at 37°C for 8 min. The trypsin 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 100 rpm in a desk top centrifuge, resuspended in growth medium, and incubated in a 100-mm Petri dish (Falcon, Oxnard, CA) 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 1.3 x 10⁶ cells/cm² on collagen-coated 100-mm Petri dishes or at a density of 2.0 x 10⁶ cells/cm² in 16 mm multiwell dishes (Costar, Cambridge, MA) containing 24 wells/unit. On the third day of incubation, the medium was changed. Unless otherwise indicated, cells were used for experiments on culture day 4.

**Media.—**Cells were grown in a modification of medium 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 HEPES (adjusted to pH 7.4 with NaOH), 5 mM glucose, 0.001% (v/v) phenol red, and 1.8 mM CaCl₂.

**Measurement of [³H]QNB Binding to Homogenates.—**The assay presented was a modification of the method of Yamamura and Snyder (12). After three rinses with ice-cold M-199, HEPES, cells were harvested in a small volume of HEPES-buffered medium M-199. After freezing at -70°C and thawing twice, the cells were homogenized in a glass on glass homogenizer and allowed to warm to 22°C prior to assay.

The final assay mixture was 0.5 ml of HEPES-buffered M-199 containing [³H]QNB (43 Ci/mol, 1 nM in [³H]QNB unless otherwise specified), drugs as indicated in the figure legends, and 0.5 ml of homogenate. At the appropriate time (1 h at 22°C unless otherwise stated), 0.5 ml of wash medium (120 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 50 mM HEPES, 10 mM Na₂HPO₄) adjusted to pH 7.4 with NaOH at 22°C was added to terminate the incubation. The reaction mixture was passed through a Whatman glass fiber (GF/C) filter, the assay tube washed 3 times, and filters dried and assayed for radioactivity in a liquid scintillation counter with 10 ml of Omnifluor (Packard). Protein was determined by the procedure of Lowry et al. (13) after precipitation of the homogenate protein with trichloroacetic acid, using bovine serum albumin as standard.

**Measurement of [³H]HMS Binding to Intact Cells.—**Cells prepared as described in 16-mm multiwell plates were fed with fresh medium containing [³H]HMS at 37°C. Total washing time was 12 s. [³H]HMS binding was found to be constant over this wash time. Wells were rinsed rapidly 3 times with 3 ml of ice-cold wash medium and 1 ml of 0.5 N NaOH added to each well to solubilize cell proteins. Aliquots, 0.8 ml, were taken from each well, neutralized with 0.5 ml of 1 M Tris, pH 7.4, and assayed for [³H]HMS and [³H]QNB. Fifty per cent of [³H]HMS binding to intact cells was achieved at each well was incubated at 37°C for 1 h.

**Measurement of [³H]HMS Binding to Intact Cells.—**Cells were prepared as described in 16-mm multiwell plates were fed with fresh medium containing [³H]HMS and [³H]QNB. Data was corrected for nonspecific binding in the presence of 0.1 mM oxotremorine.

**Computer Analysis.—**Kinetic parameters were determined by fitting data to equations describing a given binding model using a derivative free nonlinear regression analysis (14). The equation describing the time course of binding of ligand for the model of Equation 3 as "Residuals" is

\[
\sum (B_t - B) = A e^{-k_1 t} + \left( 1 - A \right) e^{-k_2 t} + \left( 1 - A \right) e^{-k_3 t}
\]

This relation is a simplification of the general solution described by Frost and Pearson (15) in which \(B_t = \text{total ligand bound at time } t\) and \(k_1, k_2, k_3\) are the pseudo-first order rate constants for each of a number of steps in the process. The pseudo-first order rate constant \(k_1\) equals \(k_1 = k_1 + k_2 + k_3\), where \(k_1, k_2, k_3\) are the rate constants for each step in the process.
the product of the second order constant $k$, and the initial ligand concentration.

In principle, the four rate constants can be evaluated simultaneously using Equation 1. However, more reproducible estimates were obtained if $k_{r1}$ and $k_{r2}$ were first determined from dissociation data and then used as fixed values in Equation 1. The time course of dissociation of bound ligand is

$$B_0/B_3 = ae^{-k_{r1}t} + (1-a)e^{-k_{r2}t},$$

where $a = k_{r1}/(k_{r1} + k_{r2})$, $[QR]_B$, and $[QR^*_0]$ represent the concentrations of the rapidly and slowly dissociating complexes at time 0 when the dissociation measurements are started.

RESULTS

**Agonist-mediated Loss of [3H]QNB Binding Sites Following Exposure to Various Concentrations of Agonist**—We have previously demonstrated that the time course of the disappearance of muscarinic receptors during exposure of intact cultured heart cells to muscarinic agonists, measured by binding of [3H]QNB to cell homogenates, is biphasic (7). The rapid loss of 26% of sites during the first minute of agonist exposure was followed by the gradual loss of an additional 43% of the total receptor population over 3 h. The experiments described here were designed to determine whether the rapid loss of receptors involved the disappearance of a subclass of high affinity receptors while the slower loss of receptors involved the disappearance of a subclass of low affinity receptors.

The results of an experiment to determine the effect of a brief (15 min) exposure of cells to various concentrations of the agonist carbamylcholine on the binding of [3H]QNB to homogenates of these cells are shown in Fig. 1. These data demonstrate a 23% maximal decrease in [3H]QNB binding sites with a half-maximal effect at $10^{-4}$ M carbamylcholine. In the same experiment, a 3-h exposure of cells to various concentrations of agonist resulted in a maximal decrease of 70% in [3H]QNB binding to homogenates with a half-maximal effect at $1.7 \times 10^{-3}$ M carbamylcholine. The data in Fig. 1 show that at low concentrations of agonist (less than $10^{-5}$ M), the loss of high affinity binding sites was complete within 15 min and no further loss could be detected following a 3-h exposure to agonist. If the subclass of receptors lost during a 15-min exposure to agonist is subtracted from the total receptor loss at 3 h, the data further indicate that during the 2 h and 45 min following rapid loss of receptors, a second subclass of 46% of receptor sites is lost with a half-maximal effect of agonist at $3.0 \times 10^{-4}$ M carbamylcholine. Analysis of the data for the effect of a 3-h exposure to various concentrations of agonist on [3H]QNB binding (Fig. 1A, open circles) by the method of Brown and Hill (16) gave a biphasic curve with Hill coefficients at low and high concentrations of agonist of 0.59 and 0.60, respectively (Fig. 1B). The Hill coefficient for the slow loss of 46% of receptor binding sites (Fig. 1A, closed triangles) was 0.69 (Fig. 1B). These data are consistent with the view that both the time course and concentration dependence of agonist-induced loss of [3H]QNB binding sites are biphasic, involving at least two separate classes of receptor sites.

We have shown that the relative effectiveness of a muscarinic agonist in mediating a decrease in [3H]QNB binding sites was proportional both to its ability to compete with [3H]QNB for receptor binding and to the relative pharmacologic potency of that agonist (7). These findings indicated that agonist modulation of receptor number involves specific binding of agonist to muscarinic receptors. Hence, the most obvious interpretation of the data in Fig. 1 is that brief agonist exposure results in loss of high affinity receptors while prolonged agonist exposure results in loss of lower affinity receptors.

**The Effects of Agonist and Guanine Nucleotides on High Affinity Muscarinic Receptors**—More direct evidence for the presence of two subclasses of muscarinic receptors and the rapid loss of high affinity sites following brief agonist exposure was obtained from replicate measurements of the affinity of the muscarinic receptor for carbamylcholine. Affinity was determined by the ability of various concentrations of agonist to compete with [3H]QNB for binding to cell homogenates. In homogenates of cells not subjected to agonist exposure prior to homogenization, such measurements of affinity were biphasic. The shoulder seen at low concentrations of carbamylcholine (Fig. 2, closed circles) suggests the presence of a subclass of high affinity receptors that reaches a maximum value of approximately 37 fmol/mg of protein. Computer analysis of these data by nonlinear regression for a two receptor class model was consistent with a subset of high affinity receptors ($R_h$), $34 \pm 3.5$ (S.D., $n = 25$) fmol/mg of protein (26% of total receptors), with an $IC_{50}$ for carbamylcholine displacement of $3.9 \pm 1.0 \times 10^{-4}$ M; and a second subset of low affinity receptors, $95 \pm 3.6$ fmol/mg of protein.
with an IC50 of 4.5 ± 0.7 × 10^{-5} M for carbamylcholine displacement of [3H]QNB.

In order to determine whether brief exposure of intact cells to agonists resulted in loss of [3H]QNB binding to the subclass of high affinity receptors, cells were exposed for 15 min to 10^{-3} M carbamylcholine. The affinity of the receptors for carbamylcholine was then determined in homogenates of these cells (Fig. 2, open triangles). Under these conditions the shoulder seen in the control curve (Fig. 2, closed circles) could not be demonstrated and total [3H]QNB binding in cell homogenates decreased by 35 ± 3% (S.D., n = 20) fmol/mg. Each point represents the mean of three replicate determinations repeated n times; error bars represent S.E. The solid lines are drawn by eye. ○, control (n = 25); ○, control plus 10^{-4} M Gpp(NH)p (n = 20); Δ, carbamylcholine pretreatment for 15 min (n = 20); □, data derived by subtracting the subclass of high affinity receptors from the control curve; ---, extrapolation of high affinity shoulder.

Further evidence for the presence of high affinity receptors and their relationship to RL can be demonstrated by the effects of guanine nucleotides on RL. Exposure of cell homogenates to 10^{-4} M Gpp(NH)p also eliminated the shoulder seen in the control curve (Fig. 2) and gave a close fit to a curve describing a single class of low affinity receptors with IC50 of 8.6 ± 0.25 × 10^{-5} M (Fig. 2, open circles). However, exposure to Gpp(NH)p had no effect on total receptor number. This value for IC50 is similar to the IC50 of 8.2 × 10^{-5} M for these receptors remaining in homogenates of cells exposed for 15 min to 10^{-3} M carbamylcholine. Similar results were obtained when homogenates were incubated with 10^{-4} GTP (data not shown).

The shift in affinity following exposure to carbamylcholine is consistent with loss of high affinity receptors. These data indicate that both the agonist-induced rapid loss of high affinity receptors and the effect of guanine nucleotides on high affinity receptors in cultured heart cells are due to the presence of high affinity receptors. The data do not support a significant direct effect of guanine nucleotides on the affinity of RL sites. Hence, both guanine nucleotides and brief agonist exposure appear to affect only the high affinity subclass of receptors. Any direct effect of these agents on the low affinity receptor is small and of doubtful significance.

The Role of the Intact Cell in the Biphasic Response of the Muscarinic Receptor to Agonist Binding—In experiments described previously intact cells were exposed to agonist and the effect on the number of [3H]QNB binding sites and affinity of receptors for agonist were measured in homogenates of these cells (Fig. 2; Refs. 6 and 7). To determine whether the effects of both short term and more prolonged agonist exposure on receptor number required an intact cell for their expression, cell-free homogenates were exposed to muscarinic agonists. The experiment summarized in Fig. 3 demonstrates the effect of a brief (15 min) exposure of cell homogenates to various carbamylcholine concentrations, followed by removal of agonist by repeated centrifugations, on both the total binding of [3H]QNB and on receptor affinity for agonist. Under these conditions 26% of receptor sites were lost and the apparent IC50 for carbamylcholine competition with [3H]QNB binding increased from 2.0 × 10^{-5} to 7.1 × 10^{-5} M. Although the shoulder at low carbamylcholine concentrations seen in Fig. 2 is not apparent in the control curve of Fig. 3 (open circles), the shift in affinity following exposure to carbamylcholine is consistent with loss of high affinity receptors. These data indicate that both the agonist-induced rapid loss of high affinity receptors and the effect of guanine nucleotides on high affinity receptors in cultured heart cells are due to the presence of high affinity receptors. The data do not support a significant direct effect of guanine nucleotides on the affinity of RL sites. Hence, both guanine nucleotides and brief agonist exposure appear to affect only the high affinity subclass of receptors. Any direct effect of these agents on the low affinity receptor is small and of doubtful significance.
affinity receptors (Fig. 2) do not require the intact cell for their expression.

Previously published results suggest that the slow phase of agonist-induced receptor loss involves microtubule function and endocytosis of the cell surface receptor (7). If this view is correct, the cell surface and its interaction with microtubules should be intact in order to mediate the slow response to agonist exposure. When cell homogenates were incubated for 3 h with 10−3 M carbamylcholine, no further loss of [3H]QNB binding sites could be detected beyond that noted after a 15-min exposure to agonist (Fig. 3, open squares). Hence, the slow phase of receptor loss does not take place in a broken cell preparation.

Binding of Muscarinic Antagonists to Intact Cultured Heart Cells—The studies to be described next were designed to elucidate the mechanism by which prolonged exposure to agonist results in a loss of receptor sites. Because the slow phase of agonist-induced receptor loss requires an intact cell membrane and does not take place in cell homogenates (Fig. 3), studies of the effects of prolonged agonist exposure on receptor number must be carried out in intact cells. Furthermore, the physiologic effects of muscarinic agonists on the heart including changes in K+ permeability (17) and the rate and force of contraction (18) require the interaction of agonist with receptors on the surface of the intact cell. Receptor number measured in heart cell homogenates may not reflect the subset of receptors in the intact cell available for agonist binding. For these reasons, we characterized the binding of muscarinic antagonists to the intact cell. We compared the binding of [3H]M and [3H]QNB to intact cells under control conditions and following exposure to muscarinic agonists. Although at pH 7.4 both [3H]M and [3H]QNB are predominantly positively charged, we determined that [3H]QNB is markedly more hydrophobic than [3H]M. A comparison of the partition of [3H]QNB and [3H]M between an aqueous phase consisting of the wash solution described under "Experimental Procedures" for ligand binding studies (pH 7.4, physiologic ionic strength) and an organic phase consisting of ether or chloroform revealed that the ether/aqueous phase partition coefficient for [3H]QNB was 10 times greater than that for [3H]M and the chloroform:aqueous partition coefficient for [3H]QNB was 30 times greater than the corresponding values for [3H]M. Our previous data (7) having suggested that loss of receptors during prolonged agonist exposure may involve an endocytic process, we postulated that this process might involve as an initial step an agonist-induced alteration in the configuration of the receptor within the cell membrane. We considered the possibility that the more hydrophobic [3H]QNB might diffuse into or through the intact cell membrane and bind to receptors that were not available for interaction with agonist at the cell surface while the less hydrophobic [3H]M might bind only to receptors on the cell surface. Hence, differences in binding of these two antagonists to the receptor in the control state might offer a probe for the study of changes in the configuration and/or localization of receptors in the cell membrane following agonist exposure.

An experiment comparing the binding of [3H]M and [3H]QNB to intact cells after a 2-h incubation with various concentrations of either [3H]M or [3H]QNB is shown in Fig. 4. The specific binding of both ligands was saturable, and demonstrated a single apparent receptor affinity class for each antagonist ligand. Seventy per cent of [3H]M and 50% of [3H]QNB binding were specific as measured by displacement by 10−4 M oxotremorine. The mean of six determinations such as that in Fig. 4 gave a Kd of 0.47 ± 0.12 nM with a maximum receptor number of 178 ± 14 fmol/mg of protein for [3H]M and a Kd of 0.11 ± 0.02 nM with a maximum receptor number of 183 ± 13 fmol/mg of protein for [3H]QNB binding. The difference between the number of receptor sites measured by the two methods was not statistically significant, suggesting that all receptors are similarly accessible to both ligands and presumably available for agonist binding at the cell surface. The data for the binding of [3H]QNB to intact cells were quite similar to those we have reported for the binding of [3H]QNB to cell homogenates. The Kd for binding of d,l-[3H]QNB to heart homogenates was 0.21 ± 0.04 nM (19) compared to a value of 0.11 ± 0.02 nM for binding of d,l-[3H]QNB to intact cells. Since at the concentrations used here the d form of the ligand does not bind to any significant extent to the receptor, these data indicate that the Kd values for binding of [3H]QNB to intact cells and cell homogenates are approximately equal. Furthermore, maximum receptor number in homogenates was 192 fmol/mg of protein determined from Scatchard analysis of binding of [3H]QNB to homogenates (7) compared to 183 fmol/mg of protein in intact cells. Taken together, these data are consistent with the view that binding of [3H]QNB identified similar receptor populations in intact cells and cell homogenates.

The specificity of [3H]M and [3H]QNB binding to intact cells was determined by comparison of the relative pharmacologic potency of muscarinic ligands with their relative ability to compete with [3H]QNB or [3H]M binding to intact heart cells. The Kd calculated from methylscopolamine competition with binding of 1 nM [3H]QNB to intact cells was 0.22 nM (data not shown). Methylscopolamine inhibited 50% of binding of 1.0 nM [3H]M with a Hill coefficient of 1.0, and the Kd calculated by assuming a simple competitive interaction with [3H]M was 0.19 nM (Fig. 5). The agonists oxotremorine and carbamylcholine inhibited 50% of 1 nM [3H]QNB binding at 8.0 and 300 μM, respectively (data not shown), while the agonists oxotremorine and carbamylcholine inhibited 50% of binding of 1 nM [3H]M at 4.2 and 190 μM, respectively, with Hill coefficients of 0.82 and 0.70 (Fig. 5). The essentially
identical values of $K_d$ for methylscopolamine measured by competition with $[^3H]MS$ or $[^3H]QNB$ and the similarity of the relative ability of carbamylcholine and oxotremorine to compete with $[^3H]QNB$ and $[^3H]MS$ constitute further evidence that both $[^3H]MS$ and $[^3H]QNB$ are binding to the same receptor.

The concentrations at which carbamylcholine and oxotremorine inhibited 50% of binding of 1 nM $[^3H]QNB$ to intact cells are significantly higher than those reported previously for competition with 1 nM $[^3H]QNB$ in cell homogenates (6, 7). For example, in homogenates $IC_{50}$ was $3 \times 10^{-5}$ compared to $3 \times 10^{-4}$ M in intact cells. One explanation for these findings is that in intact cells, during the measurement of affinity of the receptor for agonist by competition of carbamylcholine with $^3H$-antagonist, carbamylcholine is simultaneously decreasing the affinity of the receptor for further agonist binding. Thus, apparent $IC_{50}$ might be increased compared to measurements in homogenates. Alternatively, the presence of endogenous guanine nucleotides in intact cells could mediate the conversion of $R_I$ to $R_e$ in a manner similar to the effect of exogenous guanine nucleotides on homogenates (Fig. 2, open circles).

Kinetic Analysis of $[^3H]MS$ and $[^3H]QNB$ Binding to Intact Cultured Heart Cells—Data from Fig. 4 indicate that $[^3H]QNB$ has a 4-fold higher affinity for the receptor than $[^3H]MS$. In order to determine whether these differences in affinity might reflect important differences in the kinetics of binding due to preferential binding of each ligand to a different state of the receptor, a comparison of the kinetics of binding of $[^3H]QNB$ and $[^3H]MS$ was carried out. Data presented below indicate that the binding of both ligands involves sequential formation of a low affinity agonist-receptor complex followed by the conversion of this complex to a high affinity form. Although the rate constants for formation of the low affinity complex are quite similar for $[^3H]MS$ and $[^3H]QNB$, the probability of rearrangement of the $[^3H]MS$ low affinity complex to a high affinity form is much less favorable than that for $[^3H]QNB$. These data suggest that a form of the receptor does exist which preferentially binds $[^3H]QNB$.

As shown in Fig. 6, at a concentration of 2 nM binding of each ligand proceeded without a lag and reached 50% of the equilibrium value at 4.5 min for $[^3H]MS$ and 2 min for $[^3H]QNB$, reflecting a significantly lower rate of binding for the less hydrophobic $[^3H]MS$.

Data from an experiment in which initial rates of binding are plotted according to the equation for a simple bimolecular process are shown in Fig. 7. Both $[^3H]QNB$ and $[^3H]MS$ gave bimolecular curves. The simplest mechanisms that would yield the data shown in Fig. 7 would be parallel reactions of the ligands with two binding sites having different kinetic properties or a two stage sequential reaction, e.g. binding followed by a rearrangement yielding a more stable complex. The fraction of total sites reacting in the slow phase can be approximated by extrapolating the linear portion of the plots in Fig. 7 to $t = 0$. Inspection of kinetic plots such as those in Fig. 7 indicates that the fraction of receptors reacting in the rapid phase of the reaction increases from less than 10% to greater than 50% as the concentration of QNB or methylscopolamine increases (data not shown). This finding is inconsistent with the parallel reaction mechanism, in which a constant fraction of receptors should react rapidly. It is, however, consistent with a sequential reaction mechanism of the
sort shown in Equation 3. Furthermore, we have previously demonstrated that the binding of $[^{3}H]$QNB ($Q$) can be demonstrated to proceed with the formation of a rapidly reversible complex $QR^*$ which forms quickly, reaches a maximum concentration by 2 to 3 min, and then disappears. A slowly reversible complex $QR$ appears more slowly and continues to increase until nearly 90% of total QNB bound is in a slowly reversible form (19).

The data, then, are consistent with a sequential reaction mechanism

$$Q + R \stackrel{k_1}{\rightleftharpoons} QR^* \stackrel{k_2}{\rightleftharpoons} QR$$

(3)

where $k_1$ and $k_2$ are forward rate constants for the initial and late phase of the reaction, respectively, and $k_1$ and $k_2$ are the reverse rate constants for these two phases. When ligand is present in excess the data in Fig. 7 can be represented by the sum of two exponentials, as indicated in Equation 1 under "Experimental Procedures." When $In (B_{eq} - B_i)/B_{eq}$ is plotted as a function of time (Fig. 7), the limiting slopes of the rapid and slow phases of the binding curves correspond respectively to $\lambda_1$ and $\lambda_2$ of Equation 1. $B_i$ represents total ligand bound at time $t$, the sum of the rapidly and slowly reversible complexes $QR^*$ plus $QR$. $B_{eq}$ equals the value of $B_i$ at equilibrium. The data in Fig. 7 were fit to Equation 1 by a nonlinear least squares analysis (see "Experimental Procedures") and gave the fit demonstrated by the coincidence of the experimental and computer-fitted data in Fig. 7 with $k_1$ at $37^\circ C$ for binding of $[^{3}H]$QNB of $0.21 \times 10^9 M^{-1} min^{-1}$ and a $k_2$ for binding of $[^{3}H]$MS of $0.13 \times 10^9 M^{-1} min^{-1}$. The value of $k_1$ for $[^{3}H]$QNB is in close agreement with the value of $0.19 \times 10^9 M^{-1} min^{-1}$ obtained for the binding of $[^{3}H]$QNB to cell homogenates at $23^\circ C$ (6) and confirms the very rapid nature of the initial phase of binding. The rate constant $k_1$ for binding of $[^{3}H]$MS was only slightly less than that for $[^{3}H]$QNB, indicating that initial binding of $[^{3}H]$MS and $[^{3}H]$QNB to the intact cell is quite similar. However, the forward rate constants estimated from Equation 1 for the postulated second phase of antagonist binding ($k_2$) were $0.54 min^{-1}$ for $[^{3}H]$QNB and $0.04 min^{-1}$ for $[^{3}H]$MS, consistent with a markedly slower rearrangement of the initial $[^{3}H]$MS-receptor complex during the second phase of the reaction (Table I).

The dissociation of $[^{3}H]$QNB- and $[^{3}H]$MS-receptor complexes was also biphasic; dissociation rate constants were estimated from the slopes in Fig. 8 or by fitting these data to the sum of two exponentials by a nonlinear least squares analysis (Equation 2 under "Experimental Procedures"). Table I summarizes the values of $k_1$ and $k_2$ derived from such a fit of these data. Mean values for $k_1$ were 0.373 min$^{-1}$ for $[^{3}H]$QNB and 0.366 min$^{-1}$ for $[^{3}H]$MS, indicating that the dissociation rates of both ligands from the rapidly reversible complex are essentially the same. Hence, $K_d$ values for the initial rapidly reversible reaction can be estimated as $k_2/k_1$ and are quite similar: $1.8 \times 10^{-9} M$ for $[^{3}H]$QNB and $2.8 \times 10^{-9} M$ for $[^{3}H]$MS. However, the reverse rate constants for the second, more slowly reversible phase of the reaction ($k_2$ of Equation 2) were estimated to be $0.006 min^{-1}$ and $0.045 min^{-1}$ for $[^{3}H]$QNB and $[^{3}H]$MS, respectively, a nearly 8-fold more rapid reverse reaction rate for $[^{3}H]$MS. Hence, $[^{3}H]$MS not only has a slower rate of transition for the second forward phase of the reaction than $[^{3}H]$QNB, but also a more rapid rate of reversal for this reaction, $k_2$, than $[^{3}H]$QNB (Table I).

In determining $K_d$ from $k_2/k_1$ for the second phase of the reaction only a range of values could be obtained because of the propagated errors in the parameter estimates for $k_2$. Hence, the $K_d$ estimate for $[^{3}H]$MS varied from 0.5 to 1.7 while that for $[^{3}H]$QNB varied from 0.004 to 0.023. These data are consistent with a roughly 100-fold decrease in the effectiveness with which the $[^{3}H]$MS-receptor complex is converted from $QR^*$ to $QR$ (Equation 3) compared to the analogous $[^{3}H]$QNB transition. One possible interpretation of these data is that the second phase of the antagonist binding reaction involves a rearrangement of the ligand-receptor complex within the membrane. If this were the case, the more hydrophobic properties of $[^{3}H]$QNB might facilitate its interaction with a more hydrophobic domain outside the active site of the receptor and within the surrounding membrane, and account for the markedly higher efficiency with which the $[^{3}H]$QNB-receptor complex is converted to this form.

The values of the apparent $K_d$ for the binding of $[^{3}H]$QNB and $[^{3}H]$MS derived from kinetic parameters are compared to the $K_d$ for equilibrium binding for $[^{3}H]$MS and $[^{3}H]$QNB in Table I.
[\textsuperscript{3}H]QNB in Table I. The discrepancy between the kinetically derived values of $K_d$ and those derived by equilibrium binding studies reflect the uncertainties in the determination of $k_2$ and especially of $k_{-2}$. Delineation of the slow phase of dissociation involves the measurement of a small change in the small amount of residual binding seen at late times, while the slow phase of formation of the complex involves the measurement of small increments in binding at times when binding is approaching equilibrium. Hence, both of these measurements are subject to more uncertainty than the remaining kinetic data.

**Agonist-mediated Changes in the Binding of [\textsuperscript{3}H]QNB and [\textsuperscript{3}H]MS to Intact Cells**—Previously reported data support the hypothesis that the slow phase of agonist-induced receptor loss involves endocytosis of cell surface receptors (7). If this were the case, agonist-induced receptor loss might involve an agonist-mediated configurational change in the receptor followed by movement of the receptor into or across the cell membrane. If [\textsuperscript{3}H]MS and [\textsuperscript{3}H]QNB bind with markedly different affinities to a form of the receptor altered as a result of agonist interaction, we reasoned that these two ligands might distinguish between different configurations assumed by the receptor during prolonged agonist exposures. We therefore compared the binding of [\textsuperscript{3}H]MS and [\textsuperscript{3}H]QNB to intact cells following prolonged agonist exposure. Data from an experiment in which [\textsuperscript{3}H]QNB and [\textsuperscript{3}H]MS binding were compared in cells following a 6-h exposure to various concentrations of carbamylcholine is shown in Fig. 9. No difference could be detected between the binding of the two ligands. Sixty-eight percent of radioligand binding sites were lost with a half-maximal effect at $3 \times 10^{-5} \text{M}$ carbamylcholine. A similar IC\textsubscript{50} for carbamylcholine-induced receptor loss was noted previously for cell homogenates (7).

An experiment comparing the time course of agonist-induced receptor loss in intact cells as measured by the binding of [\textsuperscript{3}H]MS or [\textsuperscript{3}H]QNB is shown in Fig. 10A. The binding of [\textsuperscript{3}H]MS to intact cells (closed circles) was unaffected by a 10- to 12-min prior exposure to muscarinic agonists. However, following this lag period nearly 80% of [\textsuperscript{3}H]MS binding sites were lost over the next 6 h of agonist exposure. Half of these receptors were lost during the first 30 min of agonist exposure following the lag phase (Fig. 10A). These binding sites recovered during a 12-h incubation in fresh medium. Recovery was inhibited by cycloheximide, 2 \mu g/ml (data not shown), and hence was dependent on synthesis of protein. The time course of agonist-mediated receptor loss as measured by the
the time course of changes of $A$, $B$, and $C$ according to classical precursor-product considerations (21):

$$\frac{dA}{dt} = k_1A$$  \hspace{1cm} (5a)\n
$$\frac{dB}{dt} = k_1A - k_0B$$  \hspace{1cm} (5b)\n
$$\frac{dC}{dt} = k_0B$$  \hspace{1cm} (5c)\n
The sequential reactions in Equation 4 should not be confused with those described in Equation 3 for the two step binding of $[^3H]QNB$ and $[^3H]MS$ to the receptor. The sequential reactions in Equation 4 apply here only to cells that are first exposed to agonist for various times before binding of $[^3H]MS$ or $[^3H]QNB$ is measured.

The predicted concentrations of $A$, $B$, and $C$ at any time may be derived from the data in Fig. 10A. Computer analysis of these data by nonlinear regression for two sequential irreversible reactions described by Equations 6a-c demonstrated that $k_1$ and $k_0$ were approximately equal. Consequently, the data were fit to a model in which $k_1$ was set equal to $k_0$. The solutions of Equations 6a-c when $k_1 = k_0$ are

$$A = e^{-kt}$$  \hspace{1cm} (6a)\n
$$B = k_tA e^{-kt}$$  \hspace{1cm} (6b)\n
where $A_0$ is the number of receptor sites at time 0.

A fit of the data in Fig. 10B to Equations 6a-c gave $A_0 = 157 \pm 4$ fmol/mg of protein ($n = 10$), $k = 0.0117 \pm 0.0006$ min$^{-1}$, and total receptor number remaining at 6 h was $30 \pm 4$ fmol/mg or about 20% of receptor sites. The equivalence of $k_1$ and $k_0$ suggests either that loss of both $[^3H]MS$ and $[^3H]QNB$ binding sites are part of a continuous time-dependent process rather than two independent processes, or that $k_1$ and $k_0$ describe the rates of two independent processes which happen to be similar in rate. In any event, these data show that after a 10- to 12-min lag period $[^3H]MS$ binding sites began to decrease (closed circles) with the conversion of sites to a form which binds only $[^3H]QNB$. Sites that bind only $[^3H]QNB$ (open circles) reach a maximum at 1 h and disappear over the next 5 h. At 30 min $[^3H]QNB$ binding sites begin to decrease slowly, reaching a minimum by 6 h (open squares). These data strongly suggest that $[^3H]MS$ and $[^3H]QNB$ binding data are capable of delineating two distinct states of the receptor.

**DISCUSSION**

The studies described here comprise two parts. First, we demonstrate the presence of two receptor subtypes and provide evidence consistent with the hypothesis that almost immediately upon agonist exposure an agonist-high affinity receptor complex is formed which renders a subclass of high affinity receptors unavailable for subsequent binding of $[^3H]QNB$. The second set of experiments comprises two parts. First, we demonstrate that $[^3H]QNB$ and $[^3H]MS$ bind to the receptor by a sequential process with formation of a rapidly reversible low affinity antagonist receptor complex followed by rearrangement of this complex to a slowly reversible form. The more hydrophobic $[^3H]QNB$ formed the high affinity complex 100-fold more readily than did $[^3H]$methylscopolamine. Hence, the receptor was capable of assuming a form which preferentially bound $[^3H]QNB$. In the second set of experiments, we compared the binding of $[^3H]MS$ and $[^3H]QNB$ to receptors which had been exposed for various times to high concentrations of muscarinic agonists. In these studies, prior exposure to agonist resulted in conversion of muscarinic receptors from a form which bound both $[^3H]MS$ and $[^3H]QNB$ to a form which bound only $[^3H]QNB$. This set of studies indicates that $[^3H]QNB$ and $[^3H]MS$ recognize different forms assumed by the receptor during agonist-induced receptor down-regulation and support the hypothesis that this process involves conversion of the receptor to an altered form which may be an intermediate in the process of receptor endocytosis. In the discussion that follows, the relationship of these immediate and late effects of agonist-receptor interaction to the ability of the cultured heart cell to respond to muscarinic stimulation will be considered.

The initial response of the muscarinic receptor population to agonist is the rapid loss of a fraction of $[^3H]QNB$ binding sites (20%) which corresponds to a subclass of high affinity receptors with an IC$_{50}$ for carbamylcholine inhibition of $[^3H]QNB$ binding of 3.9 $\times$ 10$^{-7}$ M (Fig. 2). This loss of receptor sites did not require an intact cell for its expression and could be demonstrated in homogenates from intact cells which had been exposed briefly to agonist prior to homogenization (Fig. 2) or in cell-free homogenates exposed to agonist (Fig. 3) following homogenization. Our studies indicate that the loss of sites was complete as early as 1 to 15 min following exposure of cells to concentrations of carbamylcholine less than 10$^{-6}$ M (Fig. 1). The affinity for agonist of those receptors remaining in homogenates of agonist-treated cells was similar to that for $R_0$ (Fig. 2). A plausible interpretation of these data is that
rapid receptor loss involves persistent binding of agonist to $R_h$ following brief exposure to agonist, leaving the high affinity receptor occupied and hence unavailable for $[^{3}H]-$antagonist binding. Under these conditions $R_h$ would appear to be lost, leaving only $R_l$. Alternatively, brief agonist exposure could lead to assumption of a receptor configuration inaccessible to $[^{3}H]-$antagonist.

Incubation of cell homogenates with Gpp(NH)p also caused the disappearance of $R_h$, but without a decrease in total $[^{3}H]$QNB binding sites (Fig. 2). This finding is consistent with the decrease in binding of $[^{3}H]$QNB to intact cells. The half-maximal rate of decrease for $R_h$ was reached within 30 min. However, unlike studies of cell homogenates, neither $[^{3}H]$MS nor $[^{3}H]$QNB binding to intact cells demonstrated the rapid loss of a subclass of $[^{3}H]$MS receptors preceding the lag phase. However, the assay of $[^{3}H]$MS and/or $[^{3}H]$QNB binding to the intact cell differed from that in the homogenate. Following brief exposure to agonists, cells were washed and then incubated for 1 h with $[^{3}H]-$antagonist. During this incubation cells continued to contract at 140 ± 5 (S.D., n = 20) beats/min, indicating that normal energy metabolism was maintained. We have demonstrated that exogenously added guanine nucleotides mediate the recovery of $[^{3}H]$QNB binding sites in homogenates of cells which had been briefly exposed to agonist with guanine nucleotides resulting in the reappearance of the subset of 26% of receptor sites lost during brief agonist exposure. These receptors reappeared in a low affinity form. These findings suggest that persistently bound agonist was released during the guanine nucleotide-mediated conversion of $R_h$ to $R_l$.

The data in Fig. 10 revealed that the time course of agonist-induced receptor loss measured by $[^{3}H]$MS binding in intact cells is quite similar to the time course of the slow phase of receptor loss assayed in homogenates of cells exposed to agonist prior to homogenization (7). In both intact cells and homogenates a 15-min-lag period was followed by a slow receptor loss that was half-maximal at 30 min. However, unlike studies of cell homogenates, neither $[^{3}H]$MS nor $[^{3}H]$QNB binding to intact cells demonstrated the rapid loss of a subclass of 26% of receptors preceding the lag phase. However, the assay of $[^{3}H]$MS and/or $[^{3}H]$QNB binding to the intact cell differed from that in the homogenate. Following brief exposure to agonists, cells were washed and then incubated for 1 h with $[^{3}H]-$antagonist. During this incubation cells continued to contract at 140 ± 5 (S.D., n = 20) beats/min, indicating that normal energy metabolism was maintained. We have demonstrated that exogenously added guanine nucleotides mediate the recovery of $[^{3}H]$QNB binding sites in homogenates of cells which had been exposed briefly to agonist prior to homogenization. During binding of $[^{3}H]-$antagonist to the intact cell, physiologic levels of guanine nucleotides could mediate recovery of $[^{3}H]$QNB or $[^{3}H]$MS binding sites. If the rapid loss of $[^{3}H]-$antagonist binding sites were due to persistent binding of agonists to high affinity receptors, then in the intact cell endogenous GTP might mediate the release of bound agonist. Such a mechanism might play a role in the recovery of the receptor to a state available for a subsequent cycle of agonist binding.

The presence of muscarinic receptors of high and low affinity has been demonstrated by Birdsell et al. (22) in studies of direct binding of $[^{3}H]-$agonist to synaptosomal preparations from rat cerebral cortex in which 25–30% of receptors from that source existed in a high affinity form. Ehler et al. (23, 24) have studied the binding of a recently available tritium-labeled muscarinic agonist, [cis-$[^{3}H]$]methyldioxolane, to rat brain. Although levels of nonspecific binding were quite high, their data also suggested the presence of two classes of receptors of differing affinity. Several groups have demonstrated that exposure of cell homogenates to guanine nucleotides results in a decrease in the apparent affinity of the muscarinic receptor for agonist, consistent with conversion of $R_h$ to a low affinity form (7–9, 23).

The early and late events resulting from muscarinic agonist-receptor interaction may be divided into an initial rapid formation of a complex capable of stimulating a characteristic physiologic response, followed by a slow decrease in receptor number and/or affinity of receptor for agonist that would regulate the ability of subsequent agonist exposure to elicit a physiologic response. Such a dual role of agonist has been suggested for the insulin receptor, $\beta$-adrenergic receptor, luteinizing hormone receptor, and others (1). Recent evidence presented by Stadel et al. (25) suggests that a long-lived agonist-high affinity receptor complex might be formed between the $\beta$-adrenergic agonist and receptor in turkey and frog erythrocytes membranes. They demonstrated that the addition of guanine nucleotide resulted in the dissociation of agonist from the receptor with concomitant activation of adenylate cyclase. DeLean et al. (26), using computer modeling for the binding of the $\beta$-adrenergic agonist $[^{3}H]$hydroxybenzylisoproterenol to erythrocytes, demonstrated a good correlation of the data with a model in which a ternary complex is formed among agonist, receptor, and a guanine nucleotide regulatory protein. Interaction of muscarinic agonist, high affinity receptors, and guanine nucleotides described in our studies suggests the following model, which contains interesting parallels with the scheme presented by DeLean (26) for the $\beta$-adrenergic receptor.

$$R_h + A \rightarrow R_hA + G \rightarrow (R_hNG) + A \rightarrow R_l + (NG)$$

(7)

where $N$ is the guanine nucleotide regulatory protein; $A$ is the agonist; and $G$ is a guanine nucleotide (GTP under physiological circumstances). The evidence for the occurrence of the species in parentheses is thus far indirect. In this scheme, in the absence of $G$, agonists bind persistently to $R_hA$ rendering these sites unavailable for binding of $[^{3}H]$QNB. In the presence of $G$, the complex $R_hA$ would interact with $G$ with release of agonist and regeneration of the receptor to a low affinity form which would now be available for binding of $[^{3}H]$QNB. Since a guanine nucleotide regulatory protein must be present in order for the receptor to interact with $G$ (27), the absence of any significant effect of $G$ on $R_h$ suggests that $N$ is associated with the high affinity state of the receptor. Hence, conversion of $R_h$ to $R_l$ in the presence of guanine nucleotides may be associated with the release of the guanine nucleotide regulatory protein. Conversely, regeneration of $R_h$ from $R_l$ may be associated with binding of a guanine nucleotide regulatory protein to the low affinity form of the receptor. Such an association might mediate conversion of $R_l$ to $R_h$:

$$R_l + N \rightarrow R_hN$$

(8)

Whether $G$ is capable of interacting with $R_hN$ in the absence of $A$ cannot be determined from these studies, since all assays of the effect of $G$ on affinity of receptor for agonist were carried out in the presence of agonist.

The negative inotropic effect of muscarinic agonists in canine (28) and rabbit heart (29) has been shown to be due at least in part to a GTP- and Na+-dependent inhibition of both basal and $\beta$-adrenergic agonist-stimulated adenylate cyclase activity. An agonist-high affinity receptor complex, $R_hA$, might constitute an "activated" form of the receptor. One might speculate that in the presence of $G$, such a complex could mediate an inhibition of adenylate cyclase, perhaps through release of a species such as $NG$.

The second aspect of the studies reported here deals with the mechanisms by which prolonged agonist exposure decreases the number of receptors available for labeled antagonist binding. Unlike the rapid effects of agonist on the high affinity receptor associated with receptor activation, the slower effect of prolonged agonist exposure on receptor number may represent an agonist-mediated modulation of the ability of subsequent agonist binding to elicit a physiologic response, and as such would constitute an important biological control mechanism.

Muscarinic cholinergic stimulation of the heart causes decreases in the rate and force of contraction, and these changes
are accompanied by an increase in K\(^+\) permeability (16) and a decrease in the movement of Ca\(^{2+}\) into the cell via the Ca\(^{2+}\) slow channel (18, 30). We have demonstrated that prior exposure of cultured heart cells to agonist results in the loss of the ability of muscarinic agonist to decrease beating rate and to increase K\(^+\) permeability as measured by altered efflux of \(^{42}\)K\(^+\) from the cells (20). The time course of this agonist-mediated loss of physiologic response to muscarinic agonists corresponds closely to the agonist-mediated loss of \([\text{H}]\text{MS}\) binding sites shown in Fig. 10, with a 15-min lag period followed by loss of half the physiologic response after a 30-min agonist exposure. Recently, Halvorsen and Nathanson (31) reported that agonist exposure of 6 h duration markedly decreased the ability of muscarinic agonists to mediate a decrease in beating rate in intact embryonic chick hearts 8 days in ovo. This effect was associated with the loss of high affinity receptors in heart homogenates. The close coupling between the loss of \([\text{H}]\text{MS}\) binding sites and the loss of physiologic response demonstrates that a modest change in receptor number is related to a comparable change in physiologic response. Such sensitivity of physiologic response to agonist exposure suggests that agonist-mediated receptor loss could be a sensitive mechanism for modulating the level of responsiveness of the heart to muscarinic stimuli.

Our data relating loss of muscarinic receptors during preexposure to various agonist concentrations (Fig. 1) suggested that the population of receptors lost slowly during agonist exposure corresponded to a subclass of low affinity receptors. The concentration of carbamylcholine required to mediate a half-maximal loss of receptors over 3 h (Fig. 1) was \(1.7 \times 10^{-5}\) M compared to a concentration of \(1 \times 10^{-4}\) M for a half-maximal decrease in rapidly lost receptors. Furthermore, since we have shown that brief agonist exposure results in the loss of high affinity receptors and leaves only low affinity receptors with an IC\(_50\) for carbamylcholine inhibition of \([\text{H}]\text{QNB}\) binding of \(3.0 \times 10^{-5}\) M (Fig. 2), more prolonged exposure to agonist must involve the loss of a low affinity subset of receptors. Our previous studies demonstrated that a large fraction of agonist-mediated receptor loss (46% of total receptor sites) was inhibited by agents that interfered with microtubule function (7), suggesting that disappearance of this subclass of receptors involved endocytosis. In the studies reported here, the slow loss of responsiveness of the heart to agonists was demonstrated in intact cells or in homogenates of intact cells that had been exposed to agonist prior to homogenization (Fig. 3). This was also consistent with the view that the slow phase of agonist-induced receptor loss involves endocytosis of receptors and hence requires functions which take place in the intact cell.

To pursue this issue further, we postulated that following agonist exposure, but prior to irreversible degradation of the receptor, receptors might assume an altered configuration involving a more intimate association with membrane lipids. Such a change in configuration might prevent subsequent agonist binding or interfere with the ability of agonists to mediate a physiologic response. Although a functional agonist-receptor complex might not form in cells pre-exposed to agonist, we further postulated that a muscarinic ligand which was sufficiently hydrophobic might bind to such an altered form of the receptor. Hence, comparison of the binding of radiolabeled ligands with markedly differing hydrophobicities might provide a means for studying the agonist-mediated transition in the state of the receptor.

Since \([\text{H}]\text{MS}\) contains one less phenyl group than \([\text{H}]\text{QNB}\) as well as an ether linkage, \([\text{H}]\text{MS}\) should be less capable of interacting with the lipid bilayer. The relative solubility of substances in the lipid bilayer of the cell membrane may be approximated by comparison of partition coefficients between an aqueous phase at physiologic pH and ionic strength and immiscible organic solvents such as ether and chloroform. We have demonstrated the relative preference of \([\text{H}]\text{QNB}\) over \([\text{H}]\text{MS}\) for the organic phase in terms of partition with an aqueous phase. By these criteria, one might expect \([\text{H}]\text{QNB}\) to be substantially more soluble (and permeant) in the lipid bilayer of the cell membrane than \([\text{H}]\text{MS}\).

In addition to the difference in hydrophobicity, there are significant steric differences between \([\text{H}]\text{MS}\) and \([\text{H}]\text{QNB}\). \([\text{H}]\text{QNB}\) has an extra phenyl group, while \([\text{H}]\text{MS}\) has two extra methyl groups on its bridged nitrogen (Fig. 11). We carried out two sets of studies to determine whether these differences in structure and hydrophobicity between \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) could be used to study different states of the receptor. First, we compared the kinetics of binding of these two ligands to intact cultured heart cells. Second, we compared the binding of \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) to intact cells which had been subject to prior exposure to high concentrations of agonist.

Studies comparing the kinetics of binding \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) to intact cells indicated that the binding of both ligands appears to be biphasic, with initial formation of a low affinity complex followed by a conversion to a relatively high affinity state. Burgisser et al. (32) have reported data for the binding of \((\pm)\text{[H]}\text{hydroxybenzylindolol}\) to frog erythrocyte membranes which indicate that biphasic kinetics of binding and dissociation are due to the use of a racemic mixture of the optically active radiolabeled antagonists. However, both the \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) used in these and our previous studies (7) are in the levo form. Hence, the biphasic association rates we observed for muscarinic antagonists require another explanation.

The kinetic rate constants for the initial binding step \(Q + R \rightarrow QR^*\) yield similar values of \(k_1\) and \(k_{-1}\) for \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) and hence similar dissociation constants (Table 1). Acetylcholine, the physiologic muscarinic agonist, and carbamylcholine, the agonist used in these studies, are both positively charged and contain a carbonyl moiety in ester linkage to a choline moiety (Fig. 11). Given the proper juxtaposition, both the nitrogen of the choline moiety and the carbonyl appear to be necessary for agonist activity (33). The similarity of the relative positions of the carbonyl and nitrogen in \([\text{H}]\text{QNB}\) and \([\text{H}]\text{MS}\) might explain the similarity in their relative
ability to recognize the active site of the receptor and hence to form the initial complex \( QR^* \). However, our kinetic data (Table I) indicate that the \([\text{H}]\text{QNB}\)-receptor complex \( QR^* \) initially formed undergoes transition to the state designated \( QR \) in Equation 3 at a substantially more rapid rate than is the case for \([\text{H}]\text{MS}\). At the same time, the back reaction from \( QR \) to \( QR^* \) is much slower for \([\text{H}]\text{QNB} \) than for \([\text{H}]\text{MS}, resulting in a \( k_2/\alpha \) ratio roughly 100-fold more favorable to \( QR \) formation for \([\text{H}]\text{QNB} \). If the steric requirements for formations of \( QR \) favored a less bulky ligand, then \([\text{H}]\text{MS} \) might bind more readily. Since this is not the case, our tentative interpretation is that differences in hydrophobicity between the two antagonist radioligands are responsible for the differing kinetics of \([\text{H}]\text{MS} \) and \([\text{H}]\text{QNB} \) binding to intact cells.

Having established that a form of the receptor existed which could bind \([\text{H}]\text{QNB} \) more readily than \([\text{H}]\text{MS} \), we compared the binding of \([\text{H}]\text{MS} \) and \([\text{H}]\text{QNB} \) to cells which had been subject to prior exposure to high concentrations of agonists for various times. Prolonged exposure to agonist mediated conversion of the receptor from a form (designated \( A \), Equation 4) which binds \([\text{H}]\text{MS} \) and \([\text{H}]\text{QNB} \) to a form (designated \( B \), Equation 4) which binds \([\text{H}]\text{QNB} \) but which does not bind \([\text{H}]\text{MS} \), suggesting that the configuration of the receptor and/or relationship of the receptor to the plasmalemma is altered. We observed a close temporal correlation between loss of \([\text{H}]\text{MS} \) binding sites in intact cells and loss of physiologic response to agonist (20). This finding is consistent with the hypothesis that accessibility of the receptor in the intact cell to \([\text{H}]\text{MS} \) is correlated with accessibility of the receptor to agonist. Alternatively, \([\text{H}]\text{IM} \) may bind preferentially to a functioning state of the receptor. Hence, the receptors in form \( B \) do not appear to mediate a physiologic response (20). More prolonged agonist exposure resulted in conversion to form \( C \), presumably a degraded form of the receptor that binds neither \([\text{H}]\text{MS} \) nor \([\text{H}]\text{QNB} \). Hence, form \( B \) of the receptor may constitute an intermediate state of the receptor formed during the process of endocytosis. It should be noted that the experiments described cannot distinguish between differences in \([\text{H}]\text{QNB} \) and \([\text{H}]\text{MS} \) binding to an altered configuration of the receptor within the membrane (i.e. plasmalemma) and differences in access to receptor sites that have been endocytosed but not yet degraded.

It is intriguing to speculate that the agonist-mediated change in the receptor described as \( A \rightarrow B \) (Equation 4) from a form \( A \) which binds both \([\text{H}]\text{QNB} \) and \([\text{H}]\text{MS} \) to a form \( B \) which binds only \([\text{H}]\text{QNB} \) might represent a transition similar to that described in the process \( QR^* \rightarrow \frac{k_1}{k_2} QR \) (Equation 3) in which both \([\text{H}]\text{QNB} \) and \([\text{H}]\text{MS} \) bind with equal affinity to the complex \( QR^* \), but the transition to the rearranged form of the receptor-antagonist complex \( QR \) is markedly more favorable for \([\text{H}]\text{QNB} \). Both the transition designated \( A \rightarrow B \) and \( QR^* \rightarrow QR \) involve rearrangement to a state which binds \([\text{H}]\text{MS} \) less well. Both could represent rearrangement to a more hydrophobic domain and both transitions are induced by ligand binding. Although endocytosis of receptors will only take place in the intact cell, it has not been determined whether the conversion of \( A \rightarrow B \) requires an intact cell. Our previous studies with cell homogenates (6) and recent work with a solubilized form of the receptor (34) have demonstrated that the transition \( QR^* \rightarrow QR \) does take place in a cell-free system.

Because in most receptor studies the binding of antagonists is only very slowly reversible, the effect of prior exposure to antagonists on subsequent antagonist binding has not been determined. Hence, it is not known whether antagonist binding can modulate receptors number in a manner similar to the effect of agonist exposure. It seems possible that the second step in ligand binding could constitute an antagonist-mediated rearrangement similar to the change in the state of the receptor that occurs during prolonged agonist exposure. Hence, one might speculate further that exposure to any specific ligand, antagonist or agonist, is capable of mediating a change in receptor conformation and that the state \( QR \) in Equation 3 is similar to the state \( B \) in Equation 4.

In conclusion, we have used radioligand binding techniques to demonstrate a response of the muscarinic receptor to occupancy by agonist that is biphasic with respect to both time and agonist concentration. Subsets of receptors were further delineated by kinetic analysis of binding of \([\text{H}]\text{QNB} \) and \([\text{H}]\text{MS} \) to intact, beating heart cells. Our findings provide further evidence for a dual role of the muscarinic agonist-receptor interaction as mediator of immediate cellular biochemical and physiological responses and also as modulator of responsiveness of the cell to subsequent or continued agonist exposure.

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