In Vivo Regulation of Muscarinic Acetylcholine Receptor Number and Function in Embryonic Chick Heart*

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We have used the chick embryonic heart to study the regulation of the muscarinic acetylcholine receptor in vivo. Sustained activation of the muscarinic receptor in vivo with the cholinergic agonist carbachol decreases muscarinic receptor number as much as 87% as measured by the specific binding of the potent muscarinic receptor antagonist \([\text{H}]\text{quinuclidinyl benzilate}\). The decrease in receptor number is both dose and time dependent. After carbachol-induced down regulation, the receptor number recovers to control levels when further receptor activation is blocked by the muscarinic receptor antagonist atropine. When receptor number is decreased 50% in vivo, isolated atria require a 12-fold greater concentration of carbachol than controls to arrest spontaneous beating in an organ bath. Analysis of the binding of carbachol to the muscarinic receptor indicates that this shift in the dose response of atria is accompanied by a change in the relative fraction of the high and low affinity forms of the muscarinic receptor with no change in their respective affinities for carbachol. In addition, this analysis suggests that the low agonist affinity form of the cardiac muscarinic receptor is the physiologically active form.

The parasympathetic nervous system mediates its effects on the heart primarily through the release of the neurotransmitter acetylcholine which, upon binding to muscarinic ACh receptors in the heart, elicits a negative chronotropic effect. Muscarinic ACh receptors are also present in smooth muscle, the central nervous system, in autonomic ganglia, and some exocrine glands. Investigation of the mACh receptor has been greatly facilitated by the development of the potent mACh receptor antagonist \([\text{H}]\text{quinuclidinyl benzilate}\) which binds specifically and with high affinity to the mACh receptor (1). \([\text{H}]\text{QNB}\) can be used to study the interaction of agonists and antagonists with the receptor and, thus, quantitate the number of receptors present in cells and tissues.

Persistent exposure of neuronal and cardiac muscle cells in culture to muscarinic agents leads to a decrease in the number of mACh receptors on these cells (2, 3). After prolonged exposure to cholinergic agonists, the response of these cells in culture to a subsequent exposure to cholinergic agonists is reduced. Nathanson et al. (4) showed that the adenylate cyclase of neuronal cells exhibited a decrease sensitivity to the stable ACh analog carbamylcholine (carbachol) following agonist-induced down regulation of mACh receptors. Cardiac muscle cells in culture exhibit a decreased chronotropic response to carbachol following down regulation of the mACh receptor (3). Taylor et al. (5) have shown that the long term down regulation of mACh receptor number in neuronal cells is distinct from short term desensitization of receptor activity.

This agonist-induced down regulation of receptor number on cells in culture has been postulated to represent a model for the regulation of receptors by synaptic activity in vivo. In a recent study, Gazit et al. (6) reported that chronic administration of sublethal doses of organophosphate anticholinesterase compounds in vivo caused up to a 57% decrease in the total binding of \([\text{H}]\text{QNB}\) in rat brain homogenates. This apparent decrease in mACh receptor number may be the result of the anticholinesterase increasing the amount of endogenous ACh available for muscarinic receptor activation. In a preliminary report, Wise et al. (7) have presented data suggesting that mACh receptors in rat heart may also be regulated in vivo.

In this study, we use the developing chick embryonic heart to study the regulation of mACh receptors in vivo. We show here that sustained agonist activation of the mACh receptor in vivo causes up to an 87% decrease in the number of mACh receptors in the heart in a dose- and time-dependent manner. The mACh receptor-mediated negative chronotropic response of isolated sinoatrial preparations is reduced after prolonged exposure to cholinergic agonists in vivo. Analysis of the binding of carbachol to the mACh receptor by competition experiments with \([\text{H}]\text{QNB}\) is consistent with the presence of two populations of mACh receptors that have different affinities for muscarinic agonists but the same affinity for muscarinic antagonists. Down regulation of receptor number causes a change in the relative fraction of these high and low affinity forms of the mACh receptor but no change in their respective affinities for carbachol.

EXPERIMENTAL PROCEDURES

Materials—White Leghorn chick embryos were obtained locally from College Biologicals (Bothell, WA) and maintained at 38°C in a humidified incubator. Embryonic ages correspond to those described by Hamburger and Hamilton (8). The embryos were 8 days old at the beginning of all experiments unless noted.

Atropine sulfate, carbamylcholine chloride (carbachol), guanyl-5'-yldimidophosphate; mACh, muscarinic acetylcholine; PBS, phosphate-buffered saline; QNB, quinuclidinyl benzilate; \(R_0\), mACh receptor with high affinity for agonists; \(K_D\), mACh receptor with low affinity for agonists; \(F_{\text{Hi}}\), fraction of \(R_0\); \(F_{\text{L}}\), fraction of \(R_L\); \(K_D\), dissociation constant of agonist for \(R_0\); \(K_L\), dissociation constant of agonist for \(R_L\).

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Atropine sulfate, carbamylcholine chloride (carbachol), GPP(NH)P, scopolamine HCl, and d-tubocurarine were obtained from Sigma. Oxotremorine was from Aldrich and \([\text{H}]\text{quinuclidinyl benzilate}\) (specific activity, 30 Ci/mmol) was obtained from Amer-sham. All other chemicals were of reagent grade.

Administration of Drugs in Ovo—Drugs for administration in ovo

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were dissolved in phosphate-buffered saline (0.9% NaCl, 0.02% KCl, 0.12% NaH₂PO₄, 0.02% KClO₄, 0.01% MgCl₂, and 0.01% CaCl₂, w/v, pH 7.4). Administration was accomplished by making a small “window” (approximately 3 mm across) in the shell so as to not disturb the inner embryonic membranes. Drugs were then carefully layered onto this inner membrane in a volume of 100 µl or less. The window was then sealed with adhesive tape and the egg was returned to the incubator for the appropriate treatment time. Embryos treated with less than 1 µmol of carbachol by this method exhibited no increased mortality. Embryos treated with 1 and 10 µmol of carbachol had a survival rate of 75 and 25%, respectively. Only hearts from embryos alive at the time of harvest were used for these studies.

**Tissue Preparation**—Beating hearts were removed from the embryos, the great vessels removed, and residual blood rinsed away in cold phosphate buffer (50 mM NaH₂PO₄, pH 7.4). Hearts were then homogenized in fresh ice-cold phosphate buffer by hand with 15–20 strokes of a glass homogenizer. Membranes were centrifuged at 6500 × g in the cold for 10 min and washed free of drugs by 2 more successive rinses and centrifugations in cold phosphate buffer. Control experiments demonstrated that this washing procedure was sufficient to yield no inhibition of [³H]QNB binding and, thus, removed carbachol and atropine from the membranes. The membranes were then resuspended in cold phosphate buffer of the beating rate media (described below) for carbachol-[³H]QNB binding competition studies. The final protein concentration was 0.12–0.64 mg/ml. Membranes were then allowed to warm to room temperature and immediately assayed in duplicate.

**Binding Assays**—The assay procedure was a modification of the method of Yamamura and Snyder (1) and utilized the tight and specific binding of the muscarinic receptor antagonist [³H]QNB. The final assay mixture contained 150 µl of homogenate and, unless noted, 0.67 nM [³H]QNB in a final volume of 1 ml with 50 mM NaH₂PO₄, pH 7.4. The incubation time was 90 min at room temperature. The reaction was stopped with the addition of 5 ml of ice-cold phosphate buffer and each tube placed on ice. Each assay solution was then passed through a Whatman glass fiber filter (2.5 cm  G/F, C, presoaked in a 0.1% solution of bovine serum albumin in buffer) over a vacuum and washed 3 times with 5 ml of the same buffer. Filters were then placed in scintillation vials to which 4 ml of scintillation fluid (25% Triton X-100, 0.888% ethanol, 0.088% (distilled water, v/v), and 0.4% Omnifluor, w/v, in xylene) was added and counted in a liquid scintillation counter with an efficiency of 30%. Specific binding was determined as the amount of binding inhibited by 10⁻⁸ M atropine and was 90–95% of total binding. Unless noted, 2 hearts were pooled per homogenate and assayed in duplicate. Each data point is the average of 2 such homogenates which varied less than 10% from the average.

Protein was estimated by a modification of the procedure of Lowry et al. (9) after solubilization with sodium deoxycholate and trichloroacetic acid precipitation using bovine serum albumin as a standard. The density of mACh receptors was expressed as femtomoles of [³H]QNB specifically bound per mg of membrane protein. For the carbachol-[³H]QNB competition binding studies the [³H]QNB concentration was 0.32–0.34 nM (approximately 85–90% saturated) and the assay was incubated for 60 min. The atrial beating rate media without glucose (see below) was used in place of the phosphate buffer in all solutions and homogenates used in the competition binding assays. For these experiments, 25–35 atria of each test group were homogenized in 5–6 ml of media. In control experiments, 10⁻⁲ M GPP(NH)p with 1.0 mM MgSO₄ gave maximal inhibition of carbachol binding to heart membranes.

**Atrial Beating Rate Studies**—Intact beating hearts were removed from embryos and placed in the beating rate media (149 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgSO₄, 10.0 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 5.5 mM glucose, and 0.4 mM NaH₂PO₄, pH 7.4), aerated with 100% oxygen and maintained at 37 °C. Atria were separated from ventricles under a dissecting microscope (see Refs. 10 and 11), and the atria were pined onto a Sylgard-coated Petri dish which contained oxygenated media. Four such atria were placed in vials to which 4 ml of scintillation fluid (25% Triton X-100, 0.888% ethanol, 0.088% (distilled water, v/v), and 0.4% Omnifluor, w/v, in xylene) was added and counted in a liquid scintillation counter with an efficiency of 30%. Specific binding was determined as the amount of binding inhibited by 10⁻⁸ M atropine and was 90–95% of total binding. Unless noted, 2 hearts were pooled per homogenate and assayed in duplicate. Each data point is the average of 2 such homogenates which varied less than 10% from the average.

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**Analysis of Carbachol-[³H]QNB Competition Experiments**—The competition data were analyzed using a two-site model (12) for agonist binding as discussed in the text using the following relationships as developed by Schmickler and Searles (13). The total concentration of QNB binding sites is [R₂], and

\[ [R]_T = [R]_I + [R]_C \]  

where [R]_T is the concentration of high affinity agonist binding sites and [R]_I is the concentration of low affinity agonist binding sites. Because [R]_H and [R]_C have the same affinity for antagonists, for QNB,

\[ K_D = \frac{[R]_D[Q]}{[R]_D[Q] + [R]_C} \]  

and similarly for R₁:

\[ \frac{[R]_D[Q]}{[R]_D[Q] + [R]_C} = \frac{[R]_D[Q]}{[R]_D[Q] + [R]_C} \]

The fractional saturation of R₁ by [³H]QNB is:

\[ y = \frac{[R]_T[Q]}{[R]_T[Q]} = \frac{[R]_T[Q]}{[R]_T[Q]} + \frac{[R]_T[Q]}{[R]_T[Q]} \]

\[ = \frac{[Q]}{K_D} + \frac{F_T}{K_D} \]

where [Q] = [R]_D[Q]/[R]_D and F_T = [R]_T/[R]_T. The total binding of QNB to R₂ in the absence of carbachol is Y₀:

\[ Y_0 = [Q] + [Q] \]

K_D is derived from the experimental data presented in Fig. 2 and the per cent inhibition of [³H]QNB binding was experimentally determined at each concentration of carbachol. Per cent inhibition of [³H]QNB binding is:

\[ \% \text{ inhibition} = \frac{1 - (Y/Y_0)}{X} \times 100 \]

Using an iterative method, the parameters F_T, F_C, K_H, and K_C were varied until the best fit to the experimental data was obtained. The
criteria for the best fit was the minimum deviation \( (D) \) defined as follows:

\[
D = \sum_{i=1}^{n} \left[ (\text{observed inhibition at } (C_i)) - (\text{calculated inhibition at } (C_i)) \right]^2
\]

A 10% increase in \( D \) was permitted to determine the allowed variation in the parameters.

RESULTS

Time Course of Carbachol-induced Decrease in [\text{\textsuperscript{3}H}]QNB Binding—Administration in \textit{ovo} of 1 \text{ \textmu mol} of the stable muscarinic receptor agonist carbachol to 8-day-old chick embryos led to a time-dependent decrease in bound [\text{\textsuperscript{3}H}]QNB as compared to control (Fig. 1). The maximum effect was a 61% decrease which was achieved between 6 and 10 h. In the same experiment, administration of 0.33 \text{ \textmu mol} of carbachol lead to a 28% decrease in [\text{\textsuperscript{3}H}]QNB binding over a similar time course. Since both time courses have reached a steady state by 6–10 h, this increased response to the higher (1 \text{ \textmu mol}) dose is not a faster time course, but represents a greater maximum effect on [\text{\textsuperscript{3}H}]QNB binding to the mACh receptor. This decreased [\text{\textsuperscript{3}H}]QNB binding persists for at least 34 h (Fig. 5).

Kinetic analysis (14) of the rate of turnover of the receptor with several different doses of carbachol (0.33, 1.0, or 10.0 \text{ \textmu mol}) reveals no difference in the rate constant for the disappearance of activated receptor \((k_{1,2} = 1.82–1.86 \text{ h}^{-1}; \text{analysis not shown})\. If the appearance of receptor is a zero order process and the disappearance of activated receptor is a first order process, then the difference in final receptor number after different in \textit{ovo} carbachol doses is a reflection only of the different number of receptors activated by 0.33, 1.0, or 10.0 \text{ \textmu mol} of carbachol.

Saturation Analysis of [\text{\textsuperscript{3}H}]QNB Binding—The decreased binding of [\text{\textsuperscript{3}H}]QNB in membranes from carbachol-treated hearts could be due to either a change in affinity of the mACh receptor for [\text{\textsuperscript{3}H}]QNB or a decrease in the total receptor number. To differentiate between these two possibilities, the dependence of binding on [\text{\textsuperscript{3}H}]QNB concentration was studied. Embryos were pretreated in \textit{ovo} with PBS as a control or 1 \text{ \textmu mol} of carbachol for 6 h. Specific binding to heart membranes was saturable for both groups and the carbachol-treated hearts yielded a decrease in total bound [\text{\textsuperscript{3}H}]QNB (Fig. 2). Scatchard analysis of the binding data indicates no significant change in the apparent affinity of the mACh receptor for [\text{\textsuperscript{3}H}]QNB \((K_D = 6.4 \times 10^{-11} \text{ M} \text{ for control and } 5.3 \times 10^{-11} \text{ M for carbachol treated})\) and a 58% decrease in the number of specific [\text{\textsuperscript{3}H}]QNB binding sites from 660 fmol/mg of membrane protein in controls to 280 fmol/mg in the carbachol-treated hearts (Fig. 2, \textit{inset})\. Thus, the decreased binding of [\text{\textsuperscript{3}H}]QNB to heart membranes after \textit{in ovo} treatment with carbachol reflects a true decrease in mACh receptor number.

Pharmacological Specificity—To determine whether this carbachol-induced down regulation is mediated by the mACh receptor, we investigated the dependence of down regulation on carbachol concentration. Embryos were treated \textit{in ovo} with increasing concentrations of carbachol. Heart membranes prepared from these embryos exhibited a dose-dependent decrease in mACh receptor number (Fig. 3). The maximum dose used (10 \text{ \textmu mol}) led to an 87% decrease in the number of mACh receptors. The \text{ED}_{50} for down regulation was 0.5 \text{ \textmu mol}. If the carbachol were assumed to be uniformly distributed throughout the volume of the egg (approximately 50 ml), this would correspond to an \text{EC}_{50} of \(10^{-5}\) M. This carbachol concentration is within the pharmacologically active range for activation of the mACh receptor in the 8-day-old embryonic chick heart (Ref. 10 and Fig. 6).

To examine the specificity of the carbachol-induced decrease in mACh receptor number, we tested another cholinergic agonist for its ability to promote down regulation. The highly specific muscarinic ACh receptor agonist, oxotremorine, caused a 35% decrease in mACh receptor number (Table I, Experiment A). The down regulation was completely blocked by concomitant administration of the muscarinic receptor antagonist atropine (Table I, Experiment A).

To test the specificity of the antagonism of cholinergic agonists we examined a number of other ACh receptor antagonists for their ability to prevent down regulation. Embryos were treated \textit{in ovo} with 1 \text{ \textmu mol} of carbachol and one of several cholinergic antagonists. The specific muscarinic receptor antagonists scopolamine and atropine were able to block 100 and 93% of the down regulation induced by carbachol, respectively (Table I, Experiment B). The ACh receptor at the neuromuscular junction is effectively blocked by the antagonist d-tubocurarine. The 48% decrease in mACh receptor number induced by carbachol was not inhibited by d-tubocurarine (Table I, Experiment B). At the nicotinic ACh recep-

![Fig. 1. Time-course of the carbachol-induced decrease in [\text{\textsuperscript{3}H}]QNB binding. Embryos were injected \textit{in ovo} with PBS (C); carbachol, 0.33 \text{ \textmu mol} (A); or carbachol 1 \text{ \textmu mol} (O). At the indicated times, hearts were harvested and assayed for specific binding of [\text{\textsuperscript{3}H}]QNB as described under ”Experimental Procedures.” Each point represents the average of 2 different membrane preparations with 2 hearts pooled per preparation.](image-url)
carbachol for 6 h and then blocked further carbachol-mACh receptor interaction with saturating doses of atropine. The administration of atropine with no prior exposure to carbachol gives no change in receptor number over a 28-h period (Fig. 5A). If hearts are pretreated with carbachol for 6 h, a subsequent blockade of mACh receptors by atropine causes an immediate block of further down regulation. Complete recovery of receptor number to control levels occurs between 6 and 16 h after atropine administration. A subsequent experiment shows this time period in more detail and indicates that recovery is continuous over this 10-h period (Fig. 5B). Studies on cells in culture have shown that the kinetics of agonist-induced down regulation were consistent with an increase in receptor breakdown and that recovery was dependent on synthesis of new protein (2, 17). The long recovery time observed in our in vitro experiments is consistent with recovery being dependent on de novo synthesis of protein.

**Effect of Treatment on Atrial Beating Rate**—To test the physiological significance of the carbachol-induced decrease in mACh receptor number, we examined the negative chronotropic response of atria to applied carbachol in control and down regulated isolated sinoatrial preparations. Embryos were pretreated in ovo with 1 nmol of carbachol for at least 6 h and the sinoatrial tissue was dissected out as described under "Experimental Procedures." The ventricular muscle of the 8-day-old chick embryo does not respond directly to

tor in autonomic ganglia hexamethonium is an effective antagonist (15). At a dose 10 times that of atropine or scopolamine, hexamethonium was not able to block any of the carbachol-induced decrease in mACh receptor number (Table I, Experiment B). These results indicate that carbachol is specifically acting at the mACh receptor to cause a decrease in receptor number.

The antagonism by atropine was further investigated by treating a series of embryos with 1 μmol of carbachol and increasing doses of atropine. Antagonism of the decrease in receptor number was dose dependent with an IDso of 0.5 nmol (Fig. 4). This ratio of 0.5 nmol of atropine to 1 μmol of carbachol (1:2000) is consistent with the relative affinities of the mACh receptor for atropine and carbachol (16). These data are in agreement with earlier reports from cell culture experiments (2, 4) that activation of the mACh receptor by an agonist is required for down regulation to occur and that occupancy of the receptor by an antagonist is insufficient to cause down regulation.

**Reversal of Down Regulation**—In order to test whether the carbachol-induced decrease in mACh receptor number was reversible, we treated embryos in ovo with 1 μmol of

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of receptors</th>
<th>% inhibition of receptor loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>1010</td>
<td>52</td>
</tr>
<tr>
<td>Carbachol (1 μmol)</td>
<td>530</td>
<td>65</td>
</tr>
<tr>
<td>Oxotremorine (0.1 μmol)</td>
<td>660</td>
<td>85</td>
</tr>
<tr>
<td>Oxotremorine (0.1 μmol) plus atropine (0.1 μmol)</td>
<td>1010</td>
<td>100</td>
</tr>
</tbody>
</table>

*Groups of 4 embryos were treated in ovo for 6.5 h with each of the drug combinations listed below. Hearts were harvested and assayed as described under "Experimental Procedures." for specific [3H]QNB binding.

![Graph 1](image1.png)

**FIG. 3** (left). Effect of carbachol dose on mACh receptor number. Groups of four embryos were treated in ovo for 6 h with the indicated doses of carbachol (●) or PBS (○) in a volume of 100 μl in ovo. After 6 h the hearts were harvested and assayed for specific [3H]QNB binding as described under "Experimental Procedures."

**FIG. 4** (right). Effect of atropine dose on carbachol-induced decrease in mACh receptor number. Groups of four embryos were treated in ovo for 6 h with 1 μmol of carbachol plus the indicated amount of atropine (●). Control points are carbachol plus PBS (○), PBS plus atropine 10^-6 mol (×), and PBS alone (△). Two hearts were pooled per membrane preparation and assayed for specific [3H]QNB binding as described under "Experimental Procedures."
Down Regulation of Muscarinic Acetylcholine Receptor

A muscarinic agonist will elicit a negative chronotropic response in the ventricle only in the presence of β-adrenergic stimulation (18). We therefore determined the beating rate of isolated atria rather than whole hearts in order to avoid the possibility of ventricular arrhythmias which could interfere with the normal sinus rhythm.

A representative dose-response curve for the inhibition of spontaneous atrial beating by carbachol is shown in Fig. 6. The concentration range of carbachol required to elicit minimal and complete responses is very narrow. For untreated embryos, 10^{-6} M carbachol causes a 14% inhibition of beating; 3 \times 10^{-6} M, a 28% inhibition; and 5 \times 10^{-6} M, a 100% inhibition. The dose-response curve is shifted approximately 10-fold to the right after treatment with carbachol in ovo. This anomalous dose-response curve of atria to cholinergic agonists at this stage of embryonic development has been previously documented by Pappano and co-workers (10,11). Because the response more closely resembles an all-or-none phenomenon than a classical dose-response curve, we measured the concentration of carbachol required to completely inhibit atrial beating (the EC_{50}) in order to obtain a more precise and useful comparison of the response of control and treated embryos to cholinergic agonists. The accumulated EC_{50} data from these experiments in control and down regulated atria are shown in a frequency distribution plot in Fig. 7. The distributions for both control and down regulated atria are restricted to a narrow carbachol concentration range. The mean EC_{50} for treated atrias having a 52% decrease in mACh receptor number was 3.6 (±0.2) \times 10^{-5} M. This value was 12 times greater than the EC_{50} for control atria (3.0 (±0.2) \times 10^{-6} M).

A small population of treated atria appeared to form a separate subpopulation distinct from the main group (Fig. 7). They either did not respond to carbachol (4 atria) or required 1 mM carbachol (3 atria) to maximally inhibit beating. These
agonists to the \( \beta \)-adrenergic receptor on intact S49 cells resemble than expected from a simple mass action isotherm. In extensive cholinergic agonists. In membranes of untreated atria, mass action is due to a heterogeneity in the mACh receptor in the presence rather than the absence of guanyl nucleotides. Since we would expect endogenous GTP to be present in the observed 12-fold shift in the dose response of treated atria to the same medium as was used for the beating experiments, GPP(NH)P increases the IC\(_{50}\) for carbachol about 10-fold (Fig. 8). In control atria, GPP(NH)P decreased the affinity of both the high and low affinity receptors for carbachol but did not significantly alter the relative proportion of high and low affinity sites (Table II). This is consistent with data from similar competition binding experiments with rat cardiac homogenates in which GPP(NH)P increased the IC\(_{50}\) for carbachol inhibition of \(^{[3]H}\)QNB binding up to 10-fold with no change in the heterogeneity of the binding sites as evidenced by no change in the Hill coefficient (26, 22). This effect of GPP(NH)P on the binding of agonists to the mACh receptor is different from its effect on the binding of agonists to both the \( \alpha \)- and \( \beta \)-adrenergic receptors, where GPP(NH)P causes a conversion of the high affinity agonist binding sites to low affinity sites without altering \( K_a \) (23, 24).

\( K_H \) and \( K_L \) are not changed significantly when the receptor number is decreased 48\% (Table II). In the absence of GPP(NH)P, \( F_H \) and \( F_L \) are not changed significantly (0.35–0.28). When the competition experiments are performed in the presence of GPP(NH)P, however, there was a significant decrease in the fraction of higher affinity sites, from 0.40 in the presence of guanyl nucleotides such as GPP(NH)P and GTP lower the apparent affinity of the mACh receptor for cholinergic agonists. In membranes of untreated atria, GPP(NH)P increases the IC\(_{50}\) for carbachol about 10-fold (Fig. 8). In control atria, GPP(NH)P decreased the affinity of both the high and low affinity receptors for carbachol but did not significantly alter the relative proportion of high and low affinity sites (Table II). This is consistent with data from similar competition binding experiments with rat cardiac homogenates in which GPP(NH)P increased the IC\(_{50}\) for carbachol inhibition of \(^{[3]H}\)QNB binding up to 10-fold with no change in the heterogeneity of the binding sites as evidenced by no change in the Hill coefficient (26, 22). This effect of GPP(NH)P on the binding of agonists to the mACh receptor is different from its effect on the binding of agonists to both the \( \alpha \)- and \( \beta \)-adrenergic receptors, where GPP(NH)P causes a conversion of the high affinity agonist binding sites to low affinity sites without altering \( K_a \) (23, 24).

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The competition curves from control atria with and without GPP(NH)P are shown in Fig. 8. The binding curves are flatter than expected from a simple mass action isotherm. In extensive studies of the mACh receptor from rat cerebral cortex, Birdasall et al. (12) has shown that this deviation from simple mass action is due to a heterogeneity in the mACh receptor population which is most readily explained by the presence of at least two binding sites differing in affinity for agonists but not for antagonists. The competition curve for treated atria also exhibits this apparent heterogeneity in binding sites and a 17-fold shift of the IC\(_{50}\) in the presence of GPP(NH)P (Fig. 8).

In the presence of GPP(NH)P the data from the carbachol competition experiments from control atria give a Hill coefficient of 0.52 (not shown). The Hill coefficient from treated atria is closer to 1 (0.65) (not shown), suggesting the possibility of a decrease in the heterogeneity of the mACh receptor population. The lack of competition at low carbachol concentrations in the presence of GPP(NH)P further suggests a loss of high affinity binding sites in treated atria.

It is also evident from a comparison of the plus GPP(NH)P curves from Fig. 8 that the IC\(_{50}\) for carbachol is shifted from 8 \( \times 10^{-10} \) M in control atrial membranes to 32 \( \times 10^{-10} \) M in treated membranes. This represents a 4-fold decrease in the apparent affinity of the mACh receptor population for carbachol. Using the curve-fitting procedure described under “Experimental Procedures” for a two-site model for agonist binding to the mACh receptor, we investigated the possibility that the small 4-fold increase in the IC\(_{50}\) for carbachol seen in treated atria might be due to a preferential loss of the high affinity form of the mACh receptor rather than a decrease in the true affinity of the mACh receptor. From the observed data in control and treated atria (Fig. 8, A and B), we calculated the fraction of high and low affinity binding sites (\( F_H \), \( F_L \)) and the affinity of these sites for carbachol (\( K_H \), \( K_L \)).

In control atria, GPP(NH)P decreased the affinity of both the high and low affinity receptors for carbachol but did not significantly alter the relative proportion of high and low affinity sites (Table II). This is consistent with data from similar competition binding experiments with rat cardiac homogenates in which GPP(NH)P increased the IC\(_{50}\) for carbachol inhibition of \(^{[3]H}\)QNB binding up to 10-fold with no change in the heterogeneity of the binding sites as evidenced by no change in the Hill coefficient (26, 22). This effect of GPP(NH)P on the binding of agonists to the mACh receptor is different from its effect on the binding of agonists to both the \( \alpha \)- and \( \beta \)-adrenergic receptors, where GPP(NH)P causes a conversion of the high affinity agonist binding sites to low affinity sites without altering \( K_a \) (23, 24).

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### Table III

<table>
<thead>
<tr>
<th>Occupancy of high and low affinity binding sites at EC(_{50}) for negative chronotropic response to added carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupancies are calculated from parameters in Table II. The mean total receptor concentration for control atria is 580 fmol/mg of protein and for treated atria, 300 fmol/mg of protein. The fraction occupied is EC(<em>{50})/(( K_D + EC</em>{50} )). The EC(_{50}) for carbachol was 3.0 ( \times 10^{-5} ) M in control atria and 3.6 ( \times 10^{-5} ) M in treated atria.</td>
</tr>
</tbody>
</table>

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### Table II

<table>
<thead>
<tr>
<th>Agonist binding parameters calculated from two-site model</th>
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</thead>
<tbody>
<tr>
<td>The following parameters were calculated from the observed data (Fig. 8, A and B) assuming two different binding sites for muscarinic agonists as described under “Experimental Procedures.” Treated atrial membranes were exposed to carbachol (1 ( \mu )mol) for 6 h in vivo. The error estimates are the allowed variation as determined by the minimum deviation as described under “Experimental Procedures.”</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>( F_H )</th>
<th>( F_L )</th>
<th>( K_H )</th>
<th>( K_L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 0.35 (\pm 0.02) )</td>
<td>( 0.65 (\pm 0.02) )</td>
<td>( 9.0 (\pm 1.0) \times 10^{-8} )</td>
<td>( 5.0 (\pm 0.5) \times 10^{-7} )</td>
</tr>
<tr>
<td>( 0.28 (\pm 0.03) )</td>
<td>( 0.72 (\pm 0.03) )</td>
<td>( 6.0 (\pm 3.0) \times 10^{-8} )</td>
<td>( 8.5 (\pm 1.5) \times 10^{-7} )</td>
</tr>
<tr>
<td>( 0.40 (\pm 0.05) )</td>
<td>( 0.90 (\pm 0.05) )</td>
<td>( 1.5 (\pm 0.5) \times 10^{-7} )</td>
<td>( 6.5 (\pm 1.5) \times 10^{-7} )</td>
</tr>
<tr>
<td>( 0.15 (\pm 0.02) )</td>
<td>( 0.85 (\pm 0.02) )</td>
<td>( 1.4 (\pm 0.0) \times 10^{-7} )</td>
<td>( 7.5 (\pm 0.5) \times 10^{-7} )</td>
</tr>
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</table>
the controls to 0.15 in the treated atria. If we assume that competition curves of agonist versus [3H]QNB in the presence of GPP(NH)P more closely reflect in vivo agonist binding, we can calculate the occupancy of the receptor sites by carbachol at the concentration required to totally inhibit beating. Since $R_I$ and $R_T$ differ in their affinities for carbachol, they might also differ in their pharmacological response to carbachol. For example, Birdsall et al. (12) have suggested that mACh receptor-mediated contraction of the smooth muscle from guinea pig ileum may be mediated through the low affinity receptor rather than the high affinity site. We investigated this in the embryonic chick atria by calculating the occupancies of $R_H$ and $R_I$ in control and treated atria at the concentration of carbachol required for inhibition of beating.

At the EC$_{50}$ for control atria ($3.0 \times 10^{-8} \text{ M}$) the occupancy of $R_H$ is 95% and $R_I$ is 32% (Table III). This corresponds to 220 fmol/mg of protein of $R_H$ and 104 fmol/mg of protein of $R_I$ bound with carbachol when the atria stop beating. In the treated atria, the occupancy at the EC$_{50}$ ($3.0 \times 10^{-8} \text{ M}$) of $R_H$ is 100% (45 fmol/mg of protein) and the occupancy of $R_I$ is 83% (212 fmol/mg). Therefore, after down regulation the total number of high affinity sites present is too few to reach the same level of occupancy as in the control atria (Table III). If maximum inhibition of atrial beating required the occupancy of 220 fmol/mg of $R_H$, we presumably would not be able to stop the treated atria with only 45 fmol/mg of $R_H$ bound with carbachol. This suggests that occupancy by carbachol of the high affinity binding site is not uniquely required for the negative chronotropic response in atria.

After treatment with a dose of carbachol sufficient to decrease the total number of receptors by 48%, there is a 80% decrease in $R_H$ and only a 27% decrease in $R_I$ (calculated from Tables II and III). Thus, the high affinity receptors are preferentially decreased. It is this decrease in $R_H$ which causes the 4-fold increase in the IC$_{50}$ in treated as compared to control atria (Fig. 8B). From these experiments we are not able to determine whether this increased loss of $R_H$ as compared to $R_I$, is due to an increased susceptibility of $R_H$ to down regulation or whether it is a reflection of the greater occupancy of $R_H$ over $R_I$ by carbachol at the dose used.

**DISCUSSION**

The developing chick embryo is a convenient system for studying receptor activation and regulation of the mACh receptor in vivo. At 8 days the chick embryonic heart has not developed functional parasympathetic or sympathetic innervation but beats spontaneously and the atria can respond to cholinergic agonists through the mACh receptor. Drugs can be administered onto the inner membrane of the egg and are readily absorbed into the embryonic environment without otherwise disrupting the membranes or vascular system.

We have shown here that sustained activation in vivo of the mACh receptor with an agonist such as carbachol or oxotremorine can decrease the receptor number as much as 87% in a dose- and time-dependent manner. This decrease in receptor number is reversible when further carbachol-mACh receptor interaction is blocked by the muscarinic antagonist atropine. Embryonic atria with a 2-fold decreased receptor number require a 12-fold greater concentration of carbachol to arrest the spontaneous beating in an organ bath. Thus, activation of the mACh receptor in vivo can lead to a functional loss of receptors and a change in the mACh receptor-mediated negative chronotropic response of the intact tissue.

Our results are in agreement with previous studies using neuronal hybrid cells (2, 4), neuroblastoma cells (5), central nervous system cells in culture (14), and embryonic chick cardiac cells in culture (3) that have documented an agonist-induced down regulation of the mACh receptor that is both dose and time dependent. Taylor et al. (5) showed that the long term agonist-induced down regulation of receptor number was distinct from the short term agonist-induced desensitization in the cGMP response in neuroblastoma cells. Our data also indicate that desensitization and down regulation of receptor number are distinct processes. The negative chronotropic response of the atria desensitizes to added carbachol within 1 min, while down regulation of receptor number requires 6-8 h to reach steady state.

Galper and Smith (3) have shown that the long term pretreatment of cardiac cells in culture with carbachol leads to a decreased chronotropic response to subsequent carbachol exposure. In our experiments, carbachol pretreated atria had a decreased dose response to subsequent carbachol exposure, verifying this earlier finding and extending it to intact cardiac tissue.

The finding that the population of mACh receptors in the embryonic chick heart is homogeneous with respect to agonist binding (Fig. 2) and heterogeneous with respect to agonist binding (Fig. 8, Table III) is consistent with other reports of the mACh receptor in rat brain (12), rabbit heart (16), porcine heart (13), embryonic chick heart (25), ileal smooth muscle (26, 27), and rat heart (19). In a recent paper, Galper and Smith (17) reported that in embryonic chick heart cells in culture the mACh receptor number can be decreased up to 70% by exposure to carbachol. This down regulation of mACh receptor number was accompanied by a 6-fold increase in the IC$_{50}$ for carbachol inhibition of [3H]QNB binding when homogenates were assayed in the absence of added GPP(NH)P. However, the data presented do not distinguish between a real decrease in affinity of the receptors for carbachol and a differential change in the fraction of high and low affinity mACh receptors. We have found only a 2.4-fold increase in the IC$_{50}$ for carbachol inhibition of [3H]QNB binding when atrial membranes are assayed without GPP(NH)P (Fig. 8). When the carbachol competition is performed in the presence of GPP(NH)P to more closely mimic in vivo binding conditions, the IC$_{50}$ increases 4-fold (Fig. 8). This increase can be totally accounted for by a preferential decrease in the fraction of high affinity receptors from 40% in control to 15% in treated atria with no change in affinity of either $R_H$ or $R_I$ for carbachol (Table II).

It has been postulated that the variation in ED$_{50}$ for mACh receptor-mediated responses in physiological studies may be accounted for by preferential coupling of the high or low affinity receptor to specific actions (12). The mACh receptor-mediated negative chronotropic effect measured here also suggests different roles for the two sites. The occupancy of $R_H$ by carbachol in control atria when atrial beating is arrested is 5 times greater than the total number of $R_H$ present in carbachol-treated atria (Table III). This demonstrates that in order to completely inhibit beating it is not necessary to occupy the entire number of high affinity sites which are occupied by carbachol in control atria at the EC$_{50}$ and suggests that occupancy of $R_H$ alone is not sufficient to inhibit beating. Data both in the presence and absence of GPP(NH)P support this conclusion (Table III).

Because the in vivo concentration of GTP may not be saturating, we cannot assume that GTP exerts its maximum possible effect on agonist binding in situ. At the EC$_{50}$ for control atria, the occupancy of $R_I$ by carbachol decreases from 324 fmol/mg in the absence of GPP(NH)P to 111 fmol/mg in the presence of saturating GPP(NH)P (Table III). In contrast, with treated atria, the occupancy of $R_I$ at the EC$_{50}$ is increased slightly from 175 fmol/mg in the absence of GPP(NH)P to 212 fmol/mg with saturating GPP(NH)P (Ta-
Down Regulation of Muscarinic Acetylcholine Receptor

Thus, at submaximal guanyl nucleotide-induced shifts in agonist binding, the number of $R_t$ occupied at the concentrations of carbachol required to stop beating in control and treated atria become similar. This suggests that the negative chronotropic effect may be mediated via agonist binding to the low affinity receptor. The low affinity form of the mACh receptor has been postulated to be the physiologically active receptor in smooth muscle (12).

The regulatory mechanisms by which the mACh receptor is modulated remain unclear. Our in vivo results confirm previous in vitro studies suggesting a role for receptor activation in the modulation and regulation of the mACh receptor. The ability to modulate mACh receptor levels both in vivo and in vitro makes the chick embryonic heart an attractive system to study the mechanisms of receptor regulation.

REFERENCES