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In Vivo Regulation of Muscarinic Cholinergic Receptors in Embryonic Chick Brain

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SUMMARY

Prolonged treatment of chick embryos *in vivo* with the muscarinic agonists oxotremorine or carbachol leads to dose- and time-dependent decreases in the number of brain muscarinic acetylcholine receptors (mAChR) as measured by the specific binding of the potent muscarinic ligand L-[^{3}H]quinuclidinyl benzilate to brain membranes. Maximal doses of agonists reduced the number of mAChR as much as 44%. Maximal loss of mAChR occurs 4 hr after treatment, but can be prevented or totally reversed within 24 hr by blockade of agonist-receptor interactions with muscarinic antagonists. After sustained *in vivo* oxotremorine treatment, brain mAChR show a decreased apparent affinity for agonists owing to a decrease in the affinities of both the high- and low-affinity agonist binding sites.

INTRODUCTION

Nerve cell activity may be regulated via a wide array of synaptic mechanisms (1, 2). An example is the regulation of macromolecules important for synaptic transmission by the level of synaptic activity itself. Prolonged activation of neurotransmitter receptors by transmitter can induce the removal of receptors from the postsynaptic membrane and decrease the sensitivity of the postsynaptic cell to further stimulation (3, 4). This agonist-induced decrease ("down-regulation") in receptor number would thus serve as a mechanism to coordinate postsynaptic sensitivity with the over-all level of presynaptic activity.

The mAChR[1] is an example of a macromolecule that may be regulated by the chronic level of agonist present. Using the binding of specific radioligands as a means of quantitating the number of mAChR in tissues and cells, regulation of mAChR number has been demonstrated after the direct addition of muscarinic agonists to cultured neurons (5) and neural cell lines (6), where the decrease in mAChR number was accompanied by a decreased physiological response of the mAChR-associated adenylate cyclase system in the cells (7). These long-term effects have been shown to be quite distinct from short-term desensitization of receptor activity (8). Agonist-induced decreases in neuronal mAChR number have also been indirectly demonstrated *in vivo* by the administration of potent acetylcholinesterase inhibitors to chronically increase endogenous levels of synaptically released acetylcholine (9, 10). In addition, prolonged treatment *in vivo* with specific antagonists to prevent the interaction of synaptically released acetylcholine with mAChR results in increased mAChR number (11, 12), providing further indirect evidence for modulation of cellular sensitivity and function by long-term transmitter-receptor interactions. However, it has not previously been demonstrated that administration of cholinergic agonists can directly regulate neuronal mAChR *in vivo*. In this paper we show that *in vivo* administration of specific muscarinic agonists can significantly decrease mAChR number in the embryonic chick brain.

METHODS

Fertile white leghorn chicken eggs were maintained in a humidified incubator at 38°. Embryo ages correspond to those described by Hamburger and Hamilton (13). Drugs were administered *in ovo* on day 9 or 10 of development by opening a small hole in the shell and pipetting a given volume of warm physiological saline containing the appropriate drug directly onto the inner embryonic membrane. Following treatment, the holes were sealed with cellophane tape and eggs were returned to the incubator until removal of the brains for assay (usually on day 10).

Drug treatments. Drugs were dissolved in PBS, pH 7.4 (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.4 mM). Drug concentrations were adjusted so that no more than a 100-μl volume was administered per egg. For

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[1] The abbreviations used are: mAChR, muscarinic cholinergic receptor; PBS, phosphate-buffered saline; carbachol, carbamylcholine chloride; QNB, quinuclidinyl benzilate; Gpp(NH)p, 5'-guanylyl imidodiphosphate; AChE, acetylcholinesterase.

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control treatments, 100 μl of PBS were administered. At the range of concentrations used in this study, only carbachol elicited a higher mortality rate than that seen in untreated embryos (approximately 40% at a dose of 1 μmole). Only brains from those embryos surviving the drug treatments were used.

Assay for mAChR. At determined times, embryos were removed from the eggs, and the heads were amputated and kept on ice. Brains were dissected out, cleansed of external membranes and blood vessels, transected at the level of the brain stem, and placed in ice-cold assay buffer (50 mM NaH_2PO_4, pH 7.4). Brains were homogenized by hand in a Duall (Kontes) ground glass homogenizer and centrifuged in the cold at 7500 × *g* for 10 min. Membrane pellets were resuspended in fresh buffer and washed an additional five times to assure removal of all ligand from receptor. The crude washed membranes were then suspended in a final adjusted volume of assay buffer to a protein concentration of 1–2 mg/ml for subsequent assay of mAChR.

Brain membranes were assayed for mAChR using a modification of the radioligand filter binding method described by Yamamura and Snyder (14) which employs the labeled specific muscarinic antagonist [^{3}H]QNB. Under standard assay conditions, homogenates were incubated for 90 min at room temperature in a 1-ml total reaction volume containing 0.1–0.2 mg of membrane protein, 660 pM [^{3}H]QNB, and assay buffer. Nonspecific binding was determined in the presence of 1 μM atropine sulfate. For competitive binding experiments, reactions were carried out for 1 hr, the [^{3}H]QNB concentration was adjusted to 330 pM, and varying concentrations of carbachol were added to a final assay volume made up to 1 ml with the modified beating rate medium (pH 7.4) of Halvorsen and Nathanson (15) consisting of NaCl, 149 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgSO_4$, 1.0 mM; 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid, 10 mM; and NaH_2PO_4, 0.4 mM.

Reactions were stopped by addition of 5 ml of ice-cold assay buffer to the tubes, and the mixtures were rapidly filtered under vacuum onto Whatman GF/C glass-fiber discs. The filters were rapidly rinsed three times with 5-ml aliquots of cold assay buffer, placed into scintillation vials containing 4 ml of counting cocktail [Triton X-100 25%, ethanol 0.088%, distilled water 0.088% (v/v) and Omnifluor 0.4% (w/v) in xylene base] and counted at an efficiency of 30%. An aliquot of the homogenate was assayed for protein by a modification of the method of Lowry *et al.* (16), and specific binding was expressed as femtomoles of mAChR per milligram of protein. Each experiment was performed at least twice, and all homogenates were assayed in duplicate. Each data point represents the average of two such homogenate preparations.

Materials. Fertile eggs were obtained from College Biological Supplies (Bothell, Wash.). L-[^{3}H]QNB (30 Ci/mmole) was purchased from Amersham Corporation (Arlington Heights, Ill.), and Gpp[NH]p was obtained from P-L Biochemicals (Milwaukee, Wisc.). Carbachol, oxotremorine, atropine sulfate, *d*-tubocurarine chloride, scopolamine hydrochloride, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, and hexamethonium bromide were supplied by Sigma Chemical Company (St. Louis, Mo.). All other chemicals were of reagent grade.

RESULTS

Time course and dose-response properties of mAChR regulation. Administration of 0.8 μmole of the specific muscarinic agonist oxotremorine to 9-day chick embryos led to a time-dependent decrease in the binding of [^{3}H]QNB to treated brain membranes compared with those from untreated embryos (Fig. 1). The maximal decrease in binding (approximately 30%) occurred within 4 hr after treatment and was then maintained throughout the remainder of the 9-hr incubation period. We calculated a half-life of 2.1 hr for disappearance of the agonist-activated receptors from kinetic analysis of receptor turnover rate (5).

In another set of experiments we incubated embryos for 6 hr with varying doses of oxotremorine and assayed brain membranes for [^{3}H]QNB binding. Administration of 0.1–10 μmoles of oxotremorine (Table 1) led to dose-dependent decreases in specific binding compared to PBS-treated membranes. Maximal loss of binding (44%) was observed at a dose of 1 μmole of oxotremorine; higher

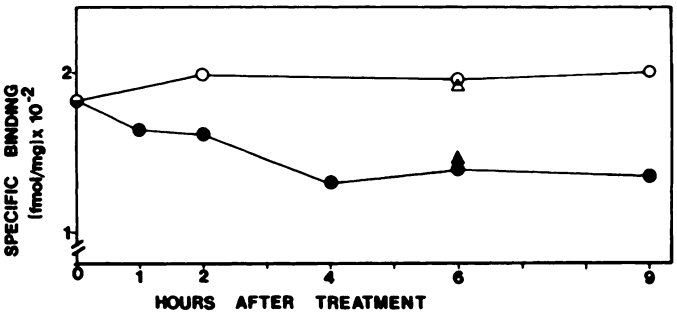

FIG. 1. *Time course for oxotremorine-induced reduction in specific [^{3}H]QNB binding*

Embryos were treated *in ovo* with either PBS (control; ○) or 0.8 μmole of oxotremorine (●). After the desired treatment periods, brains were removed and assayed for specific [^{3}H]QNB binding as described under Methods. Time-dependent decreases in binding were prevented by simultaneous addition of 0.1 μmole of atropine to embryos (△) at the time of oxotremorine treatment, but not by addition of 0.1 μmole of *d*-tubocurarine (▲). Each point represents the mean of two membrane preparations, with two brains pooled per preparation. Standard deviations are within 10% of the mean values shown.

TABLE 1

Dose dependence of decrease of mAChR number

Chick embryos were treated for 6 hr with the dose of oxotremorine shown, and brain membranes were assayed for specific [^{3}H]QNB binding as described under Methods. Values represent means (± standard deviation) of two membrane preparations, with two brains pooled per preparation.

Oxotremorine dose	Specific [^{3}H]QNB bound	% Control
μmoles	*fmoles/mg protein*	
0	246 ± 8	100
0.1	177 ± 27	72
0.5	161 ± 30	66
1.0	137 ± 7	56
10.0	145 ± 24	59

doses (up to 10 μmoles) induced no further decreases in mAChR levels. Assuming an egg fluid volume of 50 ml and homogeneous distribution of agonist throughout this volume, a 0.1-μmole dose of oxotremorine would represent an *in ovo* concentration of 2×10^{-6} M, within the range at which oxotremorine is pharmacologically and physiologically active in other preparations (17).

Pharmacological specificity. We investigated whether the oxotremorine-induced decreases in [^{3}H]QNB binding are mediated specifically via interactions with mAChR by testing the ability of various cholinergic agonists and antagonists to affect [^{3}H]QNB binding. Treatment of embryos with both oxotremorine and the structurally dissimilar long-lasting muscarinic agonist carbachol caused marked reduction in the amount of [^{3}H]QNB bound to brain membranes (Table 2). The highly specific muscarinic antagonists atropine and scopolamine completely prevented the decrease in [^{3}H]QNB binding induced by either oxotremorine or carbachol (Table 2; Fig. 1), but did not by themselves affect the levels of [^{3}H]QNB bound. In contrast, *d*-tubocurarine, the specific antagonist of nicotinic cholinergic sites in skeletal muscle, was either only partially effective (Table 2) or totally ineffective (Fig. 1) in blocking the loss of binding due to oxotremorine treatment. Hexamethonium, which blocks cholinergic sites in autonomic ganglia, was also not effective in blocking the actions of oxotremorine. Hence, these data indicate that loss of [^{3}H]QNB binding is mediated specifically by the muscarinic receptor and requires interaction between the receptor and muscarinic agonists.

TABLE 2

Pharmacological specificity of mAChR regulation

Embryos were treated overnight with the drug or combination of drugs shown, and brain membranes were assayed for [^{3}H]QNB binding and compared with PBS-treated control groups. Data were calculated from duplicate measurements of two pooled membrane preparations. All standard deviations were within 12% of the mean values shown.

Treatment	% Control specific [^{3}H]QNB binding
Oxotremorine (0.4 μmole)	66
Oxotremorine (0.4 μmole) + atropine (0.1 μmole)	108
Oxotremorine (0.4 μmole) + scopolamine (0.1 μmole)	103
Oxotremorine (0.4 μmole) + *d*-tubocurarine (0.1 μmole)	84
Oxotremorine (0.4 μmole) + hexamethonium (1.0 μmole)	63
Carbachol (1.0 μmole)	66
Carbachol (1.0 μmole) + atropine (0.1 μmole)	92
Atropine (0.1 μM)	94
Scopolamine (0.1 μM)	92

Saturation kinetics of [^{3}H]QNB binding. The observed agonist-induced decreases in specific [^{3}H]QNB binding may be due to either a decrease in the apparent affinity of mAChR for QNB or an actual loss of mAChR binding sites. To discriminate between these possibilities, we incubated brain membranes from PBS- and agonist-treated embryos in increasing concentrations of [^{3}H]QNB and analyzed the binding data. Membranes from both oxotremorine-treated and control embryos displayed similar saturating binding curves (Fig. 2), although membranes treated with either 0.8 or 4 μmoles of

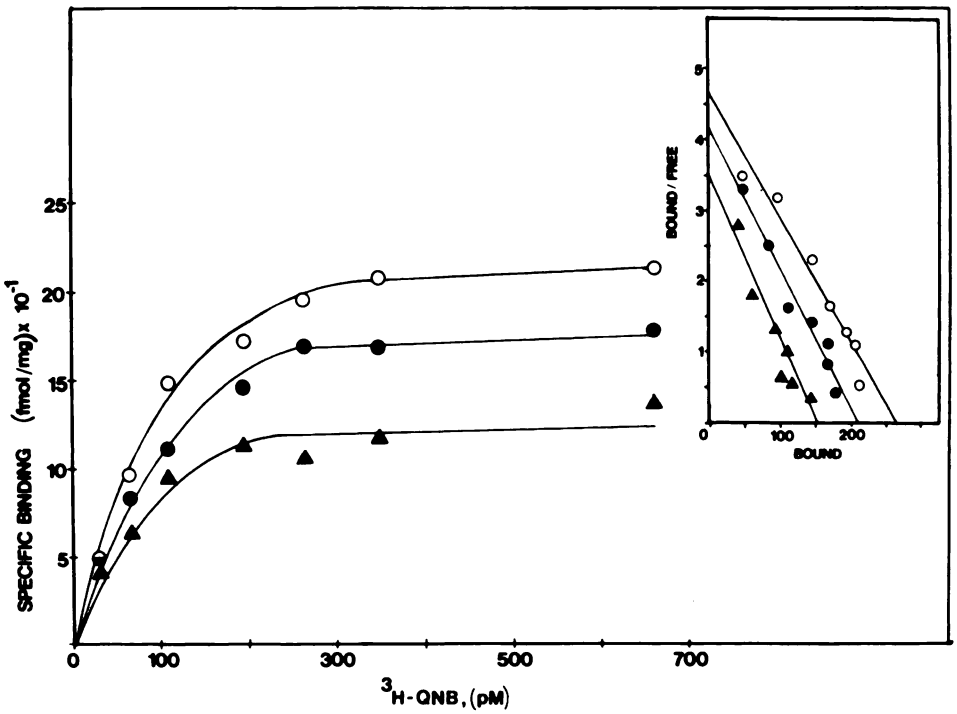

FIG. 2. *Saturation of [^{3}H]QNB binding following treatment with oxotremorine*

Embryos were treated *in ovo* with PBS (○), 0.8 μmole of oxotremorine (●), or 4.0 μmoles (▲) of oxotremorine. After 6 hr, the brains were removed and assayed for specific [^{3}H]QNB binding at varying concentrations of [^{3}H]QNB. The *inset* shows the Scatchard analysis of the binding isotherm data. Standard deviations are within 10% of mean values shown.

oxotremorine showed dose-dependent decreases in absolute levels of specific binding. Scatchard analysis of the binding isotherms (Fig. 2, *inset*) revealed that neither dose of oxotremorine altered the apparent affinities of mAChR for [^{3}H]QNB (K_D values are 5.0, 5.3, and 5.5 × 10^{-11} M for PBS-, 0.8 μmole-, and 4 μmole-treated groups, respectively.) However, the number of specific [^{3}H]QNB binding sites was substantially reduced by agonist treatment. The number of mAChRs decreased from 264 fmoles/mg of protein for PBS-treated groups to 211 and 150 fmoles/mg after treatment with 0.8 and 4 μmoles of oxotremorine, reflecting 20% and 43% losses of mAChR sites. Carbachol treatment (1 and 3 μmoles) of embryos also induced similar decreases in number of mAChRs without significant alteration of the affinity of receptors for [^{3}H]QNB (Fig. 3). These results demonstrate that agonist-induced decreases in [^{3}H]QNB binding after prolonged *in ovo* treatment are due specifically to a direct reduction of mAChR number.

Reversal of mAChR down-regulation. It is reasonable to assume that, if agonist-mediated regulation of receptor number serves a physiologically useful role in nerve cells, the process would be reversible. That is, once agonist levels are lowered and relative receptor occupancy decreases, active receptors should reappear on the membrane surface. If the decrease in receptor number results from increased active degradation or internalization of receptors (6, 18) and *de novo* synthesis of receptor molecules is required for replacement at membrane sites (6, 19), then the time course of receptor reappearance should be consistent with the processes involved in cellular biosynthetic metabolism. To test for recovery of mAChR number, we treated embryos overnight with 1 μmole of oxotremorine, then added sufficient doses of atropine to block any further agonist activation of mAChR, and continued incubation of the embryos for an additional 24 hr. Although levels of mAChR number from oxotremorine-treated groups remained significantly lower than those of saline-treated groups throughout the 24-hr interval (Fig. 4), mAChR number began to return toward control levels within 6 hr of initiating atropine blockade. Receptor levels increased throughout the time course, and by 24 hr had returned to the over-all mean control levels. Thus, *in ovo* agonist-induced loss of mAChR is fully reversible, with a time course for recovery of receptors consistent with the synthesis, transport, and insertion of new receptors into membrane sites.

Carbachol competition binding experiments. Muscarinic antagonists such as QNB bind to only one uniform population of high-affinity mAChR in a wide variety of organisms and tissues, including embryonic chick brain. In contrast, the mAChR displays heterogeneity with respect to the binding of agonist ligands (20). Agonist binding data are consistent with the presence of either two or three separate classes of sites which differ in their affinities for agonists but have the same affinities for antagonists.

We have used the displacement of [^{3}H]QNB binding by varying concentrations of the muscarinic agonist carbachol as an indirect means of characterizing the agonist binding parameters of mAChR from untreated and oxotremorine-treated embryos. The competition curves for both treated and untreated membranes were rather flat (Fig. 5); other studies of agonist binding to mAChR (20, 21) have shown that such shallow curves are usually indicative of multisite ligand-receptor interactions. Although the curves are similar in shape, agonist-induced loss of mAChR caused noticeable displacement of the

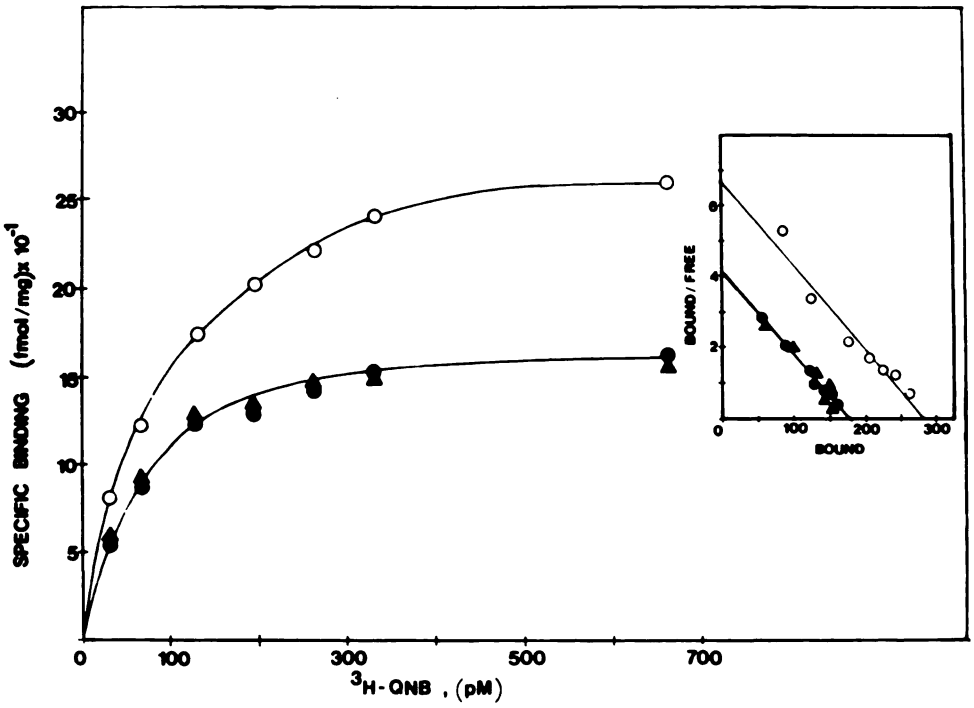

FIG. 3. *Saturation of [^{3}H]QNB binding following treatment with carbachol*

Embryos were treated as in Fig. 2 with PBS (○), 1 μmole of carbachol (●), or 3 μmoles of carbachol (▲). The *inset* shows the Scatchard analysis of the binding isotherm data. Standard deviations are within 10% of the mean values shown.

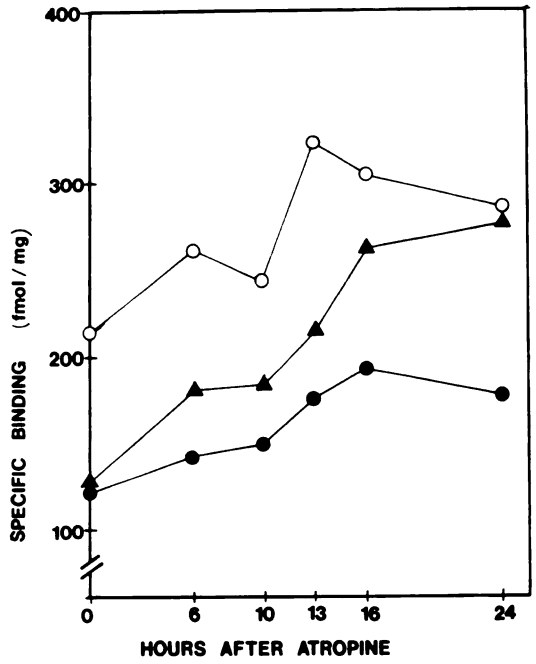

FIG. 4. *Reversability of oxotremorine-induced mAChR down regulation*

Embryos were treated overnight with either PBS or 1.0 μmole of oxotremorine, after which time some of the embryos received 0.1 μmole of atropine to block further agonist activation. Atropine was then readministered 13 hr after the first dose. At selected intervals after initial receptor blockade (time zero), brains from PBS-treated (○), oxotremorine-treated (●), and oxotremorine + atropine-treated (▲) groups were removed and assayed for specific [^{3}H]QNB binding. Each point represents the mean of duplicate membrane samples, with two brains pooled per sample. All standard deviations are within 10% of the mean values shown.

competition curve to the right and a corresponding change in the IC_{50} for carbachol from 1.6 to 3.2 × 10^{-5} M.

In order to determine whether the decrease in the apparent affinity of mAChR for carbachol following chronic oxotremorine treatment is due to an actual reduction in the population of high-affinity sites or to changes in the dissociation constants of either high- or low-affinity sites, we fit the binding data to a two-site model of receptor occupancy (see ref. 15 for details). We were thus able to compare mAChRs from untreated and treated embryos with respect to changes in the fraction of high (F_H) and low (F_L) affinity sites and their respective affinities (K_H, K_L) for agonist. The calculated curves (Fig. 5) show close agreement with the experimentally determined curves (IC_{50} values of 1.8 and 3.6 × 10^{-5} M for untreated and treated groups, respectively). The best-fit calculated binding parameters (Table 3) indicate that oxotremorine treatment does not alter either F_H or F_L but does cause at least a 2-fold decrease in the affinities of both sites (K_H, K_L) for carbachol.

DISCUSSION

We have demonstrated that prolonged treatment *in ovo* of embryonic chicks with the specific muscarinic agonists oxotremorine and carbachol leads to time- and dose-dependent decreases in brain mAChR number. This loss of mAChR can be prevented or reversed by receptor blockade with muscarinic antagonists. Our findings are in agreement with recent studies reporting agonist-induced decreases in mAChR number in a variety of other preparations, including aggregate brain cultures (5), heart cell cultures (19), cultured neuroblastoma cells (8, 22), neuronal hybrid cell lines (6, 7), rat heart (12), and embryonic chick heart (15).

Although regulation of brain mAChR number has been demonstrated directly in cultured brain cells (5) and indirectly *in vivo* following prolonged treatment with AChE inhibitors (9, 10, 23), we extend these findings by showing that direct treatment *in vivo* with cholinergic agonists induces a substantial loss of mAChR. We found that, unlike the prolonged intervals required after *in vivo* AChE inhibitor administration, decrease in receptor number occurs rapidly and reaches steady-state levels with a half-time for disappearance of about 2 hr, similar to the value reported for agonist-activated loss of mAChR in cultures of embryonic chick brain cells (5). However, using maximal agonist doses, we did not obtain as large a decrease in mAChR number *in vivo* as that reported for cultured brain cells. This may be a consequence of less accessibility of ligand for receptor imposed by an intact nervous system. Alternatively, mAChR *in vivo* may be less susceptible to regulation. Interestingly, *in vivo* treatment of rat brain with AChE inhibitors leads to decreases of mAChR levels that are similar in magnitude to our results.

Recently, Halvorsen and Nathanson (15) examined the *in vivo* regulation of mAChR in 8-day-old embryonic chick heart. Comparison of results of that study with our data for 10-day-old chick brain indicates that, although some of the basic features of agonist-induced mAChR regulation are similar in these two tissues, there are also several noteworthy differences. In both tissues, long-term agonist treatment leads to actual decreases in mAChR

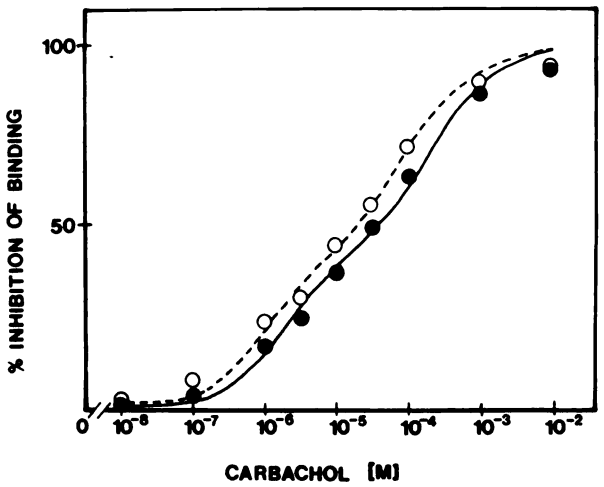

FIG. 5. *Carbachol competition of [^{3}H]QNB binding*

Brains from PBS-treated (○) and 1.0 μmole oxotremorine-treated (●) embryos were removed and assayed for [^{3}H]QNB binding in the presence of competing concentrations of carbachol (see Methods). Lines (- - -, PBS; ——, oxotremorine) indicate best-fit lines calculated by computer fit of the data to a two-site model for receptor occupancy. Points represent experimentally observed values for carbachol displacement. Values represent averaged results from three separate competition experiments and were used for data presented in Table 3. Standard deviations are within 10% of the mean values shown.

TABLE 3

Two-site analysis of agonist binding parameters

Binding parameters derived from computer fit to observed data from competition binding data shown in Fig. 5 using a two-site model for agonist binding. Mean parameter values were obtained from cumulative data of three experiments. Values in parentheses represent the range of the parameters which produces no more than a 10% allowed variation in the difference in the sum of the squares between observed and best-fit calculated data (see ref. 15 for details). F_H and F_L represent fractions of high- and low-affinity binding sites; K_H and K_L represent dissociation constants of high- and low-affinity sites.

	F_H	F_L	K_H	K_L
			$\times 10^{-7}$ M	$\times 10^{-5}$ M
Control (PBS)	0.42 (0.39–0.48)	0.58 (0.55–0.64)	1.3 (0.6–1.5)	1.2 (1.0–1.6)
Treated (oxotremorine)	0.44 (0.40–0.49)	0.56 (0.52–0.61)	2.5 (1.8–3.3)	2.8 (2.1–3.7)

number without affecting affinity of mAChR for [^{3}H]QNB. However, greater reductions in mAChR number could be induced in the heart, and the time interval required to obtain maximal receptor losses was also longer than for brain. Nevertheless, calculated half-time values for disappearance of agonist-activated mAChR were similar for both tissues, as were times for reappearance of mAChR during the reversal of down-regulation. These data suggest that, although the absolute amount of receptor loss obtainable in these tissues may differ significantly, the basic cellular mechanisms underlying mAChR removal and/or biosynthesis and transport may be analogous.

The results of carbachol competition experiments for chick brain mAChR are consistent with previous studies in other species demonstrating heterogeneity with respect to agonist binding sites (21, 24), and also agree with the data of Halvorsen and Nathanson (15) for embryonic chick heart. Long-term *in vivo* agonist treatment of brain increases the IC_{50} for carbachol 2-fold and affects the agonist binding parameters by decreasing the affinities of both high- and low-affinity agonist binding sites for carbachol without altering the relative concentrations of these sites. These findings are in agreement with the results of studies by Ehlert *et al.* (23), who reported significant decreases in the affinity of both high- and low-affinity sites in rat brain mAChR following prolonged AChE inhibitor treatment. However, our data differ from those of Smit *et al.* (9), who reported preferential loss of low-affinity sites of AChE inhibitor-treated rat brain mAChR as determined by direct, labeled agonist binding and three-site analysis. These conflicting findings are difficult to reconcile and may indicate characteristic differences between embryonic chick and rat brain mAChR with respect to susceptibility of the agonist sites to activation. Alternatively, these differences may arise from the variations in experimental protocol and methods of data assessment used in the studies.

It has been shown that guanine nucleotides may regulate agonist binding to mAChR by lowering the apparent affinity of receptor for agonists (25, 26). Thus, in the presence of the nonhydrolyzable GTP analogue Gpp(NH)p, Halvorsen and Nathanson (15) showed that embryonic chick heart mAChRs have a significantly decreased affinity for carbachol. In contrast, we were unable to demonstrate any significant effect of Gpp(NH)p on the affinity of brain mAChR for carbachol (data not shown). Previous studies have shown that regulation of mAChR by guanine nucleotides is less pronounced in brain than heart and, in fact, within the rat brain there exist major regional differences with respect to the ability of guanine nucleotides to inhibit agonist binding to mAChR (27). Chick brain and heart mAChRs may therefore share the feature of regulation via agonist activation and yet remain distinct with regard to regulation by guanine nucleotides.

The demonstration that pharmacologically active concentrations of cholinergic agonists can act directly *in vivo* to regulate mAChR levels in the brain is consistent with our previous suggestion (7) for the modulation of postsynaptic sensitivity by the alteration of the concentration of muscarinic receptors on the neuronal cell surface. Although the molecular processes involved remain largely unknown, such negative feedback regulatory interactions may play fundamental roles in interneuronal communication and synaptic plasticity.

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