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Cholinergic activity regulates muscarinic receptors in central nervous system cultures*

(synaptic plasticity/protein turnover/membranes/microfilaments)

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ABSTRACT **Muscarinic acetylcholine receptor activation induces a loss of muscarinic receptors from cultured neuroblastoma and embryonic chicken cerebrum cells. As measured by specific binding of [^{3}H]quinuclidinyl benzilate, steady-state receptor concentrations decrease 75% in response to receptor-saturating concentrations of cholinomimetic drugs. Both the degree and duration of activation determine the extent of receptor loss. A method for analyzing receptor turnover, which does not rely upon protein synthesis inhibitors, shows that activated receptors have a half-life of 1.6 hr. The regulated rate of receptor disappearance begins as soon as activators are added, and the rate is maintained as long as activators are present. The receptor blocker atropine causes an increase in receptor levels in central nervous system cultures but has no effect on receptors in cultures of adrenergic neuroblastoma cells. Because spontaneous cholinergic activity is expected only in the central nervous system cultures, the increase likely reflects blockade of endogenous regulation. Cytochalasin B blocks receptor regulation, suggesting that regulation may be mediated by a process involving microfilaments.**

Many theories of information processing in the central nervous system are based on the postulate that synaptic communication can be modified (1–4). On a cellular level, gross modification conceivably could occur through synaptic rearrangement (5). Alternatively, specific biochemical modification could occur in transmitter synthesis (6), release (7), inactivation (8), or reception (9). Because neuroreceptors play a key role in synaptic communication, and because specific markers for neuroreceptors recently have been developed (10), we have focused our attention on the regulation of neuroreceptor concentration.

Precedents for regulation of central nervous system neuroreceptors can be found in various nonneuronal cells. In general, receptor activity negatively regulates receptor density. Receptors for many polypeptide hormones thus decrease in concentration upon prolonged stimulation (11, 12). Neuroreceptors outside the nervous system behave similarly. The development of supersensitivity in muscle cells after denervation of the diaphragm is due to a 20-fold increase in nicotinic acetylcholine receptor concentration (13, 14). Direct addition of cholinomimetics to cloned muscle cells recently has been shown to decrease nicotinic receptors by as much as 50% (15). In the pineal gland β-adrenergic receptors undergo circadian changes in concentration (16), whereas in red blood cells (17, 18), and in fibroblast cell lines (19) activation of β-adrenergic receptors stimulates a loss of receptor sites beyond that due to irreversibly bound activators (20). α-Adrenergic receptor density decreases in human platelets exposed to epinephrine (21). Within the central nervous system, catecholamine receptor density reportedly changes in response to drug treatment of experimental animals (22–25).

In an earlier communication, we reported our discovery that muscarinic acetylcholine receptors (MAcChoR) were another major class of neuroreceptors that could be regulated (26). In the neuronlike hybrid cell line NG108-15, receptor activation caused a loss of binding sites for the specific muscarinic marker quinuclidinyl benzilate (QNB) (27–29) and a parallel decrease in the ability of acetylcholine to inhibit adenylate cyclase (30). Agonist-induced loss of muscarinic receptors in cultured heart cells has been noted (31). In this report, we extend our previous work to show that regulation is a common property of muscarinic receptors, including those found in the central nervous system. By using cell cultures, we have further investigated the relevance of experimentally induced receptor loss to normal synaptic communication and have measured the turnover rate of activated receptors. We have obtained preliminary evidence suggesting a role for microfilaments in the mechanism of regulation.

MATERIALS AND METHODS

Chemicals. [^{3}H]QNB was purchased from New England Nuclear (29.4 Ci/mmol, 7/78) or from Amersham (8.4 Ci/mmol, 10/76) (1 Ci = 3.7×10^{10} becquerels). Cell culture medium, serum, and antibiotic were purchased from ISI Biologicals (Cary, IL).

Cell Culture. Aggregate cultures of embryonic chicken cerebrum were prepared as described by Garber and Moscona (32). Cerebral cells from 8 to 10-day-old chicken embryos were cultured at 37°C and 70 rpm in Eagle's basal medium supplemented with 25 mM Hepes (pH 7.4), 10% newborn calf serum, and 50 μg of gentamicin per ml, and adjusted to 340 mosM.

N1E-115 neuroblastoma cells were obtained from Marshall Nirenberg (National Institutes of Health, Bethesda, MD), and were grown as described (33) in Dulbecco-Vogt modification of Eagle's minimal essential medium with 10% fetal calf serum, 10% CO_2, 44 mM $NaHCO_3$ (pH 7.4), and 50 μg of gentamicin per ml, and adjusted to 340 mosM.

Pharmacological Treatment of Cultures. All cultures, including controls, were brought to identical concentrations of added ligands immediately prior to harvesting and washing. This step controls for the possibility that some added ligand may carry over and interfere with the receptor binding assay. Unless otherwise indicated, cells were given four 5-ml washes in buffer A (50 mM Tris·HCl, pH 7.4 at 5°C Hanks' salts) and were quick-frozen in dry ice/acetone for the binding assay.

Binding Assay for MAcChoR. The number of receptor binding sites was determined from modification of the [^{3}H]QNB binding and filtration assay of Yamamura and Snyder (27) as described (28). Briefly, crude tissue homogenate was

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Abbreviations: MAcChoR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate.

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incubated at 37°C for 30 min in 0.5 ml of buffer A containing 1 nM [^{3}H]QNB. Atropine at 1 μM was added to incubations to permit determination of nonspecific binding. Triplicate samples of total and nonspecific binding were taken for each culture homogenate assayed.

RESULTS

Characterization of [^{3}H]QNB Binding to Embryonic Chicken Brain. At 37°C specific binding of [^{3}H]QNB to homogenates of embryonic chicken brain had an equilibrium dissociation constant of 0.12 nM (Fig. 1). Over the concentration range used, binding reached equilibrium by 30 min. With saturating [^{3}H]QNB, the rate of association was pseudo first-order ($k_{assoc} = k'_{assoc} \times [QNB] = 0.14\ nM^{-1}\ min^{-1}$), and the rate of dissociation was first order ($k_{dis} = 0.009\ min^{-1}$), giving a kinetically determined K_D of 0.06 nM. The total number of specific [^{3}H]QNB binding sites in whole brain was 140 fmol per mg of protein, as reported for 21-day-old embryonic chicken whole brain (29). Muscarinic ligands readily blocked [^{3}H]QNB binding, but the nicotinic ligand hexamethonium was much less effective (Fig. 2). The characteristics of [^{3}H]QNB binding to homogenates of embryonic chicken brain are consistent with [^{3}H]QNB binding to MAcChoR in other systems (27–29, 34).

Effect of MAcChoR Activation on MAcChoR Concentration. By using aggregate cell cultures of embryonic chicken cerebrum, we have found that sustained MAcChoR activation stimulated a loss of [^{3}H]QNB binding sites. Table 1 shows that 20 hr of incubation with oxotremorine, a muscarinic specific agonist, caused a 42% decrease in specific [^{3}H]QNB binding. This decrease occurred at 1 μM oxotremorine, a concentration that only half-saturates the receptor. The muscarinic specific antagonist atropine (10 nM) blocked the binding loss induced by oxotremorine. This blockade indicates that the loss of [^{3}H]QNB binding is due specifically to receptor activation, and neither to simple receptor occupancy by ligand nor to nonspecific effects of agonist on cholinoceptive cells. Scatchard analysis of data from agonist-exposed and control cultures indicates the receptor–QNB affinity is unchanged with binding loss (data not shown). The observed decrease in specific binding therefore represents an actual loss of muscarinic receptor sites.

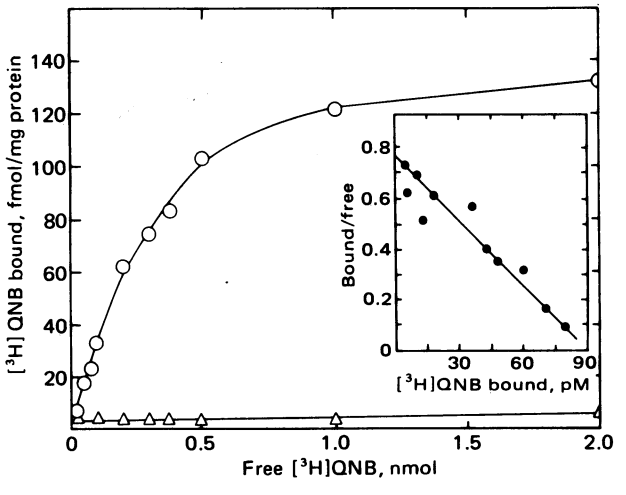

FIG. 1. Binding of [^{3}H]QNB to 21-day-old embryonic chicken brain homogenates as a function of [^{3}H]QNB concentration. Crude membrane homogenates were incubated for 30 min at 37°C with increasing doses of [^{3}H]QNB in the presence (△, nonspecific binding) or absence (○, total binding) of 1 μM atropine. Free [^{3}H]-(−)-QNB is half of the added [^{3}H]QNB minus the amount of specifically bound QNB. (*Inset*) Scatchard plot of specific [^{3}H]-(−)-QNB binding (K_D = 0.12 nM).

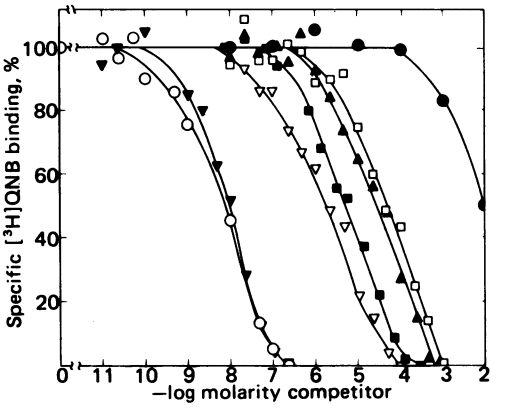

FIG. 2. Competition for [^{3}H]QNB binding sites by cholinergic drugs. Homogenates of 21-day-old embryonic chicken brain were incubated for 40 min at 37°C with 1.3 nM [^{3}H]QNB and the indicated concentrations of drugs. For acetylcholine, membranes were preincubated for 15 min with 2.5 μM eserine. ○, Atropine; ▼, scopolamine; ▽, oxotremorine; ■, acetylcholine; ▲, arecoline; □, carbachol; ●, hexamethonium.

To examine the possibility that sustained receptor activation may lower the number of receptor sites through an effect on cholinoceptive cell viability, we withdrew agonist after a period of receptor activation and tested for recovery of [^{3}H]QNB binding. With 4 days of continued incubation in 1 μM oxotremorine, cultures bound only 47% as much as control cultures (Table 2). In contrast, parallel cultures incubated for 24 hr with oxotremorine and then washed free of oxotremorine and allowed 4 days of recovery, bound 87% as much [^{3}H]QNB as controls. Because the binding loss was reversible, sustained receptor activation with oxotremorine apparently does not reduce cholinoceptive cell viability, and the binding loss is not due to destruction of cholinoceptive cells. Similarly, in monolayer cultures of cloned neuroblastoma and hybrid cells, muscarinic receptor activation has no effect on rates of cell division or RNA and protein biosynthesis (unpublished results).

Dose Dependencies for Agonist and Antagonist Effects on [^{3}H]QNB Binding Levels. The relationship between receptor levels and the degree of receptor activation was determined in aggregate cell cultures of embryonic chicken cerebrum and also in N1E-115 neuroblastoma cell cultures. In both systems, 24-hr activation with carbachol lowered [^{3}H]QNB binding as much as 80% (Fig. 3). Saturating doses of carbachol (1 mM) resulted in maximal binding loss with the half-maximal response occurring at ≈4 μM.

Atropine, because it blocks the agonist-induced receptor loss, may be expected to increase [^{3}H]QNB binding levels in cell

Table 1. Reduction of specific [^{3}H]QNB binding caused by MAcChoR activation

Growth condition	[^{3}H]QNB bound, fmol/mg protein	% of control
No addition	169	100 ± 17
Oxotremorine (1 μM)	97	58 ± 1.7
Oxotremorine (1 μM) + atropine (10 nM)	211	125 ± 22

At day 14 (conceptual age), aggregate cultures of embryonic chicken cerebrum were treated as described in *Materials and Methods*. Eighteen hours later, cells were harvested, washed, and assayed for [^{3}H]QNB binding (triplicate cultures per experimental condition). Oxotremorine-stimulated loss of [^{3}H]QNB binding is significant ($P < 0.01$, Student's t test), as is blockade of the oxotremorine effect by atropine ($P < 0.005$). ± indicates SD.

Table 2. Effect of agonist withdrawal on specific [^{3}H]QNB binding levels

Growth condition before wash	Growth condition 4 days after wash	Specific [^{3}H]QNB binding, % of control
No addition	No addition	100 ± 21
Oxotremorine	Oxotremorine	47 ± 3.5
Oxotremorine	No addition	87 ± 9.2

Aggregate cultures were first treated with 1 μM oxotremorine on day 14 (conceptual age), triplicate cultures per experimental condition. After 24 hr, aggregates were washed four times in medium and resuspended and incubated in culture flasks. Cultures thereafter were fed daily for 4 days. The increase in specific [^{3}H]QNB binding 4 days after removal of agonist is significant ($P < 0.005$). ± indicates SD.

systems that have low receptor concentrations caused by endogenous receptor activation. To test this possibility, we compared the effect of atropine on receptor levels in central nervous system cultures and N1E-115 cultures. These systems were selected because aggregate cultures form functional synapses (35, 36), whereas the adrenergic clone N1E-115 (37) does not form muscarinic synapses. Fig. 3 shows that addition to aggregate cultures of increasing doses of atropine, over the range known to saturate receptors, caused as much as a 31% increase in [^{3}H]QNB binding. The increase is statistically significant ($P < 0.005$, Student's t test). Enhancement of binding was greatest with 3×10^{-9} M atropine. This concentration is approximately 10 times greater than the calculated dissociation constant for atropine binding to MAcChoRs (ref. 34; Fig. 2). Higher atropine concentrations appear to lower receptor levels for reasons as yet undetermined. The atropine effect on aggregate cultures is in clear contrast to the effect of receptor blockade on the N1E-115 cultures. These cultures do not make muscarinic synapses, and atropine does not cause increased [^{3}H]QNB binding. Fig. 3 indicates that receptor concentration can be either increased or decreased, depending on the degree of ongoing receptor activation.

Rate of [^{3}H]QNB Binding Loss Stimulated by Receptor Activation. The extent of binding site loss is dependent on the duration of experimentally induced receptor activation. Fig. 4 shows the decrease in [^{3}H]QNB binding levels as a function

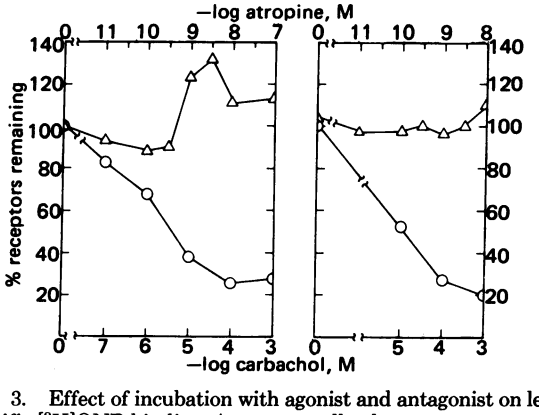

FIG. 3. Effect of incubation with agonist and antagonist on levels of specific [^{3}H]QNB binding. Aggregate cell cultures are represented on the left and N1E-115 neuroblastoma cell cultures, on the right. Cultures were incubated with carbachol or atropine for 24 hr. Each point represents the mean of three cultures exposed to atropine (△) or carbachol (○). [^{3}H]QNB binding levels are significantly less than control binding levels for all doses of carbachol given to both systems. Binding is significantly greater than control for aggregate cultures given atropine at 10^{-9} M ($P < 0.025$), 3×10^{-9} M ($P < 0.005$), and 10^{-8} M ($P < 0.05$).

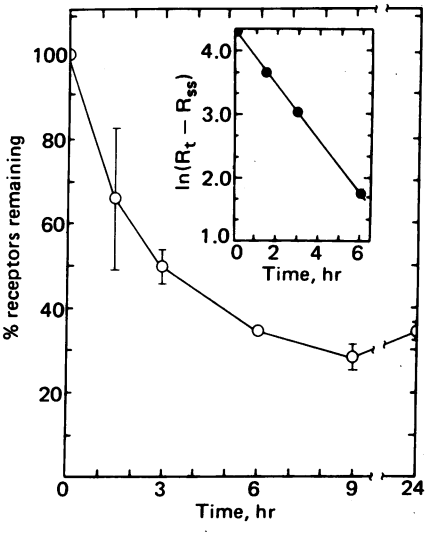

FIG. 4. Rate of [^{3}H]QNB binding loss stimulated by MAcChoR activation. Aggregate cultures, of conceptual age day 14, were incubated with 1 mM carbachol for the indicated times. Data points represent the mean of two cultures ±SD. (*Inset*) Plot of rate of binding loss with time. The steady state receptor concentration (R_{ss}) for this experiment is 28.4 fmol/mg of protein, reached after a 9-hr exposure to carbachol.

of time of exposure to agonist. A saturating dose of carbachol, 1 mM, reduced binding half-maximally by 1.6 hr and maximally (72%) by 9 hr. Continued exposure for an additional 15 hr, or for 4 days as in Table 2, did not further decrease binding sites, but did maintain receptors at the reduced level.

Method for Calculating Rate Constants for Receptor Appearance and Disappearance. We have analyzed the rate of receptor loss graphically to obtain constants for receptor appearance and disappearance without using protein synthesis inhibitors. Kinetic analysis of the turnover of induced enzymes, discussed by Schimke (38), has been applied here to the turnover of activated receptors. In this analysis, $dR/dT = A - DR$, where R is receptor concentration, A is taken to be a zeroth order rate constant for receptor appearance, and D is taken to be a first-order rate constant for receptor disappearance. If this equation holds, a plot of log ($R - R_{steady\text{-}state}$) versus time should be linear, with a slope of $-D$. Furthermore, because $R_{ss} = A/D$, the rate constant for receptor appearance also can be obtained. The *inset* to Fig. 4 shows log ($R - R_{ss}$) versus time for cultures activated with 1 mM carbachol. As the model predicts, the plot is linear ($r = 0.99$). The calculated receptor disappearance rate constant is 0.42 per hr, giving a half-life for the activated receptor of $0.69 \div 0.42$, or 1.6 hr. This is close to the $t_{1/2}$ for activated muscarinic receptors measured in the presence of cycloheximide (unpublished results). Because R_{ss} was 28.4 fmol/mg of protein, the appearance rate constant is 28.4×0.42, or 11.9 fmol/mg of protein per hr.

Cellular Mechanism of Receptor Regulation. N1E-115 cell cultures were incubated with various agents in order to discern which neuronal processes might be involved in the mechanism of regulation. Receptor regulation was blocked to varying degrees (Table 3) by the microfilament disruptor, cytochalasin B (68%), the glycolysis inhibitor, deoxyglucose (26%), and the crosslinking agent, glutaraldehyde (100%). Four conditions were used in testing each agent: (*i*) No addition, (*ii*) addition of carbachol, (*iii*) addition of agent alone, (*iv*) addition of agent for 30 min followed by addition of carbachol. The extent of carbachol-stimulated receptor loss thus was determined in the presence and absence of the perturbing agent. The effect of

Table 3. Effects of chemical treatments on receptor regulation

Agent	Specific [^{3}H]QNB binding, % control	% inhibition of receptor loss
No addition	100	—
Glutaraldehyde (1%)	74	100
Cytochalasin B (5 μg/ml)	117	68
Deoxyglucose (10 mM) + CCCP* (10 μM)	113	32
Deoxyglucose (10 mM)	126	26
Triton X-100 (0.05%)	98	7
CCCP* (10 μM)	101	0
Tetracaine (10 μM)	103	0
Colchicine (10 μM)	96	0
EGTA (5 mM)	90	−7

N1E-115 neuroblastoma cell cultures were treated with the indicated agents for 30 min. Glutaraldehyde was subsequently washed out four times with medium. For each agent tested, two cultures were activated for 30 min after agent treatment, whereas two parallel cultures were not activated as controls. Activated cultures were incubated 4 hr with 1 mM carbachol. All cultures were harvested and washed 4.5 hr after addition of agent. The degree to which each agent blocked carbachol-stimulated receptor loss was obtained by comparing the percentage of carbachol-induced binding loss in the presence and absence of agent.

* Carbonyl cyanide *m*-chlorophenylhydrazone.

each agent on [^{3}H]QNB binding to homogenates was assessed by comparing binding levels between cultures given agent alone and cultures not treated. Only glutaraldehyde substantially lowered [^{3}H]QNB binding levels (74% of control).

DISCUSSION

We have shown that muscarinic receptor activation in cells from the central nervous system causes a decrease in the concentration of muscarinic receptor binding sites. Regulation of muscarinic receptors was observed initially in the neuroblastoma–glioma hybrid cell NG108-15 (26). Coupled with recent reports on catecholamine receptors (22–25), our results suggest that neuroreceptor regulation may be a significant and general control mechanism in the central nervous system. Evidence presented here shows that neuroreceptor regulation is likely a part of normal synaptic activity. Additionally, our experiments provide quantitative analysis of receptor regulation and turnover in cells from the central nervous system, and they suggest that regulation involves a microfilament-mediated process.

Comparison of regulation in different cell types indicates that muscarinic receptors are regulated by normal synaptic activity. Because receptor loss caused by addition of activator can be blocked by atropine, we would predict that atropine should increase receptor levels in cell systems having spontaneous cholinergic activity. Atropine should have no affect on cell systems lacking synaptic activity. Our experiments confirm this prediction. In aggregate cell cultures, which make synapses (35) and have spontaneous activity (36), atropine increases receptor levels, but in the adrenergic clone N1E-115 (37), which does not make muscarinic synapses, atropine does not affect receptor levels.

The basic features of experimentally stimulated receptor loss also are consistent with regulation's being a physiological response. The ability of carbachol to regulate is commensurate with its ability to bind to the receptor (compare Figs. 2 and 4). Physiological doses of activator, equivalent to those that might occur in the synaptic cleft (39) and that are effective in regulating intracellular cyclic nucleotide concentrations (30), are effective in stimulating receptor loss. Furthermore, loss only occurs when activating ligands are used. Simple occupancy of the receptor binding site thus does not stimulate binding site loss. Additionally, receptor loss is reversible, showing that activators do not lower receptor levels by destroying cholinoceptive cells.

The turnover kinetics of activated receptors also are in harmony with a physiological role for receptor regulation. Regulation can occur in the absence of protein synthesis (unpublished results), indicating that activation increases the rate of disappearance. The half-life of the activated receptor is 1.6 hr, and the accelerated rate of disappearance is constant throughout the time activator is present (Fig. 4). Thus, even though the change from one steady state to the next takes 9 hr, the accelerated rate of receptor disappearance begins essentially upon addition of activator. This suggests a sensitive, highly responsive mechanism suitable for regulation by bursts of activity. In support of this, we have found that both nonsaturating doses of activator (Fig. 3) and intermittent activation (unpublished results), are sufficient for regulation. Although the method of analysis used here has not previously been employed in receptor studies, it appears to be a useful tool for determining turnover rate constants, especially because it obviates the need to block protein synthesis.

Ample evidence supports a correlation between receptor density and cellular responses to transmitter stimulation. We have reported that loss of muscarinic receptors in the NG108-15 hybrid cell line (26) is accompanied by a decreased ability of acetylcholine to inhibit adenylate cyclase (30). Muscarinic function in heart cells also is dependent on receptor density (31). Loss of β-adrenergic receptors in the pineal gland appears to account for the decreased ability of norepinephrine to stimulate cyclic AMP formation (16). In myasthenia gravis, a disease accompanied by nicotinic receptor loss (40), the debilitating symptom is decreased muscle responsiveness. A logarithmic relationship between nicotinic receptor density and muscle sensitivity to acetylcholine has been established (41). Furthermore, receptor density appears to correlate with synaptic responsiveness at the neurological level, because partial pharmacological blockade of receptors causes profound behavioral alterations (42, 43). Changes in synaptic strength as a result of regulation of receptor concentration thus could be significant in the integration of synaptic signals.

The molecular mechanism by which muscarinic receptors are regulated is largely unknown. Decreases in insulin and epidermal growth factor receptors appear due to internalization of receptors (44–46), consistent with regulation at the level of receptor disappearance. Our preliminary experiments suggest that internalization may occur in the regulation of muscarinic receptors as well. Regulation does not occur in broken cell preparations (unpublished results), and, most significantly, cytochalasin B blocks experimentally induced receptor loss. Preliminary experiments indicate that cytochalasin B has no effect on muscarinic ligand binding to whole cultured cells. Cytochalasin B is known to disrupt filaments and to block endocytosis (47), although it also may have nonspecific effects such as inhibition of hexose transport (48). It has been hypothesized that internalized receptors play a role in the long-term consequences of hormone action (49). Conceivably, in addition to serving as a negative feedback mechanism for synaptic communication, internalization of neuroreceptors also could be important in mediating neurotrophic phenomena.

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