Vol. 90, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 27, 1979 Pages 506-512

MUSCARINIC ACETYLCHOLINE RECEPTOR REGULATION BY ACCELERATED RATE OF RECEPTOR LOSS

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Received July 27,1979

Sustained activation of muscarinic acetylcholine receptors on neuron-like NG108-15 hybrid cells reduces the number of [^{3}H]quinuclidinyl benzilate binding sites per cell as much as 88%. The response occurs at concentrations of agonists commensurate with those needed to occupy receptors and inhibit adenylate cyclase. Decreases in steady-state receptor levels persist as long as activator remains present. Withdrawal of activator results in a slow increase in receptor levels that is blocked by cycloheximide. Activation shortens receptor half-life by a factor of nearly 4, indicating that regulation occurs at the level of receptor breakdown.

Synaptic transmission provides a basis for intercellular communication in the nervous system. For many years, it has been suggested that synaptic transmission is a regulated process (1-3). On a molecular level, modulation of synaptic transmission likely could occur through regulation of neuroreceptor concentration (4,5). In this paper, we have used cultured NG108-15 hybrid cells (6) to test the hypothesis that muscarinic acetylcholine (ACh) receptors are regulated by their own activation. Activation of muscarinic ACh receptors, which occur widely throughout the nervous system (7), causes slow changes in membrane potentials and in cyclic nucleotide concentrations (8,9). Muscarinic ACh receptors found on neuron-like NG108-15 cells bind [^{3}H]quinuclidinyl benzilate (QNB) (10) with high affinity (11,12) and respond to ACh initially by causing membrane depolarization and inhibition of adenylate cyclase (13). Sustained incubation of cells with cholinergic agonists causes total adenylate cyclase activity to increase, making the cells supersensitive to activators of adenylate cyclase such as prostaglandin E_1 (5,13).

In a preliminary communication, we reported our discovery that sustained activation of muscarinic ACh receptors decreases the number of [^{3}H]QNB binding sites per cell (5). This report confirms that discovery and provides further

Abbreviations used: ACh, acetylcholine; QNB, quinuclidinyl benzilate

0006-291X/79/180506-07$01.00/0

information on the regulation of binding sites in NG108-15 cells. Recent studies on muscarinic ACh receptors of other cultured cells (14) and on receptors of experimental animals (15) have shown that muscarinic ACh receptor regulation by receptor activation is a widespread response.

MATERIALS AND METHODS

[^{3}H]QNB was obtained from Amersham-Searle. Oxotremorine was obtained from Aldrich and all other compounds were obtained from Sigma.

NG108-15 cells of passage 15-19 were grown as described (13). To test the long-term effects of neurotransmitters and drugs on muscarinic ACh receptor concentration, replicate (2-4) cultures of confluent NG108-15 cells received the desired compound added to normal growth medium. After incubation for the indicated times, all cultures were brought to equivalent conditions, medium was removed, and cultures were washed 5 times with culture medium lacking serum at 37° over a period of 10 minutes. Cells were harvested by shaking, homogenized in 0.05 M TrisCl pH 7.8, and either assayed immediately for [^{3}H]QNB binding or first frozen and assayed at a later date. Freezing and thawing had no effect on [^{3}H]QNB binding.

A filtration assay for specific [^{3}H]QNB binding to determine muscarinic ACh receptor numbers (10) was modified as follows. Homogenates were incubated 90 minutes at 24° in 0.05 M TrisCl pH 7.8 with 5 x 10^{-10} M [^{3}H]QNB, quenched with 5 ml buffer, collected on Whatman GF/C filters, washed 3 times with 5 ml volumes of buffer, and tritium per filter determined by liquid scintillation counting. Binding in the presence of 10^{-3} M oxotremorine was taken to be nonspecific. Duplicate measurements of total and nonspecific binding were done for each culture and the differences between duplicates routinely were less than 10% of the mean. Nonspecific binding was 5% of total binding. Specific binding was linearly dependent on the amount of protein per sample.

Adenylate cyclase activity was measured as described (13).

RESULTS AND DISCUSSION

[^{3}H]QNB binding sites were measured in homogenates from NG108-15 cells grown with or without 10^{-3} M ACh for 24 hours in order to test the effect of sustained activation on muscarinic ACh receptor levels. Scatchard plots of specific [^{3}H]-QNB binding show that the maximum number of binding sites decreased 40% in cultures to which ACh had been added (Figure 1). The maximum number of binding sites, obtained by extrapolation of each plot to its abscissa intercept, was 35 fmols/mg protein in control cultures and 20 fmols/mg protein in ACh-treated cultures. While the number of binding sites in control cultures is variable between preparations, cultures harvested at the same time have nearly identical binding levels. Treatment with ACh always induces loss of binding sites irrespective of control levels of binding. As can be seen from the slopes of the Scatchard plots, growth of cells with ACh causes no significant change in the affinity of receptors for

[^{3}H]QNB. This indicates that the loss of binding sites is not due to a simple conformation change.

In experiments not shown here, we have found that various activators of muscarinic ACh receptors (acetylcholine, oxotremorine, arecoline, carbachol) all cause a dose-dependent loss of [^{3}H]QNB binding sites from cultured NG108-15 cells, but that blockers of muscarinic ACh receptors (atropine, scopolamine) cause no loss. Atropine at 3×10^{-9} M completely inhibits the loss of receptors caused by 10^{-5} M carbachol. Loss of binding sites thus is muscarinic ACh receptor-mediated and is strictly dependent on activation. Binding site loss clearly is not induced by simple occupancy of receptors by ligands or by secondary effects of activators at cellular sites other than receptors. We also have found that growth of cells in agonists has no effect on cell doubling time or on the incorporation of [^{3}H]uridine and [^{3}H]valine into RNA and protein.

A detailed study of receptor loss as a function of added carbachol concentration was done to test whether the response occurred at concentrations appropriate with respect to receptor activation. Figure 2 shows that the maximum decrease in receptor sites (88%) occurs at 10^{-3} M carbachol and that 50% of the maximum decrease occurs at 4×10^{-6} M carbachol. For comparison, Figure 2 also shows the ability of carbachol to inhibit binding of 5×10^{-10} M [^{3}H]QNB and to inhibit the basal activity of adenylate cyclase. Carbachol inhibits [^{3}H]QNB binding by 50% at 2×10^{-6} M and has a 50% maximum effect on adenylate cyclase at 10^{-5} M. In intact cells, 50% occupancy of receptors by carbachol has been calculated to occur at 4×10^{-6} M (11). These measurements show that receptor loss occurs at doses of activator commensurate with those expected to occupy binding sites and cause physiological responses.

The time course of receptor loss shows that new steady-states are attained within 4 hours of adding carbachol to cell cultures (Figure 3). These steady-states persist for at least 3 days if carbachol is present at a fixed concentration. Some variation in times needed to attain steady-state have been observed, but the maximum decrease always occurs within 9 hours. Considering the high

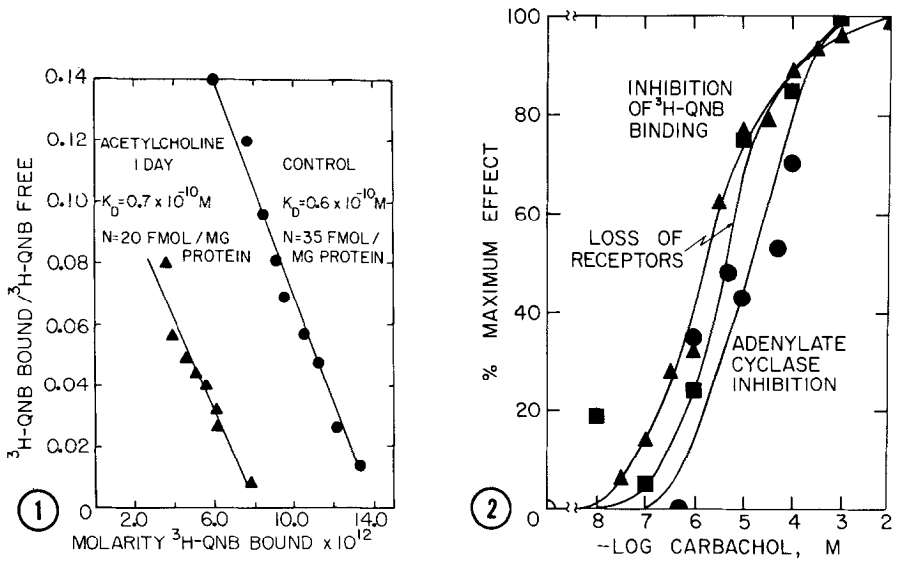

Figure 1. Decrease in Muscarinic Acetylcholine Receptor Binding Sites by Cell Growth in Acetylcholine 5 μm eserine was added to 30 confluent cultures, and after 30 minutes, 20 of these cultures also received 10^{-3} M acetylcholine. Fresh medium containing the appropriate additions was supplied 5 and 19 hours later. After 24 hours, cultures were pooled and specific binding of [^{3}H]QNB was determined as described. Concentrations of [^{3}H]QNB used to establish saturation ranged from 5 x 10^{-11} M to 10^{-9} M. Scatchard plots of specific binding to control homogenates (●—●) and acetylcholine-treated samples (▲—▲) are shown. K_Ds indicated are for the active isomer of [^{3}H]QNB.

Figure 2. Dose-Response Curves for Carbachol Inhibition of specific [^{3}H]-QNB binding to homogenates (▲—▲) and inhibition of adenylate cyclase activity in homogenates (●—●) by increasing concentrations of carbachol were measured as described. Maximum dose of carbachol completely inhibited specific [^{3}H]QNB binding and inhibited adenylate cyclase 31%, from 15 pmols cAMP formed/min/mg protein to 10.3 pmols cAMP formed/min/mg protein. Loss of receptors (■—■) due to 24 hour incubation of cells with the indicated carbachol concentrations was determined as described. Maximum carbachol decreased receptor density 88%, from 24 fmols/mg protein to 3 fmols/mg protein.

frequency of synaptic transmission found in the nervous system (16), it seems likely that sustained incubation of NG108-15 cells with agonist may represent a more physiological condition than incubation of cells without agonists. It is clear, both from Figure 2 and Figure 3, that even subsaturating doses of agonists causes a sustained and constant degree of receptor loss and that steady state receptor concentrations are highly responsive to the level of receptor activation.

Withdrawal of agonist from cultures leads to a 40% increase in receptors after 6 hours (Figure 4). By 40 hours, receptor levels are nearly double the value measured before withdrawal. This increase might be considered analogous

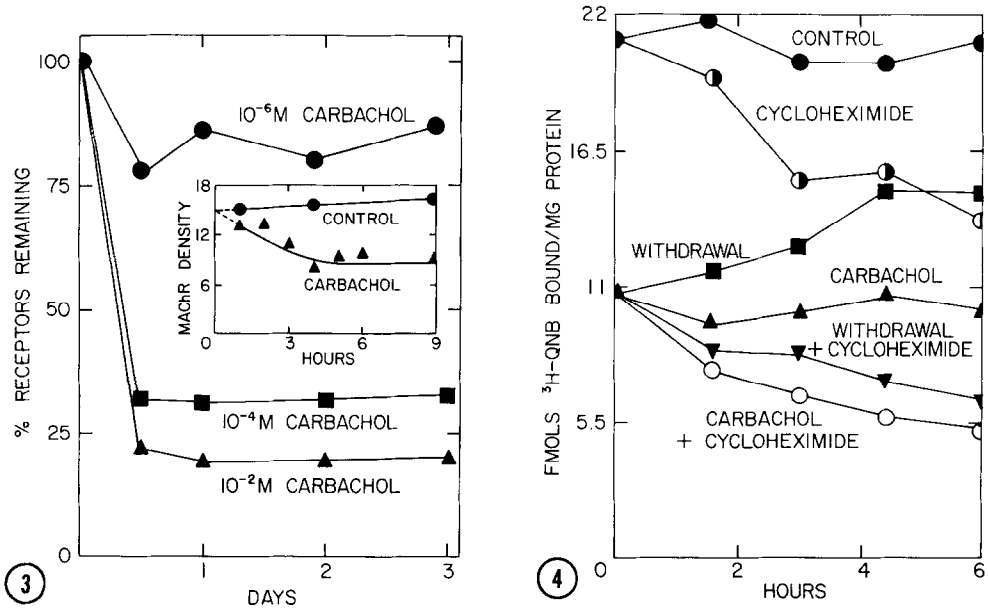

Figure 3. Long-lasting Reduction in Receptor Concentration in Presence of Carbachol Cultures were incubated with 10^{-6} M carbachol (●—●), 10^{-4} M carbachol (■—■), or 10^{-2} M carbachol (▲—▲) for periods of 0.01 to 3 days prior to harvesting. All cultures were harvested on the same day and homogenates assayed for specific [^{3}H]QNB binding as described. 100% equals 22 fmols [^{3}H]QNB bound per mg protein. INSET: Early Time Course of Receptor Loss Cultures containing 5 µm eserine for 30' were further incubated with (▲—▲) or without (●—●) 10^{-5} M carbachol for the indicated times, harvested, and specific [^{3}H]QNB binding determined.

Figure 4. Inhibition by Cycloheximide of Receptor Increase Following Carbachol Withdrawal Cultures were incubated with or without 10^{-4} M carbachol for 24 hours prior to start of experiment. At zero time, all cultures were washed 4 times with new medium containing the appropriate additions. Receptor concentrations were measured during the next 6 hours at the indicated times. Three pairs of culture conditions were employed: continued control (●—●), continued control with 180 µm cycloheximide (●—●), continued 10^{-4} M carbachol (▲—▲), continued 10^{-4} M carbachol with 180 µm cycloheximide (○—○), withdrawal by removing carbachol from the medium (■—■), withdrawal with 180 µm cycloheximide (▼—▼).

to the development of denervation-induced supersensitivity found in the central nervous system for dopamine receptors and norepinephrine receptors (17,18). The fact that receptor loss is reversible also shows that sustained activation is not lethal for a subpopulation of cholinoceptive cells, and the slow time course for recovery distinguishes the process from depolarization desensitization.

Another significant point demonstrated in Figure 4 is that the increase in receptors following withdrawal of agonist is dependent on protein synthesis. Cycloheximide completely blocks the rise in receptor levels following withdrawal.

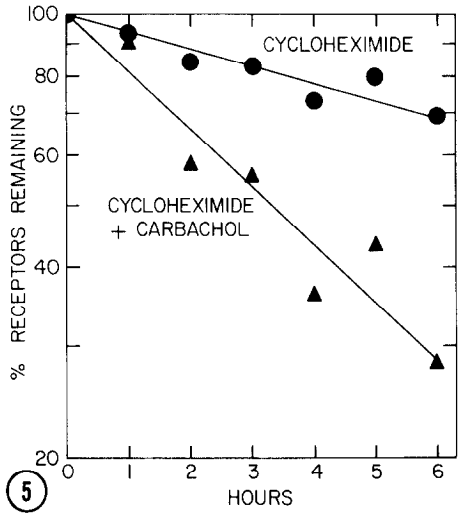

Figure 5. Stimulation of Receptor Breakdown by Carbachol Previously untreated cultures were given fresh medium containing 180 μm cycloheximide with (▲—▲) or without (●—●) 10^{-4} carbachol. Cells were harvested at the indicated times and specific [^{3}H]QNB binding determined. Receptor density without cycloheximide (100%) was 25 fmols/mg protein, determined both at the beginning and end of the experiment.

This result suggests that the decrease in [^{3}H]QNB binding sites most likely is due to an actual loss of receptor molecules. The evidence is indirect, however, and we currently are investigating the molecular nature of binding site loss in greater detail.

Changes in receptor levels could result from altered rates of receptor appearance, disappearance, or both. In order to measure the effect of receptor activation specifically on the rate of receptor disappearance, we compared the rate of receptor loss from cultures treated with cycloheximide with the rate of loss from cultures treated with cycloheximide and carbachol. Receptor levels begin to decrease immediately upon addition of cycloheximide to cultures (Figure5) We observed no lag analogous to that reported for nicotinic ACh receptors in skeletal muscle under similar conditions (19). It can be seen from Figure 5 that carbachol significantly shortens receptor half-life. In the absence of carbachol receptor half-life is approximately 11 hours, but in the presence of carbachol receptor half-life is only 3 hours. The half-life of activated muscarinic ACh receptors in cultured central nervous system cells, in the absence of cycloheximide, has been calculated to be 1.6 hours (14).

Our data show that activation of muscarinic ACh receptors in intact cells accelerates the rate of receptor disappearance, decreasing steady-state receptor concentrations by up to 88%. The number of muscarinic ACh receptors per cell thus is regulated by receptor activity. The experiments presented confirm and extend our preliminary communication in which the discovery of muscarinic ACh receptor regulation was first reported (15). Such regulation, seen here in cultured NG108-15 hybrid cells, could provide a simple molecular mechanism for the modulation of synaptic communication.

Acknowledgments: This work was supported in part by grants to W.L.K. from the Epilepsy Foundation of America and N.I.H. (1 R01 N515299-01).

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