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Regulation of adenylate cyclase activity mediated by muscarinic acetylcholine receptors

(synapse plasticity/cyclic AMP/neuroblastoma × glioma hybrid cells)

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ABSTRACT **Carbachol, an activator of muscarinic acetylcholine receptors of NG108-15 hybrid cells, inhibits adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] rapidly and reversibly and slowly evokes a 200–300% increase in adenylate cyclase activity over a period of 24–30 hr. Both the inhibition of adenylate cyclase and the gradual increase in enzyme activity are dependent on muscarinic acetylcholine receptors and the receptor activator. Withdrawal of carbachol results in a gradual return of adenylate cyclase activity to control levels over a period of 6 hr; the half-life for decay of enzyme activity is 1.6 hr. These results show that muscarinic acetylcholine receptors mediate both transient and long-lived effects on adenylate cyclase activity that resemble those of opiates.**

The possibility that cyclic AMP (cAMP) or cyclic GMP (cGMP) functions as an intermediate or a modifier of transynaptic communication at certain synapses has been considered frequently. Evidence has been reported that suggests that cAMP plays a role at synapses terminating on Purkinje neurons of the cerebellum (1, 2) and evidence for (3–6) and against (7) roles for cAMP and cGMP at synapses in sympathetic ganglia has been described.

Mouse neuroblastoma × rat glioma NG108-15 hybrid cells resemble neurons in many ways. The cells synthesize acetylcholine, have electrically excitable membranes, generate Na^+ and Ca^{2+} action potentials,‡ have clear vesicles and large dense-core vesicles (8), and form synapses with cultured striated muscle cells (9). The cells possess receptors that are coupled to the activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], such as adenosine and prostaglandin E_1 (PGE_1) receptors, and have other receptors coupled to the inhibition of adenylate cyclase, such as opiate receptors, muscarinic acetylcholine receptors, and α-adrenergic receptors (10–13). When NG108-15 cells are cultured in the presence of morphine (14, 15) or norepinephrine (13) for 10–24 hr, the specific activity of adenylate cyclase slowly increases and thus compensates for the inhibition of enzyme activity (14–16). Growth of NG108-15 cells in the presence of carbachol, a relatively stable analog of acetylcholine, was reported by Traber *et al.* (15) to result in an increase in cAMP accumulation by the cells.

In a preliminary communication (16), we reported that growth of NG108-15 cells in the presence of carbachol gradually results in an increase in the specific activity of adenylate cyclase and a decrease in the number of muscarinic acetylcholine receptors of cells. In this paper we examine the increase in adenylate cyclase mediated by muscarinic acetylcholine receptors.

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MATERIALS AND METHODS

Materials. The sources of some of the chemicals have been described (10). In addition, [α-^{32}P]ATP was obtained from New England Nuclear; atropine sulfate, arecoline hydrogen bromide, acetylcholine chloride, carbamylcholine chloride, and eserine sulfate, from Sigma; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), from Calbiochem; and oxotremorine, from Aldrich.

Cell Culture. Mouse neuroblastoma × rat glioma NG108-15 hybrid cells‡ derived by fusion of mouse C1300 neuroblastoma clone N18TG2 (17) and rat glioma C6BU-1 (18) were grown as described (19).

cAMP Assay. Confluent NG108-15 cells (3–5 mg of protein per 60-mm dish, 20 cm^2 surface area) were washed three times, each time with 5 ml of the Dulbecco–Vogt modification of Eagle's minimal essential medium (DMEM, Gibco, cat. no. H-21) supplemented with 10 mM Hepes (pH 7.4) instead of $NaHCO_3$ and adjusted to 340 mOsmol/liter with NaCl. To examine the cAMP-synthesizing capacity of the cells, 3.5 ml of DMEM (as above except 25 mM Hepes) containing 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro20-1724) and 1 μM atropine was added and cells were incubated for 10 min at 37° (atropine was added to inhibit the possible effects of traces of carbachol, present during growth, that may not have been removed by washing). Cells were incubated for an additional 5 min to test cAMP accumulation. cAMP levels were determined as described (10) by the method of Gilman (20). Values reported are corrected to 100% recovery of cAMP; each is the mean of values obtained from two or three dishes. Three or four concentrations of the extract from each dish were assayed for cAMP. The standard errors of the mean were ±10%.

Adenylate Cyclase Assays. Confluent cells were washed three times, harvested (10–13 mg of protein per 100-mm dish), suspended in 290 mM sucrose/25 mM Tris·HCl, pH 7.4, at 4–6 mg of protein per ml, frozen rapidly in small aliquots, and stored in a liquid N_2 freezer. Before use, extracts were thawed and homogenized in a ground-glass homogenizer with 10 strokes of a motor-driven pestle for 20 sec at 1200 rpm. Adenylate cyclase activity in cell-free homogenates was determined by a procedure (14) based on method C of Salomon *et al.* (21).

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PGE_1, prostaglandin E_1.

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‡ B. Hamprecht, T. Amano, and M. Nirenberg, unpublished data.

Table 1. cAMP accumulation by NG108-15 cells grown with or without carbachol

Addition for test	Growth conditions: None 43 hr	10 μM carbachol 19 hr	10 μM carbachol 43 hr
	pmol cAMP in cells and medium/mg protein		
Control	467	1258	1046
PGE_1, 10 μM	4530	6905	6280

Cells were grown with or without carbachol for the time indicated and then washed and incubated for 10 min at 37° with 0.5 mM Ro-20 1724 and 1 μM atropine sulfate. Cells were incubated for an additional 5 min with or without PGE_1 to test cAMP accumulation in cells and medium. All cells were tested and deproteinized at the same time.

Values reported are mean values obtained from two or three homogenates, prepared from separate dishes, each assayed at four protein concentrations (40–200 μg of protein per reaction mixture) with standard errors of the mean < ±10%. Where indicated, 0.5–1 μM atropine was added to reaction mixtures to inhibit possible effects of trace amounts of carbachol not removed by washing the cells.

RESULTS

As shown in Table 1, levels of cAMP in NG108-15 cells are elevated after growth of cells in the presence of carbachol, a relatively stable analog of acetylcholine. Cells were grown with or without 10 μM carbachol for 19 or 43 hr, washed, and incubated for 10 min with a phosphodiesterase inhibitor and then for an additional 5 min with or without PGE_1 to test cAMP accumulation in cells and medium. Growth of cells in the presence of carbachol for 19 hr resulted in a 300% increase in basal cAMP and a 50% increase in PGE_1-stimulated cAMP accumulation.

In Fig. 1, the relationship between the carbachol-dependent increase in specific activity of adenylate cyclase and the carbachol concentration during cell growth is shown. Growth of cells for 2 days in the presence of 0.1–100 μM carbachol resulted in increases in basal and PGE_1-stimulated adenylate cyclase activities. When cells were treated with 100 μM carbachol, basal adenylate cyclase activity was 225% higher and PGE_1-stimulated activity was 63% higher than control activities.

The muscarinic acetylcholine receptor antagonist atropine

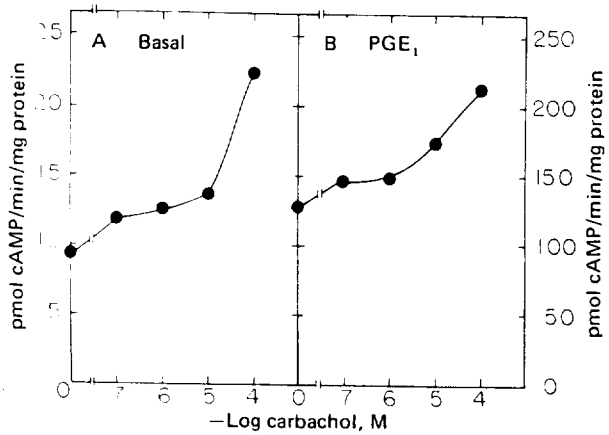

FIG. 1. Change in adenylate cyclase specific activity after 48 hr of growth of NG108-15 in the presence of various concentrations of carbachol. (*A*) Basal specific activity; (*B*) with 10 μM PGE_1. Reaction mixtures contained 0.5 μM atropine sulfate.

Table 2. Effects of growing NG108-15 cells with carbachol and atropine on adenylate cyclase activity

Cell growth conditions for 2 days	Adenylate cyclase activity: Basal	10 μM PGE_1
	pmol cAMP/min/mg protein	
Control	5	42
Carbachol, 100 μM	16	137
Atropine, 1 μM	7	61
Carbachol, 100 μM + atropine, 1 μM	5	43

Each reaction mixture contained 0.5 μM atropine sulfate.

blocked the carbachol-dependent increase in adenylate cyclase activity (Table 2). Growth of cells for 2 days in the presence of 100 μM carbachol resulted in 3-fold increases in basal and PGE_1-stimulated adenylated cyclase activities. However, the presence of 1 μM atropine had little effect on adenylate cyclase activity, which shows that occupancy of the receptor by a receptor antagonist does not result in an increase in the specific activity of adenylate cyclase. However, 1 μM atropine blocked the carbachol-dependent increase in adenylate cyclase activity, which suggests that the increase in enzyme activity is mediated by muscarinic acetylcholine receptors.

Exposure of cells to acetylcholine for 24 hr also increased basal and PGE_1-stimulated activities of adenylate cyclase 65% and 32%, respectively (Table 3). Serum was omitted in this experiment to eliminate possible effects of serum cholinesterase (EC 3.1.1.7).

The specific activities of adenylate cyclase in homogenates of cells grown for different times with or without 100 μM carbamylcholine are shown in Fig. 2. In this experiment, the specific activities of adenylate cyclase from both control and carbachol-treated cells decreased with time; however, the decrease in specific activity found with homogenates from carbachol-treated cells was less than that found with homogenates from control cells. In most experiments, however, the specific activity of adenylate cyclase increased when NG108-15 cells were treated with carbachol. The maximum difference in adenylate cyclase specific activity of cells grown with or without carbachol usually was attained in 20 to 30 hr.

NG108-15 cells were grown in the presence of carbachol for 24 hr and then for 8 hr in the absence of carbachol. As shown in Fig. 3. the increased specific activity of adenylate cyclase returned to control values in 5–8 hr. The half-life of the car-

Table 3. Adenylate cyclase activity of NG108-15 cells grown with or without acetylcholine

Additions during growth	Adenylate cyclase assay: Basal	10 μM PGE_1
	pmol [^{32}P]cAMP/min/mg protein	
Control	15	204
Eserine sulfate, 2.5 μM	16	210
Acetylcholine chloride, 1 mM + eserine sulfate, 2.5 μM	27	272

Cultures of NG108-15 cells, approximately 85% confluent, were washed twice with medium without serum and then cultured for an additional 24 hr (with two changes of media) with the indicated compounds in the absence of serum to eliminate possible effects of serum cholinesterase. Adenylate cyclase was assayed as described in *Materials and Methods*, in the presence of 0.5 μM atropine sulfate.

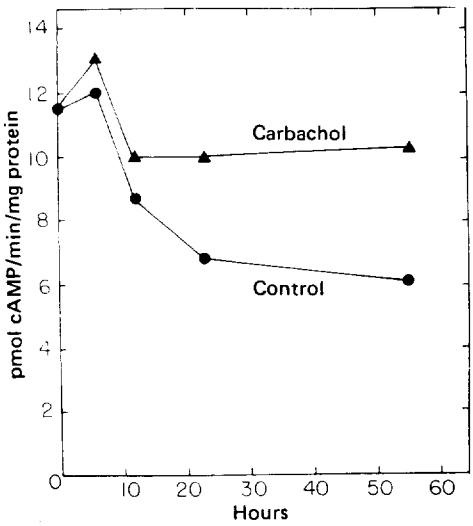

FIG. 2. NG108-15 cells were grown with or without carbachol and, at the times shown, homogenates were prepared and assayed for adenylate cyclase activity. ●, 0.9% NaCl; ▲, 100 μM carbachol chloride dissolved in 0.9% NaCl. Adenylate cyclase reaction mixtures contained 0.5 μM atropine sulfate.

bachol-dependent enzyme activity in the absence of carbachol was 1.6 hr; enzyme activity decreased exponentially with an estimated first-order rate constant (k) for loss of enzyme activity of 0.007 min^{-1} (Fig. 3 *inset*). Thus, the carbachol-dependent increase in adenylate cyclase activity is acquired slowly and is relatively long-lived compared to hormone- or neurotransmitter-dependent activation or inhibition of the enzyme.

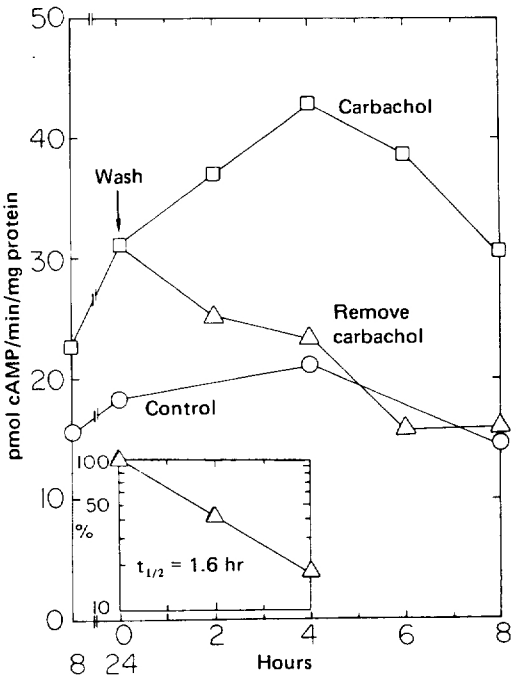

FIG. 3. Decrease in adenylate cyclase activity after carbachol withdrawal. NG108-15 cells were grown for 24 hr without carbachol (○) (control cells), or with 100 μM carbachol (□). At 24 hr (zero time for withdrawal of carbachol), cells were washed twice and the medium was replaced with fresh medium without carbachol (○, control cells; △, carbachol-treated cells), or with 100 μM carbachol (□, carbachol-treated cells). (*Inset*) Rate of decay of the carbachol-dependent increase in adenylate cyclase activity due to withdrawal of carbachol.

Table 4. Decreased sensitivity of adenylate cyclase to carbachol in NG108-15 cells grown in the presence of carbachol

Cell growth conditions	Adenylate cyclase assay: 0.5 μM atropine	100 μM carbachol	% inhibition
	pmol cAMP/min/mg protein		
Control	44	32	27
Carbachol, 100 μM, 0.25 hr	43	31	28
Carbachol, 100 μM, 24 hr	81	74	9

Adenylate cyclase assays were performed as described in *Materials and Methods*, except that the pH of reaction mixtures was 8.0, which increases both the activity of adenylate cyclase and the sensitivity of the enzyme to inhibition by carbachol.

Adenylate cyclase from NG108-15 cells exposed to carbachol for 24 hr was less sensitive to inhibition by carbachol than was the enzyme from control cells (Table 4). In contrast, treatment of cells with carbachol for 15 min did not reduce the sensitivity of adenylate cyclase to carbachol. This phenomenon, first observed by S. Sharma,[§] probably results from a gradual loss, over 5–10 hr, in the number of muscarinic acetylcholine receptors when NG108-15 cells are grown in the presence of carbachol (16).

DISCUSSION

The results show that carbachol, an activator of muscarinic acetylcholine receptors of NG108-15 hybrid cells, inhibits adenylate cyclase rapidly and reversibly and also slowly evokes an increase in adenylate cyclase activity over a period of 24–30 hr. Both the inhibition of adenylate cyclase and the gradual increase in enzyme activity are dependent on muscarinic acetylcholine receptors and the receptor activator, and both effects are blocked by 1 μM atropine. However, the carbachol-dependent increase in adenylate cyclase activity, once acquired, can be expressed independently in the absence of carbachol.

NG108-15 cells also possess opiate receptors and α-adrenergic receptors that similarly are coupled to an inhibition of adenylate cyclase and a gradual increase in enzyme activity. The cells seem to acquire tolerance to the inhibitor but instead become dependent on the inhibitor to maintain normal cAMP levels because withdrawal of the inhibitor can result in a long-lived increase in adenylate cyclase activity. Withdrawal of carbachol from NG108-15 cells that had been grown for 24 hr in the presence of carbachol resulted in an increase in adenylate cyclase activity for 4–5 hr. Carbachol-dependent adenylate cyclase activity decayed with a half-life of 1.6 hr. These and previous results show that acetylcholine and norepinephrine (13) have biological activities that resemble those of opiates (22, 23). The results also suggest that other species of receptors mediating an inhibition of adenylate cyclase also may function to increase the specific activity of the enzyme.

Thus far, NG108-15 cells have been shown to respond to activators of muscarinic acetylcholine receptors in five ways: cells depolarize,[‡] intracellular cGMP levels increase slightly,[¶] adenylate cyclase is inhibited and thus cAMP levels fall (10, 12), a receptor-mediated increase in adenylate cyclase activity is slowly evoked, and the number of muscarinic acetylcholine receptors slowly decreases over a period of hours in the presence of carbachol (16). Carbachol-dependent depolarization of cells and the increase in cGMP levels can desensitize completely in

[§] S. Sharma and M. Nirenberg, unpublished data.
[¶] H. Matsuzawa and M. Nirenberg, unpublished data.

1 min. In contrast, carbachol-dependent inhibition of adenylate cyclase is not desensitized under these conditions. It seems likely that the desensitized form of the muscarinic acetylcholine receptor, with respect to depolarization and cGMP accumulation, may be the active form with respect to inhibition of adenylate cyclase.

Adenylate cyclase in homogenates from NG108-15 cells grown in the presence of carbachol for 24 hr is inhibited less by carbachol than is adenylate cyclase from control cells. The decreased sensitivity of adenylate cyclase to carbachol probably is due to a carbachol-dependent decrease in muscarinic acetylcholine receptors (16). The recovery of receptors requires protein synthesis and a 24- to 48-hr incubation. Thus, an activator of the muscarinic acetylcholine receptors of NG108-15 cells can decrease cell responsiveness to that compound and, in addition, can regulate cell responsiveness to ligands that activate other species of receptors coupled to adenylate cyclase. For example, the acquisition of supersensitivity to PGE_1 was shown to be mediated by muscarinic acetylcholine receptors.

In summary, we find that activators of excitatory acetylcholine receptors of NG108-15 cells have both transient and long-lived effects on macromolecules that may be involved in transmission across certain synapses.

N.M.N. is a postdoctoral fellow of the Muscular Dystrophy Association.

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