

Receptor-mediated shifts in cGMP and cAMP levels in neuroblastoma cells

(acetylcholine receptor/prostaglandin E₁ receptor/adenosine receptor/synapse/neurotransmitter)

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ABSTRACT 3':5'-cGMP levels of neuroblastoma N1E-115 cells increase as much as 200-fold upon activation of muscarinic acetylcholine receptors, resulting in intracellular cGMP concentrations >600 pmol/mg of protein. The cells also have receptors for adenosine which mediate an increase in 3':5'-cAMP levels. Unexpectedly, prostaglandin E₁ was found to increase the concentrations of both cGMP and cAMP. Carbamylcholine, adenosine, and PGE₁ were added to cells separately and in pairs to determine the effect of one compound on cell responses to another. Reciprocal inhibition, unilateral inhibition, additive, and nonadditive responses were observed with respect to cGMP and cAMP levels when different pairs of receptors were activated simultaneously.

The response of neurons to transmitters frequently can be modified by other species of [transmitter-receptor] interactions; thus, two types of synaptic stimuli may potentiate or inhibit one another. The pharmacology and electrophysiology of such phenomena have been studied extensively but much remains to be learned about the mechanisms which couple the activities of different species of receptors. In part, the difficulty in defining the reactions associated with receptor activation stems from the heterogeneity of neural tissues with regard to cell type. One approach to this problem is to define receptor-mediated responses of relatively homogeneous, clonal neuroblastoma cells which have excitable membranes and respond to chemical and electrical stimuli.

In this report the effects of activators of inhibitory muscarinic acetylcholine receptors, adenosine receptors, and prostaglandin E₁ (PGE₁) receptors (1-5) on 3':5'-cGMP and 3':5'-cAMP levels of neuroblastoma cells are described. Muscarinic acetylcholine receptors frequently mediate slow, prolonged responses which may be excitatory or inhibitory; in contrast to the nicotinic acetylcholine receptor, which in higher organisms mediates fast excitatory responses. Activation of the inhibitory or excitatory muscarinic acetylcholine receptors of cardiac (6, 7) and smooth muscle (7) results in an increase in cGMP concentration. We find that cGMP levels of neuroblastoma N1E-115 cells are elevated as much as 200-fold upon activation of muscarinic acetylcholine receptors. When different pairs of receptors were activated simultaneously, reciprocal inhibition, unilateral inhibition, additive, and nonadditive responses were observed with respect to cGMP and cAMP levels.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: carbamylcholine chloride, atropine sulfate, and *d*-tubocurarine

Abbreviations: cGMP, guanosine 3':5'-cyclic monophosphate; cAMP, adenosine 3':5'-cyclic monophosphate; PGE₁, prostaglandin E₁; IBMX, 3-isobutyl-1-methyl-xanthine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DMEM, Dulbecco-Vogt modification of Eagle's minimal essential medium.

chloride from Sigma; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), Calbiochem; 3-isobutyl-1-methyl-xanthine (IBMX) from Aldrich Chem. Co.; PGE₁, a gift from Dr. John Pike, the Upjohn Co.; adenosine, Nutritional Biochem. Corp.; cAMP, Schwarz/Mann; cGMP, JEM Research Products; [G-³H]cAMP (22.1 Ci/mmol), New England Nuclear; [8-³H]cGMP (15 Ci/mmol), Amersham/Searle Corp.; cAMP-dependent protein kinase, Sigma; cGMP antiserum and succinyl-cGMP ¹²⁵I-tyrosine methyl ester, Collaborative Research, Inc.; fetal bovine serum, Colorado Serum Co.; and DMEM (the Dulbecco-Vogt modification of Eagle's minimal essential medium), GIBCO, Cat. no. H-21. Other chemicals were of reagent grade purity.

Cell Culture. The following cell lines were used: mouse neuroblastoma C-1300 clones N1E-115 and N18 (8), mouse neuroblastoma × mouse L cell hybrid clone NL1F (9), and rat glioma clone C6BU-1 (10). Neuroblastoma cells were grown in 100 mm petri dishes (Falcon, 55 cm² surface area) in 15 ml of 90% DMEM-10% fetal-bovine serum (340 mOsm/kg) in a humidified atmosphere of 10% CO₂-90% air at 37°. Hybrid cells were grown in the same medium supplemented with 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine (HAT). Cells usually were grown to confluency (1 to 3 × 10⁵ cells, 0.1 to 0.3 mg of protein per cm²) prior to use.

Incubation of Cells. Each plate was washed three times with 7 ml portions of solution A (DMEM minus NaHCO₃ with 25 mM Hepes adjusted to pH 7.4 with NaOH and to 340 mOsm/kg with NaCl). Unless stated otherwise, 10 ml of solution A supplemented with 0.5 mM IBMX were added to each plate and cells were equilibrated for 30 min at 37°. Reactions were initiated by the addition of 0.1 ml of a solution of the compound to be tested dissolved in 0.17 M NaCl, or 0.1 ml of a PGE₁ solution (1 mM PGE₁, 0.153 M NaCl, and 10% ethanol). The final concentration of ethanol in the petri dish, 0.1%, did not alter cGMP or cAMP levels of cells. Cells were incubated at 37° for the times specified, then the medium was removed by aspiration (discarded unless specified otherwise) and 4 ml of cold 5% trichloroacetic acid was added to the monolayer to terminate reactions and extract nucleotides. A solution (0.05 ml) containing 10,000 cpm of [³H]cGMP (0.67 pmol) and/or [³H]cAMP (0.45 pmol) was added to each dish and cells were detached by scraping. The suspension was transferred to a tube, the dish was washed with 1 ml of cold 5% trichloroacetic acid, and the combined suspension and wash was centrifuged for 15 min at 20,000 × *g* at 3°.

Purification of cGMP and cAMP. A rapid method was devised for separating cGMP from cAMP and removing trichloroacetic acid and endogenous interfering compounds from cyclic nucleotide fractions. The supernatant solution of the deproteinized sample in 5% trichloroacetic acid was applied to an ion exchange column (0.7 × 8 cm) of AG50W-X4

(200–400 mesh, hydrogen form, Bio-Rad) previously washed and equilibrated with H₂O. The column was washed with 2 ml of H₂O (eluates discarded) and then with 2 additional ml of H₂O. The latter eluate (2.0 ml) which contains cGMP was collected on a neutral alumina (WN-3, Sigma) column (0.7 × 3 cm) previously washed with 20 ml of 200 mM sodium acetate, pH 6.2, and then with 10 ml of 5 mM sodium acetate, pH 6.2. The eluate was discarded, and the column was washed with 3 ml of 5 mM sodium acetate buffer, pH 6.2, and then with 1 ml of 200 mM sodium acetate buffer, pH 6.2 (eluates discarded). Then cGMP was eluted with 2 ml of 200 mM sodium acetate, pH 6.2, and portions of the eluate were assayed for cGMP concentration and for recovery. The average recovery of cGMP was about 50%; <1% of the cAMP of the sample was present in the cGMP fraction.

Cyclic AMP fractions were obtained as follows: after cGMP was eluted from the AG50W-X4 column, the column was washed with 2 ml of H₂O (eluate discarded) and cAMP was eluted with 3 additional ml of H₂O. Portions of the eluate were assayed for cAMP concentration and recovery. The cAMP fraction contained <0.2% of the cGMP of the sample prior to fractionation. The average recovery of [³H]cAMP was 70%.

Each AG50W-X4 column was regenerated by washing with 8 ml of 1 N HCl and, prior to use, with 30 ml of water.

Assay of cGMP and cAMP. The cGMP concentrations of purified alumina column eluates were determined by the radioimmune method of Steiner *et al.* (11) modified as follows: each 0.5 ml reaction mixture contained 200 mM (in some experiments 140 mM) sodium acetate, pH 6.2 (solution B); 17 fmol of succinyl-cGMP ¹²⁵I-tyrosine methyl ester (10,000–20,000 cpm); cGMP antibody; and sample (0–20 pmol of cGMP). After incubation (14–18 hr, 3°) each reaction mixture was diluted with 2 ml of solution B at 3°; 10 min later the sample was passed through a Millipore filter (0.45 μm pore size, 25 mm diameter, HAWP 02500) previously rinsed with solution B and washed three times with cold solution B (4 ml each wash). Each filter was placed in a scintillation vial with 1 ml of 2-methoxyethanol; after filters dissolved (0.5–1 hr) 15 ml of Aquasol (New England Nuclear) were added. Radioactivity was determined 12–24 hr later in a Beckman liquid scintillation counter (³H plus ¹⁴C isoset).

Cyclic AMP concentrations of samples purified as described above were determined by the method of Gilman (12). Each cGMP or cAMP sample was assayed at three or four concentrations. Each value shown corresponds to 100% recovery of cyclic nucleotide and is the mean of duplicate dishes. Protein was determined by a modification of the method of Lowry *et al.* (13).

RESULTS

A rapid method was devised for separating cGMP from cAMP and removing trichloroacetic acid and endogenous factors which interfere with the cGMP assay. The procedure, based upon the *Materials and Methods* section, is based upon the methods of Salomon, Londos, and Rodbell (14) and White and Zenser (15); cGMP and cAMP are recovered in separate fractions in high yield and in sufficiently high concentration to assay directly without lyophilization. One hundred samples can be purified in 2–3 hr.

The effect of carbamylcholine, a relatively stable analog of acetylcholine, upon intracellular cGMP levels of neuroblastoma and other cell lines in the absence of a 3':5'-cyclic nucleotide phosphodiesterase inhibitor is shown in Fig. 1. Cyclic GMP levels of neuroblastoma clones N18 and N1E-

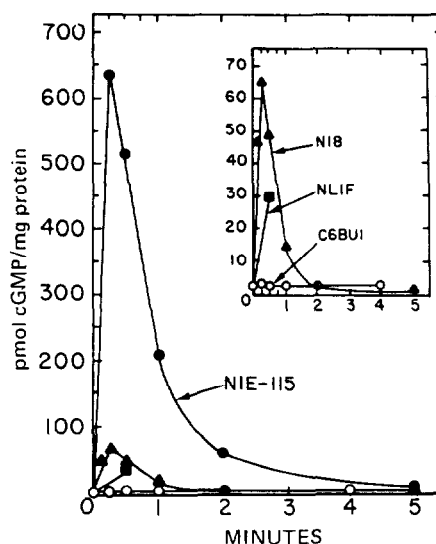


FIG. 1. Effect of 1 mM carbamylcholine on intracellular levels of cGMP in neuroblastoma clones N1E-115 and N18, neuroblastoma × L cell hybrid clone NL1F, and glioma clone C6BU-1. Cultures were confluent; the average protein content was: 11.5, 16.4, 7.6 and 6.9 mg of protein per 100 mm petri dish for N1E-115, N18, NL1F, and C6BU-1, respectively. A 3':5'-cyclic nucleotide phosphodiesterase inhibitor was not present.

115 rose 40- and 210-fold, respectively, upon incubation with carbamylcholine for 15 sec, and then fell rapidly to normal levels (50% decrease in cGMP at 45 sec). Carbamylcholine also elicited a 6-fold increase in the cGMP concentration of the neuroblastoma × L cell hybrid, NL-1F, but had no effect upon cGMP levels of rat glioma clone C6BU-1. Thus, two types of cell lines were found with respect to acetylcholine-receptor-mediated reactions which increase cGMP levels: cell lines which are sensitive to carbamylcholine and a line which is insensitive.

The effect of IBMX, a 3':5'-cyclic nucleotide phosphodiesterase inhibitor, upon the rate of disappearance of intracellular cGMP after stimulation with carbamylcholine is shown in Fig. 2A. In the absence of IBMX, maximum cGMP levels were attained 15 sec after the addition of carbamylcholine, and then rapidly decreased (50% decrease at 45 sec; Figs. 1 and 2A). In the presence of 0.5 mM IBMX, maximum cGMP levels were attained at 30 sec and cGMP levels then decreased at a slower rate (50% decrease at 3 min).

The effect of IBMX upon carbamylcholine dependent cGMP accumulation also was studied with N1E-115 cells adapted to grow in suspension culture. The assay was modified so that both intra- and extracellular cGMP was determined. As shown in Fig. 2B, carbamylcholine elevated cGMP levels of suspension cells and in the presence of IBMX cGMP levels remained constant during the 10 min incubation period. These results suggest that part of the cGMP formed by cells may be excreted into the medium. Other 3':5'-cyclic nucleotide phosphodiesterase inhibitors such as 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-20-1724) and papaverine were not as effective as IBMX in maintaining cGMP levels of N18 cells.

The relation between carbamylcholine concentration and cGMP accumulation in N1E-115 cells is shown in Fig. 3. The maximum increase in cGMP concentration was obtained with 10⁻³ M carbamylcholine; 50% of the maximum increase in cGMP concentration was evoked by 10⁻⁴ M carbamylcholine.

The effects of atropine and *d*-tubocurarine, selective inhibitors of muscarinic and nicotinic acetylcholine receptors,

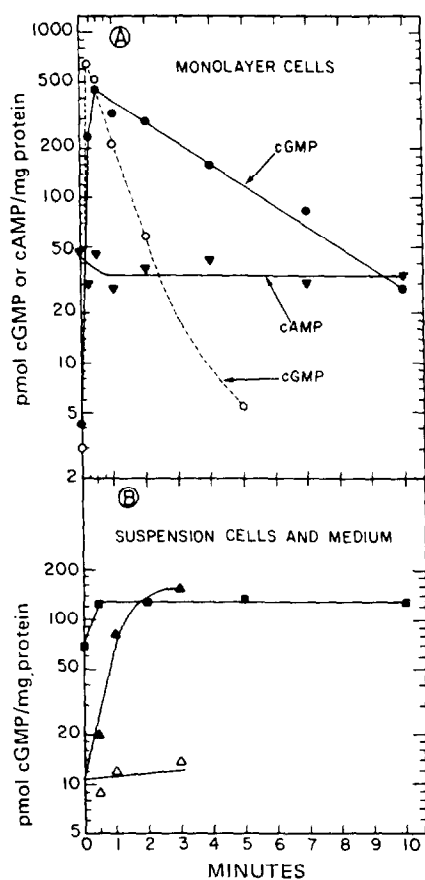


FIG. 2. Effect of 1 mM carbamylcholine in the presence of 0.5 mM IBMX, a 3':5'-cyclic nucleotide phosphodiesterase inhibitor, on cGMP levels of neuroblastoma N1E-115 cells. (A) Cells were grown for 9 days to confluence; the average cellular protein was 12.2 mg/100 mm dish. The broken line corresponds to the data for N1E-115 shown in Fig. 1 (cells were incubated in the absence of IBMX). (B) Cells were grown in suspension culture in DMEM with 10 mM NaHCO_3 , 25 mM HEPES, and 2% fetal bovine serum (pH 7.3, 340 mOsm/kg). Cells were washed and 1 ml portions (4×10^6 cells) were preincubated in the presence of 0.5 mM IBMX for 15 min with gentle shaking. Two experiments are shown. Cells were incubated for 3 min or less (\blacktriangle) with 1 mM carbamylcholine; or (\triangle) without this compound. In another experiment (\blacksquare) cells were incubated for 0 to 10 min with 1 mM carbamylcholine. In both experiments reactions were terminated by the addition of 4 ml of cold 6% trichloroacetic acid to each tube. Hence the combined intra- and extracellular cGMP concentration was determined.

respectively, upon carbamylcholine-dependent accumulation of cGMP in N1E-115 cells are shown in Fig. 4. Atropine, at 1×10^{-7} M, inhibited the carbamylcholine-dependent increase in cGMP concentration by 50%; 97% inhibition was obtained with 1×10^{-6} M atropine. In contrast, *d*-tubocurarine did not inhibit appreciably even at 1×10^{-4} M; in fact, in other experiments *d*-tubocurarine potentiated the carbamylcholine-dependent elevation of cGMP. The inhibition by atropine suggests that the increase in cGMP elicited by carbamylcholine is mediated by the muscarinic acetylcholine receptors.

The effects of PGE_1 upon cAMP and cGMP levels of N1E-115 cells are shown in Fig. 5. Cyclic AMP levels were found to increase markedly upon the addition of $10 \mu\text{M}$ PGE_1 . Unexpectedly, the level of cGMP also increased 6-fold in the presence of PGE_1 . The maximum concentration of cAMP attained in this experiment was 9-fold higher than that of cGMP; however, in other experiments, the concentration of cGMP dependent on PGE_1 increased to 250 pmol

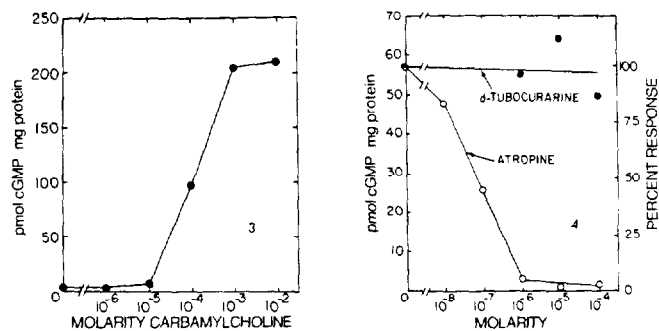


FIG. 3 (left). Relation between carbamylcholine concentration and cGMP levels of neuroblastoma N1E-115 cells. Cultures were grown for 9 days to confluence; the average cellular protein was 13.5 mg/100 mm dish. Cells were incubated for 30 sec in the presence of the indicated concentrations of carbamylcholine and IBMX.

FIG. 4 (right). Effect of atropine and *d*-tubocurarine concentration on the carbamylcholine dependent increase in cGMP level of N1E-115 cells. Cultures were grown for 9 days to confluence; the average cellular protein was 14.6 mg/100 mm dish. Cells were incubated for 30 sec in the presence of 1 mM carbamylcholine and the indicated concentrations of atropine or *d*-tubocurarine.

of cGMP per mg of protein, approximately 60% of the concentration attained by cAMP in the presence of PGE_1 . In other experiments not shown here, the increase in cGMP concentration elicited by PGE_1 was shown to be insensitive to atropine. Thus, the effect of PGE_1 upon cGMP is not mediated by the muscarinic acetylcholine receptor. The maximum cGMP concentration was achieved 30 sec after the addition of PGE_1 ; cGMP levels decreased during subsequent incubation. In contrast, the maximum cAMP level was achieved 4 min after the addition of PGE_1 .

The effects of carbamylcholine, PGE_1 , and adenosine upon cGMP and cAMP levels of N1E-115 cells plotted on a logarithmic scale to illustrate inhibitory as well as stimulatory effects are shown in Fig. 6A and B, respectively. In the presence of carbamylcholine or PGE_1 , cGMP levels increased and reached maximum levels within 1 min. The rates of disappearance of cGMP during the subsequent incubation period were linear. In contrast, the basal level of cGMP was reduced approximately 50% in the presence of 0.1 mM adenosine. As shown in Fig. 6B, cAMP levels increased markedly in the presence of PGE_1 or adenosine, but decreased 30% in the presence of carbamylcholine.

Various combinations of carbamylcholine, PGE_1 , and adenosine were added simultaneously to determine the effect of one compound upon cell responses to another (Fig.

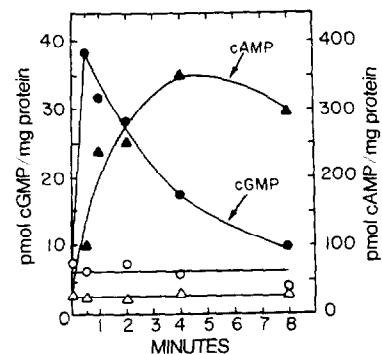


FIG. 5. Effect of PGE_1 on intracellular levels of cGMP and cAMP in neuroblastoma N1E-115 cells. Cultures were grown for 8 days; the average cellular protein was 6.8 mg/100 mm dish. Closed and open circles correspond to cGMP levels with and without $10 \mu\text{M}$ PGE_1 , respectively; closed and open triangles correspond to cAMP levels with and without $10 \mu\text{M}$ PGE_1 , respectively.

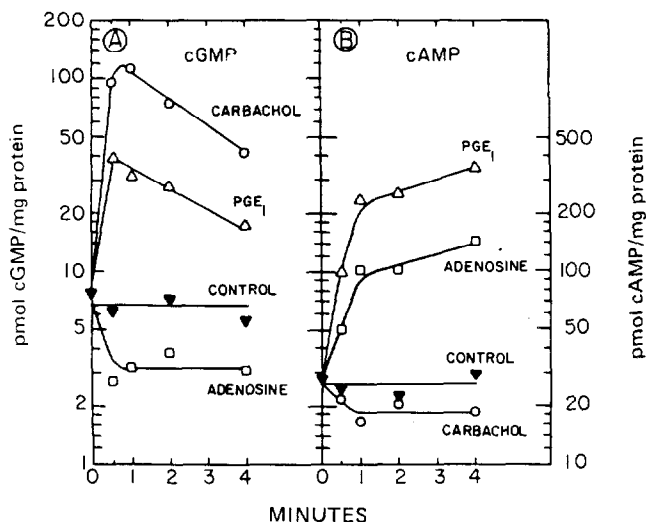


FIG. 6. Effect of 1 mM carbamylcholine (carbachol), 0.1 mM adenosine, or 10 μ M PGE₁ on intracellular levels of cGMP (A) and cAMP (B) of neuroblastoma N1E-115 cells. Culture conditions were identical to those described in the legend to Fig. 5.

7). The effect of exposing cells to both carbamylcholine and adenosine upon cGMP and cAMP levels is shown in Fig. 7A and B, respectively. Adenosine inhibited the carbamylcholine-dependent increase in cGMP concentration 25 and 60% when incubations were 30 and 60 sec, respectively.

In Fig. 7B, the effect of carbamylcholine upon the adenosine-dependent increase in cAMP concentration is shown. Carbamylcholine did not inhibit the adenosine-stimulated increase in cAMP during the first 30 sec of incubation even though the maximum increase in cGMP concentration evoked by carbamylcholine was achieved at 30 sec. However, during further incubation, carbamylcholine inhibited almost completely the adenosine-dependent elevation in cAMP concentration.

Carbamylcholine and PGE₁ added separately increased cGMP levels as indicated by the broken lines in Fig. 7C. The effects of carbamylcholine and PGE₁ added simultaneously to cultures were additive or more than additive. However, carbamylcholine inhibited the PGE₁-induced increase in cAMP concentration, particularly during the latter part of the incubation period (Fig. 7D). Thus, carbamylcholine may have a delayed inhibitory effect upon both adenosine- and PGE₁-dependent increases in cAMP concentrations. Atropine completely blocked the inhibitory effect of carbamylcholine upon adenosine- or PGE₁-dependent increases in cAMP levels (data not shown).

The effect of activating receptors for adenosine and PGE₁ separately and simultaneously upon cGMP and cAMP levels is shown in Fig. 7E and F, respectively. Adenosine inhibited slightly the rise in cGMP levels elicited by PGE₁. The results show that the increases in cAMP levels elicited either by adenosine or PGE₁ are not additive. In other experiments, the sequence of addition of carbamylcholine and either adenosine or PGE₁ was varied, but the results did not differ significantly from those found when two compounds were added simultaneously.

DISCUSSION

Two types of cells were found with respect to muscarinic acetylcholine receptor activity: cells sensitive to carbamylcholine and cells insensitive to this compound. The degree of response also varied widely from one clone to another. Activation of the muscarinic acetylcholine receptors of neuro-

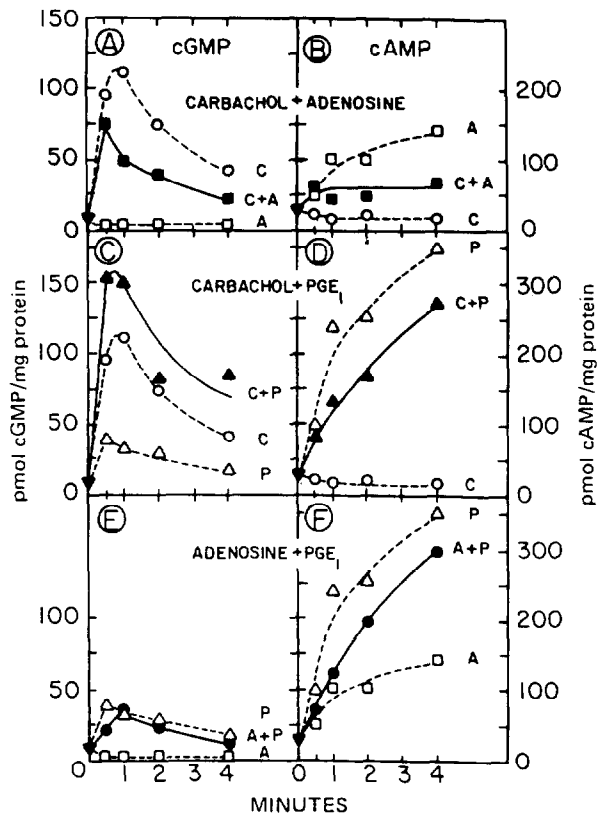


FIG. 7. Effect of various combinations of 1 mM carbamylcholine, 0.1 mM adenosine, and 10 μ M PGE₁ on intracellular levels of cGMP (panels A, C, and E) and cAMP (panels B, D, and F) of neuroblastoma N1E-115 cells. Culture conditions were identical to those described in the legend to Fig. 5. The broken lines correspond to the effect of each compound added separately (data shown in Fig. 6); C, A, and P represent carbamylcholine, adenosine, and PGE₁, respectively.

blastoma N1E-115 cells evoked up to a 200-fold increase in cGMP concentration, yielding levels in excess of 600 pmol of cGMP per mg of protein. These cells have high tyrosine hydroxylase activity (8), generate action potentials in response to electrical stimuli (8), have neurosecretory vesicles, and also possess PGE₁ receptors and adenosine receptors which upon activation lead to elevated cAMP levels (2, 3).

Adenosine, carbamylcholine, and PGE₁ were added to cells separately and in pairs to determine the effect of one compound upon cell responses to another. The results, summarized in Table 1, show that in the presence of adenosine cAMP levels increase and cGMP levels decrease; whereas, in

Table 1. Summary of receptor-mediated shifts in cGMP and cAMP levels in neuroblastoma cells

Addition	cGMP	cAMP
	percent	
None	100	100
Adenosine	40	500
Carbamylcholine	1500	70
PGE ₁	500	1250
Adenosine + carbamylcholine	650	250
PGE ₁ + carbamylcholine	2050	1000
PGE ₁ + adenosine	300	1100

100% corresponds to 7.5 pmol of cGMP/mg of protein or 27.5 pmol of cAMP/mg of protein. The data are from the experiments shown in Figs. 6 and 7.

the presence of carbamylcholine, cGMP levels increase and cAMP levels decrease. In addition to the inverse relationship between cGMP and cAMP concentrations, a novel phenomenon was observed; that is, in the presence of PGE₁ the levels of both cGMP and cAMP increase. The increase in cGMP elicited by carbamylcholine was not inhibited by PGE₁ in spite of the fact that PGE₁ elicits a marked increase in cAMP levels. Prostaglandin-E₁- and carbamylcholine-dependent increases in cGMP were fully additive, and in some experiments, were greater than additive. In contrast, the elevated cAMP concentrations induced either by PGE₁ or by adenosine were not additive when both compounds were added simultaneously to cells.

We conclude that activation of a species of muscarinic acetylcholine receptor inhibits adenosine- and PGE₁-receptor-mediated reactions which elevate cAMP levels; whereas, activation of adenosine receptors inhibits reactions mediated by muscarinic acetylcholine receptors and PGE₁ receptors which elevate cGMP levels. In contrast to these results, activation of PGE₁ receptors did not affect acetylcholine- or adenosine-receptor-mediated reactions which affect cGMP or cAMP levels. Thus, reciprocal inhibition, unilateral inhibition, additive, and nonadditive responses were observed when different pairs of receptors were activated simultaneously.

Relatively little is known at the molecular level about the mechanisms which couple one receptor with another. The observed phenomena are obviously complex and may reflect changes in membrane permeability, metabolism of cyclic nucleotides, and so forth. Other examples of reciprocal coupling of cGMP and cAMP levels and possible mechanisms of coupling have been discussed by Goldberg *et al.* (22, 23).

Blume *et al.* (5) have reported that acetylcholine inhibits the increase in cAMP levels induced by PGE₁ or adenosine in mouse neuroblastoma cells. Acetylcholine also has been shown to inhibit the PGE₁-dependent elevations of cAMP levels, the inhibition mediated by excitatory muscarinic acetylcholine receptors in neuroblastoma × glioma hybrid cells (16). Acetylcholine and carbamylcholine also have been reported to stimulate mouse neuroblastoma adenylate cyclase activity (17).

The inhibitory effect of carbamylcholine on reactions mediated by other species of receptors shows that some N1E-115 cells with muscarinic acetylcholine receptors also have adenosine or PGE₁ receptors. Some cells also have both adenosine receptors and PGE₁ receptors. Although N1E-115 is a clonal cell line, single cell analysis by electrophysiologic methods reveals two acetylcholine receptor phenotypes; cells sensitive and cells insensitive to carbamylcholine. All N1E-115 cells sensitive to acetylcholine or carbamylcholine exhibit hyperpolarizing responses to these compounds, frequently -25 mV in magnitude and 10-15 sec in duration. Hence, N1E-115 cells have inhibitory muscarinic acetylcholine receptors.

The activity of PGE₁ in eliciting increases in both cGMP and cAMP levels should be considered in context with the different actions of PGE₁ on various tissues which have been reported. For example, PGE₁ elevates cAMP levels in some tissues and reduces hormone-dependent elevation of cAMP levels in others (18), activates adenylate cyclase activity in

homogenates (19), including those prepared from neuroblastoma × glioma hybrid cells (20), and inhibits the vasopressin-dependent activation of adenylate cyclase (21).

Prostaglandin E₁ elicits a transitory, 30 sec, increase in cGMP accumulation and a prolonged increase in cAMP accumulation. The difference in the duration of cGMP and cAMP accumulations may reflect the activities of different enzymes which catalyze the synthesis and perhaps the hydrolysis of the nucleotides, or alternatively, may indicate the presence of two species of PGE₁ receptor, one mediating an increase in cAMP concentration, the other, an increase in cGMP. Preliminary results which support the latter hypothesis will be described elsewhere.

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