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Regulation of Adenosine 3',5'-Monophosphate Content in Human Astrocytoma Cells by Isoproterenol and Carbachol

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SUMMARY

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Carbachol, a typical cholinomimetic, inhibited isoproterenol stimulation of intracellular adenosine 3',5'-monophosphate (cAMP) levels in a dose-dependent fashion, causing a maximal 60-70% decrease at 0.1 mM. The blocking action of carbachol was evident by 30 sec, and reached a maximum between 5 and 7 min. Neither lidocaine, a local anesthetic, nor ascorbic acid and thiourea, reducing agents, had any effect on the action of carbachol. Carbachol inhibited the accumulation of cAMP caused by isoproterenol, adenosine, and prostaglandin E_1. Neither hexamethonium bromide, tetraethylammonium chloride, nor phentolamine affected the regulation of cAMP levels by carbachol; in contrast, 1.0 μM atropine completely blocked its effects. Ca^{2+}-free incubation medium eliminated the inhibitory effect of carbachol on isoproterenol-stimulated accumulation of cAMP, but did not affect isoproterenol stimulation alone. These observations suggest that the astrocytoma cell line has a muscarinic, Ca^{2+}-dependent cholinergic receptor, the stimulation of which results in inhibition of cAMP accumulation with isoproterenol stimulation.

INTRODUCTION

Considerable effort has been directed toward elucidating the roles of the cyclic nucleotides adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in the central nervous system, especially in neurotransmission processes in the brain. It has been reported that the regulation of cyclic nucleotide levels in mammalian brain slices was a primary effect of electrical stimulation (1) and depolarization by various agents (2-6). It was significant, therefore, that putative neurotransmitters of the central nervous system (7-11) have also been shown to influence intracellular cyclic nucleotide levels in the brain. For example, Ferrendelli *et al.* (11) have shown that, in slices of mouse cerebellum, norepinephrine elevates levels of both cAMP[3] and cGMP. Additionally, the putative neurotransmitters adenosine (12, 13), γ-aminobutyric acid, glutamate, and glycine (14) are capable of increasing intracellular levels of cAMP and/or cGMP in brain tissue. Lee *et al.* (9) have demon-

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[3] The abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; cGMP, guanosine cyclic 3',5'-monophosphate; PGE_1, prostaglandin E_1; TCA, trichloracetic acid.

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strated that the stimulation of muscarinic cholinergic receptors in mammalian cerebral cortex elevates levels of cGMP, leaving the cAMP levels unaltered or slightly decreased. Ferrendelli *et al.* (8) have reported increased cGMP levels in mouse cerebral cortex and cerebellum in response to the cholinomimetic oxotremorine. Atropine was shown to block the action of the compound specifically.

Matsuzawa and Nirenberg have recently shown that carbachol reduces the PGE_1 and adenosine stimulation of cAMP accumulation while itself increasing the levels of cGMP in cultured mouse neuroblastoma (15). Thus much evidence exists to suggest that both cAMP and cGMP are in some way involved in cholinergic action and presumably neurotransmission; however, there is as yet no direct evidence in support of this role.

The complexity of the brain's actions and the presence of many cell types in brain slices complicate the formulation of any general model for the role of the cyclic nucleotides in the mediation of putative neurotransmitter action in the brain. It is not known, for example, whether hormone effects on cyclic nucleotide metabolism are pre- or postsynaptic (or both), or whether, in fact, their site of action is in glia as well as in neurons. This series of experiments was undertaken to determine the extent to which carbachol regulates intracellular cyclic nucleotide levels in the cultured human astrocytoma cell line 132-1N1. The responses of these cells to catecholamines, adenosine, and other cAMP agonists have been described elsewhere (13, 16, 17).

The data presented demonstrate that carbachol and isoproterenol have opposite effects on intracellular cAMP levels, carbachol inhibiting the isoproterenol-stimulated increase in cAMP in a Ca^{2+}-dependent manner. This lends further support to the hypothesis that glial cells play an active role in brain function involving the cyclic nucleotides; specifically, they may be involved in the functioning of both adrenergic and cholinergic pathways.

MATERIALS AND METHODS

l-Isoproterenol *d*-bitartrate was purchased from Sterling-Winthrop Laboratories; tetraethylammonium chloride, from Aldrich, and carbachol, cAMP phosphodiesterase, atropine sulfate, and hexamethonium bromide, from Sigma Chemical Company. Lidocaine (xylocaine) was purchased from Astra Chemical Company. Adenosine was obtained from Calbiochem, and prostaglandin E_1 was a gift from the Upjohn Company.

Cell cultures. The human astrocytoma cell used in this study, 132-1N1, was isolated in 1972 as a clone of the tumor astrocyte line 118MG (18). The details regarding the origin and growth characteristics of the 118MG line have been described elsewhere (18, 19). The 132-1N1 line has been demonstrated to be free of *Mycoplasma*.

Stock cultures were maintained in T-75 Falcon flasks in Eagle's minimal essential medium containing Earle's salts plus glutamine (Grand Island Biological Company) with 10% fetal calf serum (Microbiological Associates). These were kept in a humidified incubator under an atmosphere of 95% air–5% CO_2 at 37°. The medium was buffered at pH 7.0–7.4 with $NaHCO_3$, 2.2 g/liter of minimal essential medium. The cells were allowed to grow to near confluence, at which point they were treated with trypsin (0.25% trypsin in minimal essential medium) and divided 1:20 into new flasks. Fresh medium was supplied on the following day, and on every third day thereafter.

For experiments, cells were seeded into 60- or 100-mm Falcon plastic Petri dishes at concentrations of approximately 1–2 × 10^5 or 5–6 × 10^5 cells/5 or 10 ml of minimal essential medium, respectively. The cells were supplied with fresh medium on the fourth day in culture and were used for experimentation 18–24 hr later. At this stage cells were not yet confluent, ranging from 0.3 to 0.5 mg of protein per 60-mm dish (28.6 cm^2). We had previously shown that cells at this density respond maximally to norepinephrine, epinephrine, and isoproterenol with regard to cAMP accumulation (16).

Experimental conditions. The medium from the experimental cultures was aspirated, and the attached cells were washed twice (total wash volume equaling seeding

volume) in fresh medium lacking serum and including *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer at a concentration of 20 mM. The cultures were then returned to the incubator for 20 min (37°, 95% air–5% CO_2 atmosphere). Subsequent to this incubation, test agents were added simultaneously and incubations were carried out for the appropriate time intervals. For experiments in which the medium was to be Ca^{2+}-free, the 20-min preliminary incubations were performed in Ca^{2+}-free medium and were followed by two more washings and a 10-min incubation, after which the agents to be tested were added. The agents were dissolved in water or 95% ethanol; control cultures received equal volumes of the appropriate diluent. Incubations were stopped by aspirating the medium and adding 5% TCA, which quantitatively extracts the cAMP while leaving the cell sheet intact. The cells were then suspended in 0.3 N KOH and assayed for protein by the method of Lowry *et al.* (20).

Measurement of cAMP. In order to calculate recovery from the purification steps, approximately 10,000 cpm (1 pmole) of [^{3}H]cAMP was added to the TCA extracts, which were then applied to 0.4 × 4 cm columns of Dowex 50 (Bio-Rad AG50W-X8, H^+ form) equilibrated with 0.1 N HCl. The ATP-ADP fraction was eluted with 1.5 ml of 0.1 N HCl, followed by 1.5 ml of H_2O. Then 4 ml of H_2O eluted the cAMP fraction. These samples were made 10 mM in Tris buffer, pH 7.4, and applied to 10 mM Tris-washed columns (0.6 × 5 cm) of Dowex 2 (Bio-Rad AG2-X8, H^+ form). The purified cAMP fractions were eluted in 6 ml of 0.05 N HCl after washing with 3 ml of the Tris buffer. Samples were lyophilized, reconstituted in 1.0 ml of H_2O, and assayed by the method of Gilman (21). In order to assay the cAMP in the medium, the medium was made 5% in TCA, divided into 2-ml aliquots, and purified as described, except that the ATP-ADP fraction on the Dowex 50 columns was eluted after 1.0 ml of 0.1 N HCl and 1.0 ml of H_2O. Recoveries of the cell extracts were on the order of 50–70% over both columns, and in this respect were no different from 5% TCA blanks containing only [^{3}H]cAMP. Recoveries from the medium were generally between 30% and 40%.

After aspiration, no more than 0.5% of the incubation medium remained on the cell sheet. The contribution of cAMP from the medium was therefore considered negligible (e.g., this amounted to approximately 0.25 pmole in control cultures). In all samples, degradation of the cAMP with phosphodiesterase produced amounts in the assay that were equivalent to column blanks.

RESULTS

Regulation of intracellular cAMP levels by carbachol. Carbachol, when present in the incubation medium in concentrations as high as 0.1 mM, had no apparent effect on basal cAMP levels, although basal levels of cAMP are approximate, ranging from 10 to 20 pmoles/mg of protein. However, carbachol did markedly inhibit the stimulatory effects of isoproterenol on cAMP accumulation. When carbachol was added with 0.1 μM isoproterenol (a concentration which produced an approximately half-maximal increase in cAMP), inhibition was evident at 1 μM and was dose-dependent, resulting in a maximal 70% decrease in cAMP at 0.1 mM (Fig. 1).

Figure 2 shows the effect of carbachol on the time course of isoproterenol stimulation. Levels of cAMP rose quite rapidly, to over 1600 pmoles/mg of protein, during isoproterenol stimulation, reaching a maximum between 5 and 6 min. Carbachol considerably attenuated this swift initial increase in cAMP. The effect of carbachol was greatest on the initial burst of cAMP accumulation, after which the cAMP level in isoproterenol-treated cells fell more rapidly relative to the cells with isoproterenol plus carbachol.

Effect of 0.1 mM ascorbic acid–1 mM thiourea. The addition of 0.1 mM ascorbic acid–1 mM thiourea to the incubation medium effectively prevents oxidation of catecholamines and any resultant loss of activity.[4] In order to determine whether the

[4] R. Barber and R. McGuire, unpublished observations.

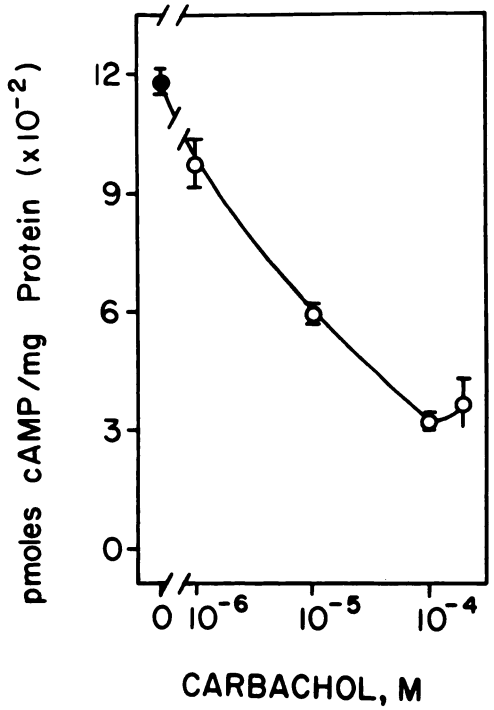

FIG. 1. *Carbachol inhibition of isoproterenol-induced increases in intracellular cAMP levels*

Carbachol was added simultaneously with 0.1 μM isoproterenol for 10-min incubations under standard conditions. Each point is the mean of triplicates ± standard error. The basal cAMP levels for this experiment were 9.4 ± 1.2 pmoles/mg of protein. Cultures were used at an average protein density of 16 μg/cm². (●) 0.1 μM isoproterenol, no carbachol.

inhibition of isoproterenol in the presence of carbachol was due to more rapid oxidation of the compound, time course experiments were conducted with and without the ascorbic acid-thiourea solution added to the medium at the start of the incubation.

Figure 3 shows the effect of ascorbic acid-thiourea on samples incubated with 0.1 μM isoproterenol plus 100 μM carbachol. In both cases no significant effects of the reducing agents were seen until 15 min; ascorbic acid-thiourea did not, therefore, affect the rapid inhibitory actions of carbachol on isoproterenol, although its effects on the preservation of isoproterenol were marked.[4]

Effect of lidocaine. Since carbachol exerted discernible effects at 1 μM concentrations, it was considered doubtful that the observed inhibitory effect was toxicological; however, it was of interest to test the effect of an anesthetic (lidocaine) on the inhibitory action of carbachol.

First the effect of lidocaine on 0.1 μM isoproterenol was examined (Table 1). Lidocaine at 1 μM caused a slight, statistically insignificant increase in cAMP levels over isoproterenol alone; 0.1 mM lidocaine caused a slight, barely significant depression. Lidocaine alone did not alter cAMP levels in concentrations as high as 0.1 mM.

The action of various concentrations of lidocaine on the carbachol inhibition of cAMP levels was then tested. By subtracting the effects of lidocaine on isoproterenol, it can be seen that the action of carbachol on cAMP levels was not affected by lidocaine.

Effect of cholinergic blockers and the alpha adrenergic blocker phentolamine. To clarify further the mode of action of

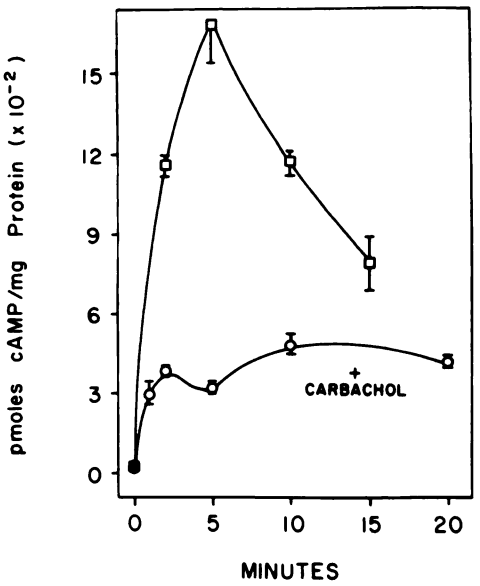

FIG. 2. *Action of carbachol on time course of isoproterenol-induced increase in intracellular levels of cAMP*

□——□, stimulation by 0.1 μM isoproterenol; ○——○, course of isoproterenol stimulation with 100 μM carbachol. Cultures were used at an average protein density of 15 μg/cm². Each point is the mean of triplicates ± standard error. Unstimulated basal cAMP levels were 7 ± 2.0 pmoles/mg of protein (■).

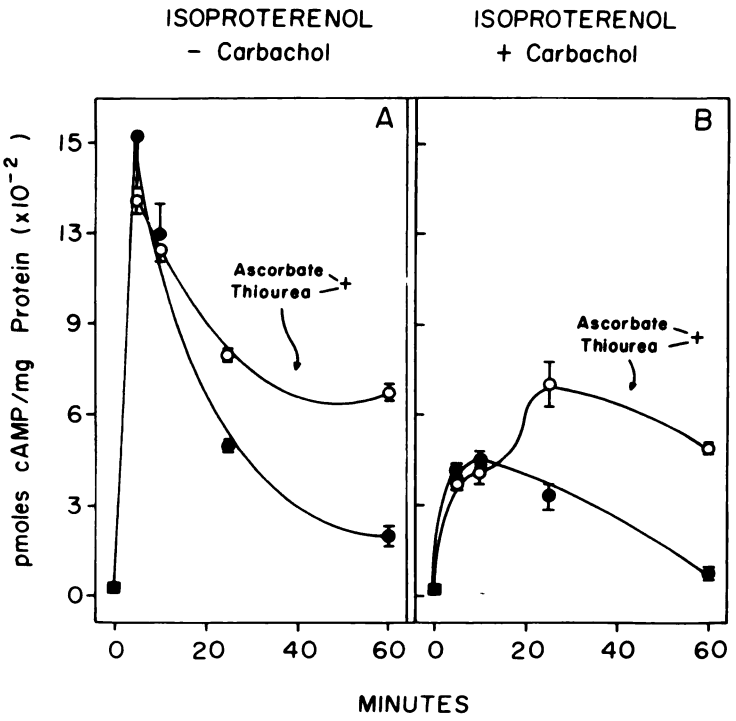

FIG. 3. *Effect of ascorbic acid and thiourea on time course of intracellular cAMP accumulation in response to isoproterenol and isoproterenol plus carbachol*

A. Cultures were incubated with 0.1 μM isoproterenol either with (○——○) or without (●——●) 0.1 mM ascorbic acid–1 mM thiourea. B. Cultures were incubated with 0.1 μM isoproterenol plus 0.1 mM carbachol either with (○——○) or without (●——●) 0.1 mM ascorbic acid–1 mM thiourea. Cultures were grown to an average protein density of 16 μg/cm². All points represent the means of triplicates ± standard errors. Basal cAMP levels in this experiment were 30 ± 2.3 pmoles/mg of protein (■).

carbachol, the effects of three cholinergic blockers were determined. Neither 0.1 mM tetraethylammonium chloride nor 0.1 mM hexamethonium blocked the actions of carbachol; atropine, however, nearly completely blocked the action of carbachol at a concentration of 1 μM (Table 2). None of the compounds affected isoproterenol-induced increases in cAMP accumulation to any significant extent in the absence of carbachol. It should be pointed out that tetraethylammonium chloride, which can act as a cholinergic agonist in some tissues, did not act in a manner similar to that of carbachol. The action of carbachol in this system is therefore selectively blocked by the muscarinic cholinergic blocker atropine.

Phentolamine, an *alpha* adrenergic blocker, was added simultaneously to samples with 0.1 μM isoproterenol and 0.1 mM carbachol at levels as high as 40 μM. It had no effect whatsoever on the actions of carbachol (not illustrated).

Effect of carbachol on agonists other than isoproterenol. The 1321N1 cells respond to a variety of different compounds with increased cAMP levels (see ref. 16). Table 3 illustrates the effects of carbachol on adenosine- and PGE_1-induced increases in cAMP levels. Carbachol caused a 50% decrease in adenosine-stimulated levels, and a 90% reduction in PGE_1-induced levels. The agonists were used at concentrations which produced about 75% of their maximal stimulation of cAMP levels.

Effect of Ca^{2+}-free incubation medium. Because of the many reports of the effects of carbachol and other cholinomimetics on the cGMP systems of various tissues (e.g., refs. 8, 9, 22, 23), and because of the known Ca^{2+} requirements by, and Ca^{2+} stimula-

TABLE 1

Effect of lidocaine on actions of isoproterenol alone and in combination with carbachol

Cultures were grown to an average protein density of 22 μg/cm². Values are the means of triplicates ± standard errors. All incubations were performed for 5 min under standard conditions.

Additions			cAMP
Isoproterenol	Carbachol	Lidocaine	
μM	μM	μM	pmoles/mg protein
			11.6 ± 3
0.1			1530 ± 25
	100		11.3 ± 4
		100	8.5 ± 3
0.1	1.0		1389 ± 110
0.1	100		617 ± 50
0.1		1.0	1596 ± 180
0.1		100	1230 ± 80
0.1	1.0	1.0	1803 ± 60
0.1	1.0	100	1152 ± 71
0.1	100	1.0	545 ± 73
0.1	100	100	362 ± 7

TABLE 2

Inhibition by atropine of the effect of carbachol on isoproterenol-stimulated accumulation of cAMP

Experimental cultures were grown to an average protein density of 15 μg/cm². Values are the means of triplicates ± standard errors. All incubations were performed for 5 min under standard conditions.

Additions			cAMP
Isoproterenol	Carbachol	Atropine	
μM	μM	μM	pmoles/mg protein
			5.9 ± 1.1
0.1			1420 ± 89
0.1	100		370 ± 20
0.1		1	1509 ± 90
0.1		100	1250 ± 42
0.1	100	1	1180 ± 117
0.1	100	10	1405 ± 128
0.1	100	100	1280 ± 112

tion of, guanylate cyclase (24–26), it was considered of interest to determine the effects of removing free Ca^{2+} from the incubation medium on the action of carbachol.

Figure 4A shows that removal of Ca^{2+} essentially eliminated the carbachol inhibition of the isoproterenol-stimulated accumulation of cAMP over the entire range of carbachol concentrations tested. Figure 4B shows that the effect of removing Ca^{2+} was immediate. The accumulation of cAMP in the absence of Ca^{2+} was essentially identical with the levels reached by stimulation with isoproterenol alone (see Fig. 2). The lack of Ca^{2+} is therefore viewed as exerting specific effects on the actions of carbachol on cAMP, leaving isoproterenol-induced changes in cAMP levels unaltered.

DISCUSSION

The following evidence indicates that carbachol acts through a specific mechanism: (a) lidocaine, a membrane anesthetic, at levels as high as 0.1 mM, had no substantial effect on isoproterenol-stimulated cAMP levels, or any effect on the ability of carbachol to block the rise in cAMP levels produced by isoproterenol; (b) carbachol inhibited the ability of 0.1 μM isoproterenol, 0.1 mM adenosine, and 11.4 μM PGE_1 to increase levels of cAMP, causing, at a concentration of 0.1 mM, inhibition of 70%, 50%, and 90%, respectively; (c) the absence of Ca^{2+} from the incubation medium specifically abolished the effect of carbachol on the accumulation of cAMP, but in no way altered the ability of isoproterenol to raise levels of cAMP; and (d) the effect of carbachol was specifically blocked by atropine, while tetraethylammonium chloride and hexamethonium bromide had no effect on the action of either carbachol

TABLE 3

Inhibition by carbachol of adenosine- and PGE_1-stimulated intracellular accumulation of cAMP

Experimental cultures were grown to an average protein density of 22 μg/cm². Values are the means of triplicates ± standard errors. All incubations were performed for 5 min under standard conditions.

Additions[a]	cAMP
	pmoles/mg protein
None	11.6 ± 3
Adenosine	207 ± 14
Adenosine + carbachol	112 ± 12
PGE_1	1095 ± 117
PGE_1 + carbachol	118 ± 24

[a] Adenosine, 0.1 mM; PGE_1, 11 μM; carbachol, 0.1 mM.

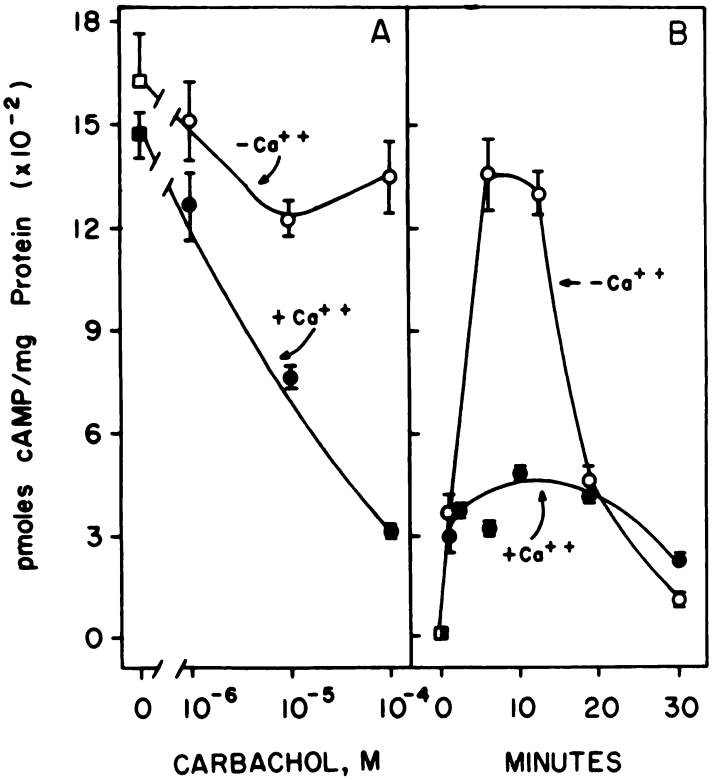

FIG. 4. *Ca^{2+} dependence of carbachol inhibition of isoproterenol-stimulated increases in cAMP levels*

A. Effect of varying concentrations of carbachol on the stimulation by 0.1 μM isoproterenol with (●——●) and without (○——○) Ca^{2+}; 0.1 μM isoproterenol, no carbachol, either with [■] or without [□] Ca^{2+}. Incubations were performed for 5 min. B. Time course of 0.1 μM isoproterenol stimulation in the presence of 0.1 mM carbachol with (●——●) and without (○——○) Ca^{2+}. Cultures used were grown to an average protein density of 16 $\mu g/cm^2$. Control levels of cAMP were 10 ± 1.0 pmoles/mg of protein (◨). All points represent the means of triplicates ± standard errors.

or isoproterenol. Our data are thus consistent with those of other groups who have demonstrated carbachol-mediated inhibition of adrenergically induced (23) and PGE_1- and adenosine-induced (15) accumulation of cAMP in other tissues.

Carbachol probably does not act at an *alpha* adrenergic receptor, as phentolamine did not alter its regulation of intracellular cAMP levels. The possibility that carbachol exerts its effects by directly or indirectly blocking the action of isoproterenol at a *beta* adrenergic receptor is inconsistent with the following data: (a) the structures of the two compounds are so radically different that, a priori, the binding of carbachol at a *beta* adrenoceptor seems to be highly unlikely; (b) the blocking action of atropine is highly specific and sensitive, maximal blockage by 0.1 mM carbachol occurring at 1 μM atropine; and (c) the removal of free Ca^{2+} does not affect the adrenergic stimulation of cAMP accumulation. The data cited support the conclusion that carbachol acts at a specific extracellular muscarinic binding site. The nature of this receptor is not yet known, nor are the mechanisms by which it mediates the actions of carbachol.

Our preliminary evidence indicates that both isoproterenol and carbachol increase intracellular levels of cGMP; this is in agreement with the work of Butcher *et al.* (23) and Durham *et al.* (27) with isoproterenol in parotid gland, Ferrendelli *et al.* (11) in mammalian brain slices, and Mat-

suzawa and Nirenberg (15) in cultured neuroblastoma cells. We have found that isoproterenol, at a concentration as low as 0.1 μM, increased intracellular cGMP levels from 0.3 to 0.8 pmole/mg of protein. This transient response had apparently reached a maximum by the earliest time sampled, 2 min, and returned to basal levels by 5 min. Carbachol (0.1 mM) also was found to increase cGMP levels approximately 5-fold, and, when added in combination with isoproterenol, produced supra-additive levels. This response reached a maximum by 1 minute, and did not return to basal values by 15 min. The removal of free Ca^{2+} from the incubation medium eliminated the effects of isoproterenol and carbachol on intracellular cGMP levels.

The mechanism by which isoproterenol increases levels of cGMP is unclear, as is the role of cGMP once formed, either by isoproterenol or carbachol. It is possible that regulation of cAMP levels by carbachol may be mediated via cGMP, with carbachol directly or indirectly causing elevated cGMP levels and, in turn, increased cAMP phosphodiesterase activity (28). It is equally plausible that the rise in cGMP levels is not the primary cAMP-regulating event, but rather is due to another action of carbachol, e.g., depolarization-mediated influx or mobilization of Ca^{2+}. The increased concentration of Ca^{2+} could cause the decrease in cAMP levels either by stimulation of phosphodiesterase or by inhibition of adenylate cyclase,[5] or by a combination of these mechanisms.

Our studies have previously identified adenosine, adrenergic compounds, and prostaglandins as agents which stimulate accumulation of intracellular cAMP levels in 132-1N1 cells. The present data indicate that a fourth class of compounds, the cholinergics, are regulators of both cyclic nucleotides, and that the adrenergics may also regulate cGMP levels. This suggests that glia may be a site for the various effects of these regulatory compounds. Finally, the data stress the importance of interpreting data on cAMP levels in the light of similar studies on cGMP and Ca^{2+}.

[5] R. B. Clark, unpublished observations.

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