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NEUROTRANSMITTER MODULATION OF PROSTAGLANDIN E_1-STIMULATED INCREASES IN CYCLIC AMP

I. CHARACTERIZATION OF A CULTURED NEURONAL CELL LINE IN EXPONENTIAL GROWTH PHASE

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Abstract—Two sympathetic ganglion cell X neuroblastoma somatic cell hybrids (TCX 11 and TCX 17) were found to have a PGE_1-sensitive adenylyl cyclase which was inhibited in whole cells by carbachol, norepinephrine and dopamine. Serotonin and morphine were without effect, the latter despite the presence of opiate receptors. In the TCX 17 clone, carbachol produced a greater degree of inhibition (30 per cent of control levels) than norepinephrine or dopamine (55–65 per cent of control). The ED_{50} for inhibition was of the order norepinephine > dopamine > carbachol (10^{-7}, 3×10^{-7} and 10^{-6} M respectively). The inhibition by carbachol could be reversed by the muscarinic antagonists scopolamine and atropine, while the nicotinic antagonists α-bungarotoxin and *d*-tubocurarine were without effect. The inhibition by norepinephrine and dopamine possessed the following properties: (1) the inhibition was mimicked by phenylephrine but not by isoproterenol, nor by dopaminergic agonists apomorphine and ET495; (2) the α-antagonists phenoxybenzamine and phentolamine reversed the inhibition by norepinephrine and dopamine; and (3) chlorpromazine reversed the inhibition of cyclic AMP formation by dopamine. Other phenothiazines tested, trifluoperazine, and fluphenazine, had no effect. It is concluded that the TCX 17 clone expresses two classes of receptors capable of modulating PGE_1. The cholinergic receptor is muscarinic and the catecholamine receptor has α-adrenergic properties.

Delineating the mechanisms of neurotransmitter function through membrane receptors (e.g. membrane permeability, excitability and metabolism) has been difficult to achieve in the central nervous system (CNS) because of the organizational complexity and heterogeneity of cells. One alternative approach has been to utilize cells grown in culture which are derived from tumors of nervous tissue. Such continuously dividing cells can be grown in an easily controlled environment as homogeneous populations relatively free of synaptic interactions and have been shown to express a variety of electrical and biochemical properties characteristic of nerve cells [1].

Neurotransmitter-sensitive adenylyl cyclase has been implicated as a mediator of synaptic transmission in both the peripheral [2] and central nervous system [3]. In the cases of dopaminergic transmission in the superior cervical ganglion and β-adrenergic transmission involving Purkinje cells in the cerebellum, increases in cyclic AMP levels can be correlated with hyperpolarizing responses of postsynaptic elements [2, 3]. Prostaglandin E_1 (PGE_1) has been shown to modulate adenylyl cyclase activity in many tissues [4]. In neural tissue it is thought to play a neuroregulatory function by affecting intracellular cyclic AMP levels [5].

The present study was undertaken with exponential growth phase cells (1) to investigate the ability of various putative neurotransmitter substances to modulate 3′,5′-cyclic adenosine monophosphate (cyclic AMP) metabolism and (2) to pharmacologically characterize the presumptive receptors which mediate these effects. Somatic cell hybrids derived from an embryonic mouse sympathetic ganglion cell and N18TG2, a subclone of the C1300 mouse neuroblastoma, were studied. In a companion paper [6] the receptor-mediated effects on cyclic AMP metabolism described here are compared with those which occur in cells that have been "differentiated" by treatment with dibutyryl cyclic AMP. In addition, the possible relevance to membrane conductance changes elicited by neurotransmitters [7, 8] with these cells is discussed.

In this report, we describe two somatic cell hybrids in which cyclic AMP levels are increased in the presence of PGE_1. Norepinephrine, dopamine and the cholinergic agonist, carbachol, inhibit PGE_1-stimulated increase in cyclic AMP levels as well as basal cyclic AMP levels. Morphine has no effect on cyclic AMP levels, despite the presence of opiate receptors [9]. A preliminary report of some of this work has already appeared [10].

METHODS

Materials. [^{3}H]cyclic AMP (20 Ci/m-mole) was purchased from New England Nuclear, Boston, MA. Morphine sulfate was acquired from Mallinkrodt Chemical Works, St. Louis, MO., and apomorphine was purchased from Merck, West Point, PA. The following drugs were kindly provided:

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PGE_1 (Dr. J. Pike, Upjohn, Kalamazoo, MI), R020-1724 (Hoffmann-LaRoche, Nutley, NY), chlorpromazine and phenoxybenzamine (Smith, Kline & French, Philadelphia, PA), phentolamine (Geigy, Ardsley, NY), propranolol (Ayerst, New York, NY), fluphenazine (Squibb, Princeton, NJ), phenylephrine (Winthrop, New York, NY), ET495 (piribedil), (LeSevier, Orleans, France), bublocapnine (Dr. W. Hunt, AFRRI, Bethesda, MD) and trifluoperazine (Dr. J. Kebabian, NIH, Bethesda, MD). All other chemicals were purchased from Sigma, St. Louis, MO.

Cell culture. Cells used in these studies (TCX 17 and TCX 11) were derived as subclones (P. Myers and W. Shain, manuscript in preparation) from the somatic cell hybrid clone (NX31)[11]. Cells were grown in modified F12 medium[12] supplemented with 5 per cent fetal calf serum (GIBCO) to which the additional modifications were made: (1) removal of all tyrosine, (2) increases in hypoxanthine (30–100 μM) and thymidine (3 μM increased to 16 μM) and (3) addition of aminopterin (0.4 μM) and carbachol (100 μM). Tyrosine was omitted from the media to select for cells with high tyrosine hydroxylase activity. Aminopterin and increased concentrations of thymidine and hypoxanthine were included to maintain at least a portion of the hybrid genome in the cell line. Carbachol was added to select against cells with physiologically active cholinergic receptors (P. R. Myers and W. Shain, manuscript in preparation). The parent line has been shown to express electrophysiologically nicotinic cholinergic receptors[13]. Cells were grown on Falcon plastic dishes at 37° in a 5% CO_2:95% air atmosphere saturated with water.

Drug incubation studies. Two days prior to an experiment, 3 to 5 × 10^5 cells were seeded into 60-mm dishes. The medium was then changed to modified F12 without supplements 12–18 hr prior to the drug incubation studies. To inhibit phosphodiesterase activity, cells were preincubated for 1 hr with 10^{-4} M R020-1724. The concentration of R020-1724 was chosen by measuring the levels of cyclic AMP produced at a given concentration of PGE_1 (10^{-8} M) as a function of R020-1724 concentrations. Since increasing R020-1724 from 10^{-4} to 5 × 10^{-4} M did not cause an appreciable further elevation of cyclic AMP, the former concentration was used in all subsequent studies. Neurotransmitter agonists were added at the same time as PGE_1 while neurotransmitter antagonists were added to cells 3 min prior to PGE_1 or agonists. At the end of the incubation the medium was removed by aspiration and 200 μl of 0.4 N $HClO_4$ were added to the plate. Cells were collected with the aid of a Teflon policeman and the plate was washed with an additional 200 μl $HClO_4$. The combined extracts were briefly sonicated, neutralized with 2 N KOH and centrifuged to remove debris.

Assay for cyclic AMP. Aliquots of the neutralized extract were assayed in duplicate using the cyclic AMP-binding assay described by Brown *et al.*[14]. To correct for reagent effects on the binding assay, an equal volume of reagent blank ($HClO_4$ neutralized with KOH) was added to each of the standards[15]. In preliminary experiments, cyclic AMP was determined after purification from neutralized extracts by the method of Mao and Guidotti[16] and compared with those values obtained from neutralized extracts. Values for cyclic AMP levels using purified and crude extracts were comparable (40.5 ± 7.2 *p*moles/mg of protein in purified extracts, five plates; 46.7 ± 1.7 *p*moles/mg in crude extracts, three plates) and neutralized perchloric acid extracts were used directly for subsequent cyclic AMP determinations. Radioactivity was determined by liquid scintillation using a toluene–Triton X-100–Liquaflor (New England Nuclear) (184:100:16) counting solution. Two or three plates of cells (3 to 6.5 × 10^5 cells/plate; 200–400 μg protein) were used for each experimental condition and each plate was assayed in duplicate for cyclic AMP content. Protein was determined by the method of Lowry *et al.*[17]. Results are expressed as *p*moles/mg of protein ± the range of error, for two plates, or ± the S.D. for three plates. All experiments were repeated at least twice.

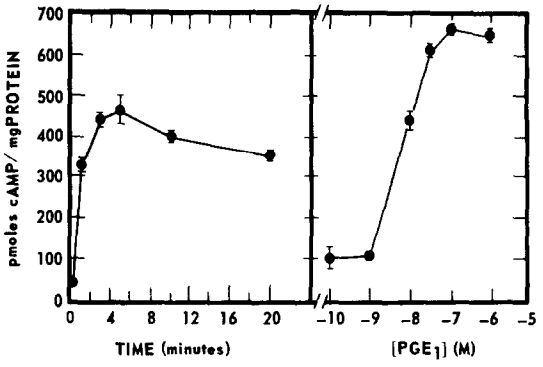

Fig. 1. Effect of PGE_1 as a function of time (left panel) and concentration (right panel) on cyclic AMP levels in the TCX 17 clone.

RESULTS

PGE_1 stimulation of cyclic AMP levels. Cyclic AMP levels rose rapidly in response to 10^{-8} M PGE_1 in TCX 17 cells, reaching maximum levels by 3 min (Fig. 1). Levels of cyclic AMP decreased slowly with longer incubations but were still 75 per cent of the maximum values after 20 min. Saturation of the cAMP response occurred with 3 to 10 × 10^{-8} M PGE_1. Half-maximal stimulation was achieved at 3

Table 1. Effect of selected compounds on both basal and PGE_1-stimulated increases in cAMP*

Additions	Basal	PGE_1(10^{-8} M)
None	46.75 ± 1.70	356 ± 32
Carbachol	24.90 ± 0.15	120 ± 5
l-Norepinephrine	37.40 ± 1.90	195 ± 8
Dopamine	42.50 ± 0.90	215 ± 14
Serotonin	47.30 ± 3.00	394 ± 10
Histamine	60.80 ± 22.70	436 ± 13
Morphine	43.50 ± 3.50	332 ± 25

* Drugs were incubated at 10^{-5} M as described in Methods.

Values are expressed ± S.D. for three plates of cells.

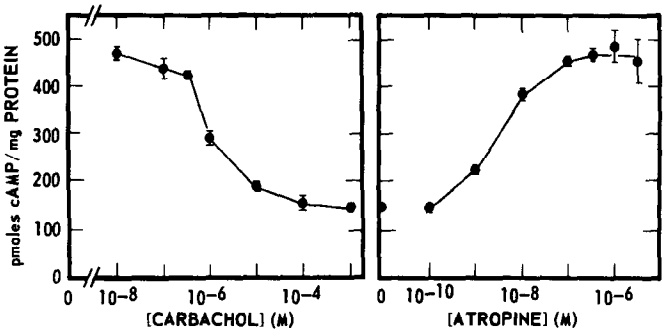

Fig. 2. Left panel: the effect of varying concentrations of carbachol co-incubated with 10^{-8} M PGE_1 on cyclic AMP levels. Right panel: the effect of varying concentrations of atropine in the presence of 10^{-5} M carbachol and 10^{-8} M PGE_1 cyclic AMP levels. The value for cyclic AMP in the presence of 10^{-5} M atropine and PGE_1 was 480 ± 10 *p*moles/mg of protein. For 10^{-5} M carbachol and PGE_1, the cyclic AMP levels were 140 ± 12 *p*moles/mg of protein.

to 10×10^{-9} M PGE_1 (Fig. 1). Based on these results, 3-min incubations with 10^{-8} M PGE_1 were used for all the following studies.

Inhibition of cyclic AMP formation. Carbachol, norepinephrine and dopamine at 10^{-5} M inhibited both basal and PGE_1-stimulated increases in cyclic AMP levels. In a representative experiment depicted in Table 1, carbachol produced a greater degree of inhibition (67 per cent) than either norepinephrine or dopamine (40 and 45 per cent respectively) on cyclic AMP levels in the presence of PGE_1. These drugs also depressed basal levels while serotonin, histamine and morphine (all 10^{-5} M) had no inhibitory effect in the presence or absence of PGE_1. Subsequent studies were done in the presence of PGE_1 because of the relatively greater ease in quantitating cyclic AMP levels.

The inhibition of PGE_1-stimulated cyclic AMP levels by carbachol, norepinephrine and dopamine exhibited dose dependency (Figs. 2 and 3). The ED_{50} for inhibition was determined to be 10^{-6} M for carbachol, 3×10^{-7} M for dopamine, and 10^{-7} M for norepinephrine. Thus norepinephrine was the most potent inhibitor while carbachol produced the greatest degree of inhibition. The effect of varying concentrations of morphine on the cyclic AMP levels was also tested (Fig. 4). No statistically significant inhibition could be observed between 10^{-8} and 10^{-5} M.

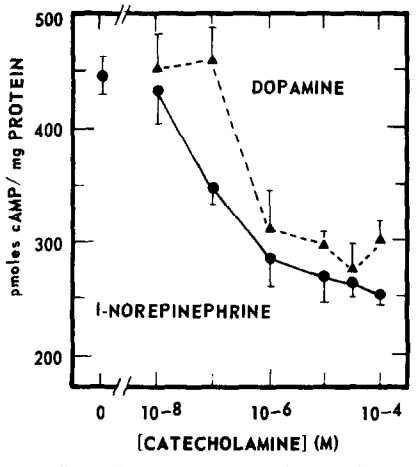

Fig. 3. Effect of preincubation with cholinergic and adrenergic antagonists on cyclic AMP levels in the TCX 17 clone in the presence of 10^{-5} M carbachol and 10^{-8} M PGE_1.

Reversal of the carbachol inhibition. Atropine was found to be a potent antagonist of carbachol (Fig. 2, right panel), with 50 per cent inhibition of the carbachol effect at 3×10^{-9} M. Scopolamine also caused a complete reversal of the carbachol inhibition at 10^{-6} M (Fig. 5). α-Bungarotoxin and *d*-tubocurarine (nicotinic antagonists), propranolol (β-adrenergic antagonist), and chlorpromazine were ineffective (Fig. 5). These data suggest that carbachol is acting through a muscarinic receptor.

Reversal of the norepinephrine inhibition. The inhibition by norepinephrine could be mimicked by phenylephrine, an α-agonist, but not by the β-agonist isoproterenol (Table 2). A number of α- and β-antagonists were also tested in the presence of 10^{-6} M norepinephrine and 10^{-8} M PGE_1. Both phentolamine and phenoxybenzamine at 10^{-6} M reversed the norepinephrine inhibition while the β-antagonists dichloroisoproterenol and propranolol had no effect at similar concentrations. Chlorpromazine, a phenothiazine, also reversed the norepinephrine effect and appeared to be more potent than the other α-antagonists (Table 2).

Inhibition by dopamine. The PGE_1-stimulated increases in cyclic AMP levels were not inhibited significantly by the dopaminergic agonists apomorphine and ET495 at concentrations from 10^{-7} to 10^{-5} M (Table 3, values for 10^{-5} M shown). Of a number of dopaminergic antagonists including fluphenazine,

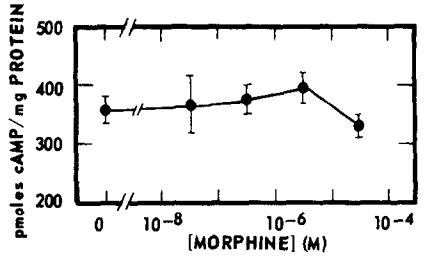

Fig. 4. Effect of varying concentrations of morphine on the levels of cyclic AMP in the presence of 10^{-8} M PGE_1.

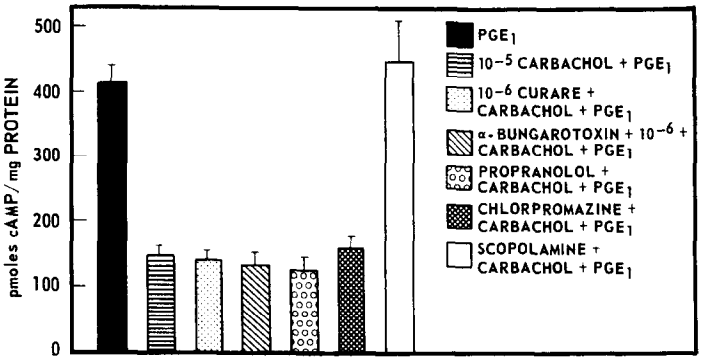

Fig. 5. Effect of co-incubation of dopamine or *l*-norepinephrine with 10^{-8} M PGE_1 on cyclic AMP levels in TCX 17 cells. The value for PGE_1 alone was 440 ± 20 *p*moles/mg of protein.

trifluoperazine, chlorpromazine and bulbocapnine, only chlorpromazine could reverse the depression of cyclic AMP levels induced by 5×10^{-6} M dopamine (Table 3). Phenoxybenzamine was also an effective antagonist of dopamine (Table 3), suggesting that dopamine and norepinephrine may both be acting at the same or similar receptors. This was supported in experiments to test for additive inhibitory effects of the two drugs. When norepinephrine concentrations were held constant (10^{-6} M) and the concentration of dopamine was varied from 10^{-8} to 10^{-5} M, no further inhibition of PGE_1-stimulated cyclic AMP levels was seen.

Effect of neurotransmitters on cyclic AMP levels in the TCX 11 clone. As seen in Table 4, carbachol, norepinephrine and dopamine were effective inhibitors in the sister clone TCX 11. Unlike the TCX 17 clone, all three drugs produced approximately the same degree of inhibition (45–53 per cent).

Table 2. Effect of adrenergic agonists and α- and β-antagonists in the presence of norepinephrine on PGE_1-stimulated cyclic AMP levels*

Additions	Concn (M)	Per cent of control
Control (PGE_1)	10^{-8}	100
Norepinephrine	10^{-6}	64 ± 8
Phenylephrine	10^{-7}	105 ± 8
	10^{-6}	74 ± 4
Isoproterenol	10^{-7}	120 ± 13
	10^{-5}	117 ± 5
Norepinephrine	10^{-6}	
+ Phentolamine	10^{-7}	75 ± 17
	10^{-6}	105 ± 10
+ Phenoxybenzamine	10^{-8}	59 ± 5
	10^{-7}	72 ± 6
	10^{-6}	89 ± 4
+ Chlorpromazine	10^{-8}	67 ± 8
	10^{-7}	110 ± 10
+ Dichloroisoproterenol	10^{-6}	60 ± 10
+ Propranolol	10^{-6}	65 ± 9

* Cells were incubated with adrenergic agonists in the presence of PGE_1 and phosphodiesterase inhibitor as described in Methods. Adrenergic antagonists were preincubated for 3 min prior to addition of PGE_1 and norepinephrine. The effect of each drug was measured at three concentrations (10^{-8} to 10^{-6} M) with two or three plates used for each concentration. Each drug was tested in at least two separate experiments. Where no effect was observed, the highest concentration used is reported. Values are expressed as per cent of control, the value for PGE_1-stimulated increases in cyclic AMP in a given experiment taken to be 100 per cent. In the absence of norepinephrine, values for antagonists were (expressed at per cent of control): 10^{-6} M dichloroisoproterenol, 104 ± 14; 10^{-6} M propranolol, 97 ± 8; 10^{-6} M phenoxybenzamine, 93 ± 7; and 10^{-6} M chlorpromazine, 96 ± 7.

DISCUSSION

The cyclic AMP levels in the two clones studied were found to be sensitive to stimulation by low concentrations of PGE_1. This is presumably the result of direct stimulation of adenylyl cyclase since the experiments were done in the presence of high concentrations of phosphodiesterase inhibitor. The inhibition by norepinephrine, dopamine and carbachol does not seem to be the result of competition with PGE_1 for a receptor site since the three drugs

Table 3. Effect of dopaminergic agonists and adrenergic and cholinergic antagonists in the presence of dopamine on PGE_1-stimulated cAMP levels*

Additions	Concn (M)	Per cent of control
Control (PGE_1)	10^{-8}	100
Dopamine	5×10^{-6}	60 ± 5
Apomorphine	10^{-5}	110 ± 2
ET 495	10^{-5}	89 ± 24
Dopamine		
+ Fluphenazine	10^{-6}	57 ± 4
+ Trifluoperazine	10^{-6}	62 ± 9
+ Bulbocapnine	10^{-6}	55 ± 9
+ Chlorpromazine	10^{-8}	75 ± 5
	3×10^{-7}	86 ± 4
	6×10^{-7}	96 ± 9
+ Phenoxybenzamine	10^{-6}	95 ± 5
+ Propranolol	10^{-6}	55 ± 5
+ Scopolamine	10^{-6}	65 ± 5

* Drugs were incubated with cells as described in Methods and in the footnote to Table 2. Each drug was tested at three concentrations (10^{-8} to 10^{-6} M) in at least two separate experiments. Values for antagonists in the absence of dopamine (expressed as per cent of control) were: 10^{-6} M fluphenazine, 86 ± 7; 10^{-6} M trifluoperazine, 95 ± 5; and 10^{-6} M scopolamine, 97 ± 8. Values for other antagonists are in the footnote to Table 2.

Table 4. Effect of neurotransmitters on PGE_1 stimulation of cAMP levels in the TCX 11 hybrid clone*

Additions	cAMP (*pmoles*/mg protein)
Basal	78.8 ± 8.4
PGE_1 (10^{-8} M)	253 ± 32
PGE_1 + dopamine (10^{-5} M)	122 ± 10
PGE_1 + norepinephrine (10^{-5} M)	113 ± 23
PGE_1 + carbachol (10^{-5} M)	133 ± 34
PGE_1 + serotonin (10^{-5} M)	313 ± 22

* Drugs were incubated with cells as described in Methods.

Results are expressed as ± S.D. for three separate plates of cells.

also depress basal levels of cyclic AMP in the presence of phosphodiesterase inhibitor (Table 1). In addition, norepinephrine, dopamine and carbachol inhibit adenosine-induced increases in cyclic AMP levels in the TCX 17 clone (Blosser, unpublished results). The most direct interpretation is that these drugs inhibit adenylyl cyclase at sites distinct from those of agents which stimulate cyclic AMP. However, the depression of cyclic AMP levels by activation of a phosphodiesterase which is relatively insensitive to phosphodiesterase inhibitor remains a possibility.

The depression of cyclic AMP levels by carbachol appears to be mediated by a muscarinic receptor. Both scopolamine and atropine (Figs. 2 and 5) are potent antagonists of carbachol while the nicotinic antagonists α-bungarotoxin and *d*-tubocurarine are effective at concentrations a hundred times higher than those needed for complete reversal by the muscarinic antagonists.

Acetylcholine has been reported to inhibit cyclic AMP synthesis via muscarinic receptors in other neuronal hybrid and neuroblastoma clones [18, 19] and to increase cyclic AMP levels in one neuroblastoma line [20].

The inhibition by norepinephrine and dopamine could be reversed by the α-antagonists phenoxybenzamine (Tables 2 and 3) and phentolamine (Table 2) while the β-antagonists propranolol and dichloroisoproterenol were without effect. Chlorpromazine was also a potent antagonist of both norepinephrine and dopamine (Tables 2 and 3). Although chlorpromazine is a well-known dopaminergic antagonist in the CNS [21], its α-antagonist properties have been documented for peripheral tissues [22]. Since the TCX clones are derived from a sympathetic ganglion cell and a neuroblastoma clone of peripheral origin [9], chlorpromazine may be acting in the latter fashion. The lack of effect of fluphenazine and trifluoperazine on the action of dopamine may reflect the widely variable α-antagonist properties of different phenothiazines [22]. Both of these drugs are effective antagonists of dopamine-stimulated adenylyl cyclase [23] and specific dopamine binding to the dopamine receptor in the CNS [24]. Bulbocapnine, another dopamine antagonist, was also without effect on the inhibition by dopamine (Table 3).

Several lines of evidence indicate that norepinephrine and dopamine are acting through an α-like adrenergic receptor. First, the inhibition is not additive. This could result from saturation of a common receptor or, alternatively, by saturation of the response shared by two separate receptors. Second, α-adrenergic antagonists reverse the inhibition by both catecholamines. Third, an α-antagonist, but not dopaminergic or β-adrenergic agonists, mimics the inhibition by norepinephrine and dopamine (Tables 2 and 3).

It may be of significance that these exponential growth phase cells are in an undifferentiated state. The companion paper presents evidence that differences exist between the pharmacology of the NA and DA response in cells treated with dbcAMP. Thus, we cannot rule out the possibility that the catecholamine receptor in these exponential growth phase cells is non-specific for NA and DA.

Dopamine and norepinephrine have been shown to affect cyclic AMP levels in other cell lines of neuronal origin. Traber *et al.* [25] have also reported norepinephrine and dopamine inhibition of PGE_1 stimulation of cyclic AMP levels in glioma × neuroblastoma hybrids. Prasad and Gilmer [26] found dopamine-induced increases in cyclic AMP in a neuroblastoma clone. Presumably, such opposite effects are a function of the cell line being examined.

Despite the presence of opiate receptors in the TCX 17 clone [9], morphine had no inhibitory effect on basal or PGE_1-sensitive adenylyl cyclase. This is in contrast with reports describing an inhibitory effect by morphine in neuroblastoma × glioma hybrid cells [27, 28]. The effect of morphine on adenylyl cyclase in the CNS is not yet clear. Although opiates have been reported to inhibit adenylyl cyclase *in vitro* [29], efforts to demonstrate this phenomenon in brain slices [30] *in vivo* [31] or with brain homogenates [32, 33] by other investigators have proven difficult.

An alternative speculation is that opiate receptors (or different kinds of opiate receptors) could be coupled to different membrane functions. In this regard, morphine has been shown to block a dopamine depolarization response in the TCX clones (P. Myers, unpublished results). Opiate receptors linked to different functions would not be unlike neurotransmitters, such as acetylcholine, which affect distinct physiological processes through different receptors (i.e. nicotinic or muscarinic receptors). The possibility of different types of opiate receptors is suggested by the difference in relative potencies of opiates, Met-enkephalin and Leu-enkephalin in different peripheral assays [34].

The mechanism by which the cyclic AMP levels are depressed by the cholinergic and adrenergic agonists is unknown. The suggestion by Goldberg *et al.* [35] that cyclic GMP mediates physiological events that are antagonistic to those which are mediated by cyclic AMP provides one possible lead. In this regard, Matzuzawa and Nirenberg [19] have observed that carbachol, which stimulated cyclic GMP levels, can inhibit adenosine stimulation of cyclic AMP levels.

These results described here clearly demonstrate

the ability of neurotransmitters to depress basal and PGE_1-stimulated adenylyl cyclase. This may hold significance in understanding the mechanism of neural function of these neurotransmitter agents in more complex preparations.

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