Biochemical Pharmacology, Vol. 27, pp. 1173–1177.

0006-2952/78/0415-1173$02.00/0

# NEUROTRANSMITTER MODULATION OF PROSTAGLANDIN $E_1$-STIMULATED INCREASES IN CYCLIC AMP

## II. CHARACTERIZATION OF A CULTURED NEURONAL CELL LINE TREATED WITH DIBUTYRYL CYCLIC AMP

PAUL R. MYERS,* JAMES BLOSSER† and WILLIAM SHAIN
Neurobiology Department, Armed Forces Radiobiology, Research Institute, Bethesda, MD 20014, U.S.A.

(*Received* 22 *April* 1977; *accepted* 8 *July* 1977)

**Abstract**—The ability of selected neurotransmitters to modulate $PGE_1$-stimulated increases in cAMP was tested in the somatic cell hybrids TCX 17 and TCX 11 differentiated by growth in dibutyryl cAMP. $PGE_1$ was shown to cause an increase in cellular cAMP. Carbachol, noradrenaline and dopamine inhibited the effect of $PGE_1$, while 5-hydroxytryptamine had no effect. The carbachol inhibition is mediated by a muscarinic receptor since nicotinic antagonists failed to block carbachol while scopolamine reversed its effect. The noradrenaline inhibition was reversed by the antagonists phenoxybenzamine and phentolamine, but not by the $\beta$-antagonists propranolol and dichloroisoproterenol. The dopamine inhibition was reversed by chlorpromazine and trifluoperazine. The dopamine agonist ET495 mimicked dopamine while apomorphine had little or no effect. These results obtained from differentiated cells are compared to those reported for exponential growth phase cells of the same cell line. Distinct differences were found with respect to the pharmacology of the noradrenaline and dopamine inhibition. Finally, the biochemical results are compared to the electrophysiological results reported for the cell lines. Neurotransmitter agents that modulate $PGE_1$ effects do not necessarily elicit membrane conductance changes, and, similarly, neurotransmitters that elicit an electrophysiological response do not inhibit $PGE_1$-stimulated increases in cAMP. Dopamine elicits an electrophysiological response and inhibits the effects of $PGE_1$. The possibility exists that a single receptor is mediating two cellular events.

It is well documented that prostaglandins function in modulation of nervous system activity [1, 2]. The mechanism by which this occurs is, for the most part, unknown. One approach to understanding events at the cellular level is the utilization of cultured neural cell systems. Such preparations have proven useful for investigating the ability of opiates to antagonize $PGE_1$-stimulated adenylyl cyclase [3, 4] and to study the modulation of $PGE_1$ activity by neurotransmitters [5]. Two aspects involved in the use of cultured neural cells have not been comprehensively studied: (1) the differences that may exist between exponential growth phase cells and cells that have been "differentiated", and (2) the existence of a relationship between the electrophysiological characteristics of neurotransmitter receptors and the ability of these neurotransmitters to alter the $PGE_1$-stimulated adenylyl cyclase. Such data would be helpful in understanding the interactions of small molecules and $PGE_1$ from both a developmental and a mechanistic standpoint.

We have utilized two somatic cell hybrids differentiated by growth in dbcAMP to investigate the effect of selected neurotransmitters on cyclic AMP production stimulated by $PGE_1$. The purpose was to ascertain possible differences in the sensitivity of the cells to $PGE_1$ and the selected neurotransmitters and to construct a pharmacological profile of the neurotransmitter responses that could be compared with results using exponential growth phase cells reported in a companion paper [6]. In addition, the biochemical results reported here are discussed in light of the previously reported electrophysiological results obtained from the cells [7].

### MATERIAL AND METHODS

Two somatic cell hybrid lines were used in this study, TCX 17 and TCX 11. TCX 17 and TCX 11 are subclones of NX 31, a cell line that resulted from the fusion of cells from the neuroblastoma N18TG2 and sympathetic ganglion cells from 13-day-old mouse embryos [8]. The selection and growth of the cell lines are described elsewhere (P. Myers and W. Shain, manuscript in preparation). The data from experiments on TCX 11 are reported for comparison to TCX 17 and also for purposes of discussion regarding the relationship between neurotransmitter modulation of $PGE_1$-induced changes in cyclic AMP levels and the electrophysiological results from separate studies.

The growth of cells in 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma Co., St. Louise, MO, grade II) has been previously described [7]. Cells (2 to $3 \times 10^5$) were seeded into 60-mm Falcon dishes using regular growth medium. The following day, this medium was replaced with the medium supplemented with 1 mM dbcAMP. The incubation procedure

* Present address: Central Research and Development Department, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, DE 19898.
† Present address: Dept. of Neurology, Baylor College of Medicine, Houston, TX 77030.

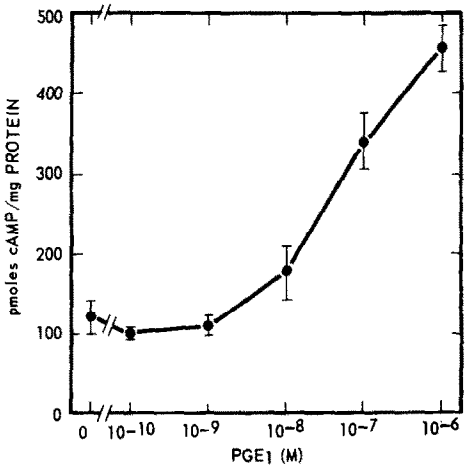


Fig. 1. Ability of $PGE_1$ to stimulate increases in cyclic AMP levels in TCX 17 differentiated by growth in dibutyryl cyclic AMP. Medium containing dibutyryl cyclic AMP was removed 3 hr prior to experiments and replaced with a conditioned growth medium. These data, and all other data presented, are the result of triplicate determinations (I.S.D.). All experiments were repeated at least twice. Subsequent experiments were done at $6.6 \times 10^{-8}$ M $PGE_1$, unless stated otherwise.

and source of drugs are identical to those in the companion paper. After growth in dbcAMP for 6 days, medium was removed and the cells were washed twice with 5 ml of warm (37°) growth medium without dbcAMP. The last wash was replaced using conditioned medium. Conditioning was attained by exposure of the medium to exponential growth phase cell cultures for 48 hr. The washing procedure removed dbcAMP that would interfere with measurement of cyclic AMP that was produced as a result of incubation with $PGE_1$. A 3-hr time period was allowed after the wash procedure prior to experiments. Use of a non-conditioned medium with or without dbcAMP resulted in a temporary loss of biochemical and electrophysiological responsiveness by cells. Approximately 4–6 hr are required to regain sensitivity to effectors. Use of

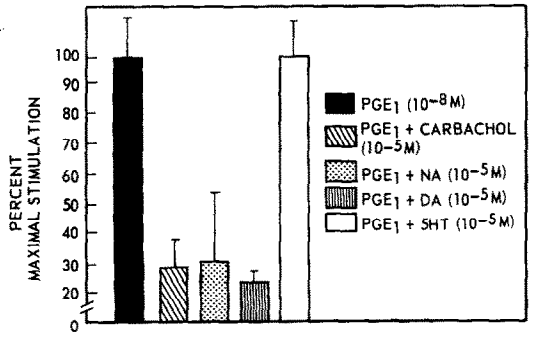


Fig. 2. Effects of selected neurotransmitters or neurotransmitter agonists on $PGE_1$-stimulated increased in cyclic AMP (± S.D.) in TCX 17 differentiated by growth in dibutyryl cyclic AMP. In this representative experiment, the basal level of cyclic AMP was 62 ± 9 pmoles/mg. $PGE_1$ ($10^{-8}$ M) raised cyclic AMP levels to 172 ± 23 pmoles/mg; $PGE_1$ + DA ($10^{-5}$): 91.5 ± 2.4 pmoles/mg; $PGE_1$ + NA ($10^{-5}$): 96 ± 21 pmoles/mg; $PGE_1$ + carbachol: 89.9 ± 11 pmoles/mg; and $PGE_1$ + 5-HT: 172 ± 22 pmoles/mg.

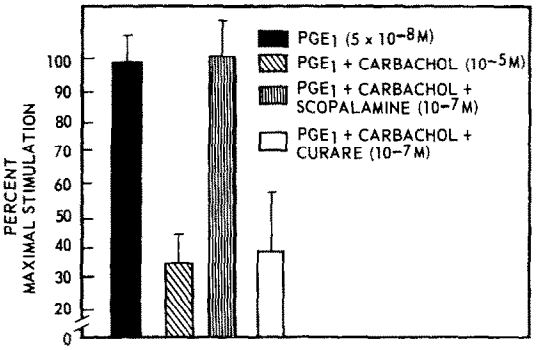


Fig. 3. Reversal of the carbachol inhibition in differentiated TCX 17 cells by the muscarinic antagonist scopalamine but not by the nicotinic antagonist curare (± S. D.).

conditioned medium prevents loss of these responses. Cyclic AMP was assayed, as in the companion paper, by methods described in Ref. 9.

## RESULTS

An incubation time of 3 min was chosen for all experiments. At this time period, maximal stimulation was observed with exponential growth phase cells (see companion paper). Figure 1 shows that TCX 17 grown in dbcAMP responds to $PGE_1$ by production of cyclic AMP. A concentration of $6.6 \times 10^{-8}$ M $PGE_1$ was used in subsequent experiments unless otherwise indicated. The highest concentration of $PGE_1$ tested was 1 $\mu$M and the response was not saturated.

If either carbachol, dopamine or noradrenaline was added simultaneously with $PGE_1$, cyclic AMP production was inhibited (Fig. 2). In contrast, 5-hydroxytryptamine was not effective in inhibiting the $PGE_1$-stimulated adenylyl cyclase. Pharmacological experiments were done in order to further characterize the inhibition by neurotransmitters of $PGE_1$ action (Table 1).

The inhibition by carbachol ($10^{-5}$ M) was blocked by the muscarinic antagonist scopalamine ($10^{-7}$ M) but not by the nicotinic antagonist *d*-tubocurarine ($10^{-7}$ M) (Fig. 3).

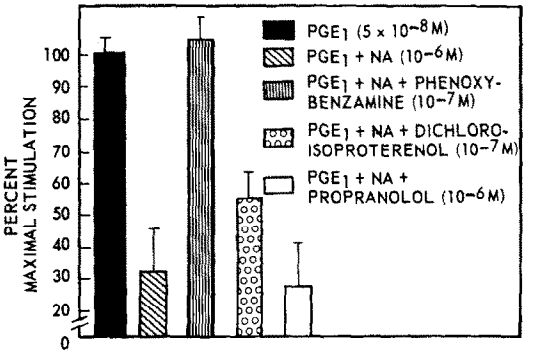


Fig. 4. Reversal of the noradrenaline (NA) inhibition of $PGE_1$-stimulated increases in cyclic AMP in differentiated TCX 17 by the $\alpha$-antagonist phenoxybenzamine. Phentolamine also reversed the noradrenaline effect while $\beta$-adrenergic antagonists were ineffective.

Table 1. Inhibition of $PGE_1$ activity*

| | Exponential growth phase | Differentiated | Electro-physiological response |
|---|---|---|---|
| Dopamine | + | + | + |
| Noradrenaline | + | + | + |
| 5-Hydroxytryptamine | − | − | + |
| Carbachol | + | + | − |

* Biochemical results from exponential growth phase TCX 11 cells vs differentiated TCX 11 cells and the ability of selected neurotransmitters to inhibit $PGE_1$-stimulated increases in cyclic AMP are compared to the ability of these compounds to elicit an electrophysiological response. In the electrophysiological experiments acetylcholine, not carbachol, was used. See text for experimental details.

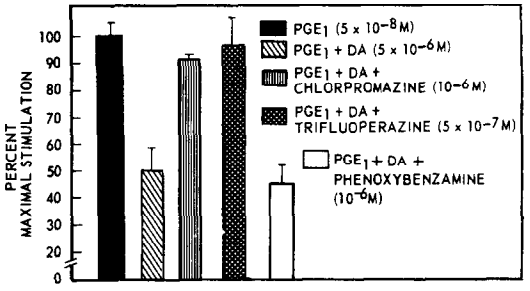


Fig. 5. Reversal of dopamine inhibition of $PGE_1$-stimulated increases in cyclic AMP by chlorpromazine and trifluoperazine, but not phenoxybenzamine, in differentiated TCX 17 cells (± S. D.).

The pharmacological results on the noradrenaline inhibition were consistent with the presence of an α-like receptor. Phenoxybenzamine ($10^{-7}$ M) reversed the noradrenaline ($10^{-6}$ M) inhibition while propranolol ($10^{-6}$ M) had little or no effect and dichlorisoproterenol ($10^{-7}$ M) had a small but significant effect (Fig. 4). Phentolamine ($10^{-6}$ M) (not shown) also reversed the noradrenaline inhibition.

The phenothiazines chlorpromazine ($10^{-6}$ M) and

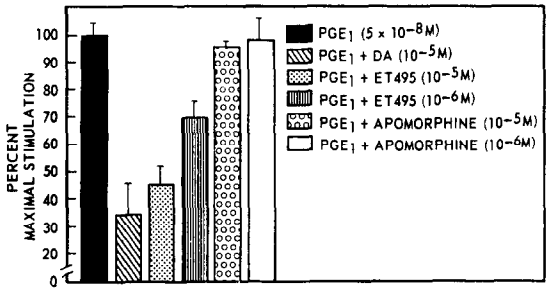


Fig. 6. Ability of the dopamine agonist ET495 to mimic the inhibition by dopamine of $PGE_1$-stimulated increases in cyclic AMP in differentiated TCX 17 cells. Apomorphine appeared to have little, if any, effect. In this experiment, $PGE_1$ ($5 \times 10^{-8}$ M) increased cyclic AMP 270 ± 28 *p*moles/mg over basal levels; $PGE_1$ + DA ($10^{-5}$ M): 85 *p*moles/mg; $PGE_1$ + ET495 ($10^{-6}$ M): 165 *p*moles/mg; $PGE_1$ + ET495 ($10^{-5}$ M):110 *p*moles/mg; $PGE_1$ + apomorphine ($10^{-6}$ M): 229 *p*moles/mg; and $PGE_1$ + apomorphine ($10^{-5}$ M): 225 *p*moles/mg. Values are the mean of four determinations.

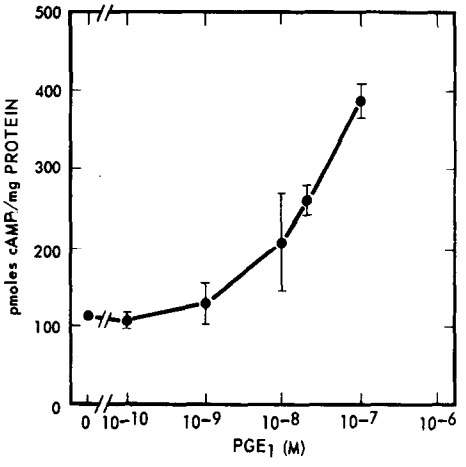


Fig. 7. Effects of $PGE_1$ on cellular cyclic AMP levels in TCX 11 cells differentiated by growth in dibutyryl cyclic AMP. As with experiments with TCX 17, medium containing dibutyryl cyclic AMP was removed 3 hr prior to experiments and replaced with conditioned medium. Data are expressed as the mean (± S. D.) and were determined in triplicate. Most experiments with TCX 11 were repeated at least twice.

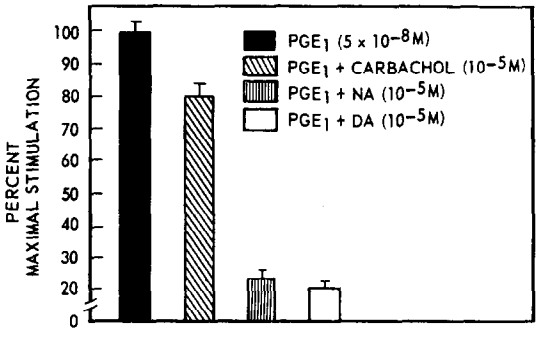


Fig. 8. Inhibition of $PGE_1$-stimulated increases in cyclic AMP by carbachol, noradrenaline and dopamine in TCX 11 cells differentiated by growth in dibutyryl cyclic AMP. Error bars denote standard deviation. $PGE_1$ ($6.6 \times 10^{-8}$ M) increased levels of cyclic AMP 659 *p*moles/mg above basal levels; $PGE_1$ + carbachol ($10^{-5}$ M): 532 *p*moles/mg; $PGE_1$ + NA ($10^{-5}$ M): 161 *p*moles/mg; and $PGE_1$ + DA ($10^{-5}$ M): 137 *p*moles/mg. Values are the mean of four determinations.

trifluoperazine ($5 \times 10^{-7}$ M) reversed the inhibition by dopamine of the $PGE_1$-stimulated adenylyl cyclase (Fig. 5). Phenoxybenzamine ($10^{-6}$ M) failed to reverse the dopamine effect. Likewise, the dopamine agonist ET495 was an effective inhibitor of cyclic AMP production stimulated by $PGE_1$ (Fig. 6). The dopamine agonist apomorphine appeared to have little or no activity.

Experiments were also done on the cell line TCX 11. $PGE_1$ stimulated the production of cyclic AMP (Fig. 7) and this stimulation was inhibited by carbachol, noradrenaline and dopamine (Fig. 8), but not 5-hydroxytryptamine (not shown). Carbachol was not as effective in inhibiting the $PGE_1$-stimulated adenylyl cyclase in TCX 11 cells as it was in TCX 17 cells. In TCX 17 cells, $10^{-5}$ M carbachol inhibited $PGE_1$ stimulation of cyclic AMP by 70 per cent, but in TCX 11 cells, $10^{-5}$ M carbachol inhibited only 20 per cent. The degree of inhibition by dopamine and norepinephrine was comparable with that seen in TCX 17 cells.

## DISCUSSION

These results demonstrate the presence of a $PGE_1$-sensitive adenylyl cyclase in the cell lines TCX 17 and TCX 11 after the cells were differentiated by growth in dbcAMP. Furthermore, production of cyclic AMP is inhibited by the neurotransmitters carbachol, noradrenaline and dopamine, but not 5-hydroxytryptamine. Pharmacological experiments suggested that the cholinergic effect was mediated by a muscarinic receptor and that the noradrenaline effect had pharmacological properties consistent with those of an $\alpha$-receptor. The dopamine effect was inhibited by two phenothiazines, compounds known to be dopamine receptor antagonists.

Growth of cells in dbcAMP has been reported to induce both biochemical and morphological changes [10–13]. Chalazonitis and Greene [14] have reported that growth of NX31, the parent cell line to TCX 17 and TCX 11, in dbcAMP results in significant electrophysiological changes. The rate of cell division in TCX 17 and TCX 11 decreases, and the cells extend processes to become neuronal in appearance in response to growth in medium containing dbcAMP. The membrane potential also increases from $-10$ to $-20$ mV (unpublished observations) to an average value of $-58$ mV [7]. Thus with reference to these properties and those reported in the literature for other diverse cell lines, these cells are characteristic of a "differentiated" state. The remainder of this discussion will (1) address the pharmacological changes which occur between cells in exponential growth phase and differentiated cells and (2) relate the biochemical results to electrophysiological results from these cells as to whether a single receptor is mediating two cellular events (i.e. modulation of $PGE_1$ and also membrane conductance changes).

Distinct differences occur between TCX 17 in exponential growth phase and those exposed to dbcAMP with regard to (1) the sensitivity to $PGE_1$, (2) the degree of inhibition by the neurotransmitters tested and (3) the pharmacology of the neurotransmitter inhibition. Half-maximal stimulation of cyclic AMP production by $PGE_1$ in exponential growth phase cells is at $5–6 \times 10^{-9}$ M (see companion paper) whereas in differentiated cells the value appears to be shifted to a higher concentration. $PGE_1$ concentrations up to 1 $\mu$M were tested but the response is not saturated at this high concentration. This reflects a significant shift to the right of the dose–response characteristic in dbcAMP-treated cells. The degree of inhibition of $PGE_1$-stimulated increases in cyclic AMP in TCX 17 by NA and DA changed when cells were grown in dbcAMP. In exponential growth phase cells the degree of inhibition was approximately 30–40 per cent for NA and DA but in dbc-treated cells the inhibition of $PGE_1$ increased to 70 per cent. Carbachol inhibition remained unchanged. In TCX 11 cells, there was a slight increase in inhibition of $PGE_1$ activity by NA and DA when cells were treated with dbcAMP. Interestingly, the inhibition by carbachol decreased from a value of approximately 45–50 per cent in exponential growth phase cells to 15–20 per cent inhibition in dbcAMP-treated cells.

The pharmacological results clearly demonstrate that the cholinergic receptor is muscarinic in both the exponential growth phase and differentiated cells since muscarinic antagonists blocked the carbachol effect but nicotinic antagonists did not.

Results obtained from the companion paper on exponential growth phase cells have indicated that noradrenaline and dopamine are acting at a common or similar receptor(s). This is based primarily on the reversal of both the noradrenaline and dopamine inhibition of $PGE_1$-stimulated adenylyl cyclase by the same antagonists ($\alpha$-antagonists and chlorpromazine). However, the results reported for differentiated cells contrast significantly in three regards: (1) the $\alpha$-antagonist phenoxybenzamine blocks the noradrenaline effect but not the dopamine effect, whereas in exponential growth phase cells it blocked both noradrenaline and dopamine; (2) trifluoperazine did not antagonize the dopamine inhibition in exponential growth phase cells but did inhibit the dopamine effect in differentiated cells; (3) the dopamine agonist, ET495, was ineffective in exponential growth phase cells but mimicked dopamine in differentiated TCX 17 cells. Apomorphine had little or no effect on the $PGE_1$-stimulated accumulation of cyclic AMP in either exponential growth phase or differentiated cells. Therefore, with cells in exponential growth phase, the responses to dopamine and noradrenaline appear similar pharmacologically but in differentiated cells the data indicate that the receptor pharmacology has changed. There is now pharmacological specificity of the dopamine and noradrenaline responses, suggesting that separate receptors are expressed for these catecholamines.

This observation is of interest from a developmental standpoint. A catecholamine receptor may have been expressed in an "immature" state in the exponential growth phase cells. Upon differentiation of the cells, biochemical or structural alterations conferred specificity upon the receptors, permitting pharmacological agents to distinguish them. Alternatively, growth in dbcAMP could induce the expression of dopaminergic receptors not previously

present in any form. Of course, the question remains if in the exponential growth phase cells the catecholamines themselves were sharing a common receptor or separate receptors and that the pharmaceuticals simply could not distinguish these receptors.

Electrophysiologically, the compounds dopamine, noradrenaline and 5-hydroxytryptamine elicit responses in the cell line TCX 11, but acetylcholine does not [7]. Dopamine and noradrenaline also elicit a response in TCX 17 (unpublished observations; 5-hydroxytryptamine was not tested). Based upon parallel experiments, the electrophysiological response to dopamine in TCX 17 is identical pharmacologically and physiologically to that in TCX 11 (unpublished results). The electrophysiological evidence from both TCX 11 and TCX 17 strongly suggests that dopamine and noradrenaline are acting at a common receptor. This is primarily based upon observations that (1) dopamine is more potent than noradrenaline, and (2) dopamine desensitizes the membrane to noradrenaline and noradrenaline desensitizes the membrane to dopamine [7]. The electrophysiological experiments were conducted on cells grown in medium containing dbcAMP.

In light of the results reported in this paper which show that dopamine, noradrenaline and carbachol, but not 5-hydroxytryptamine, inhibit $PGE_1$-stimulated adenylyl cyclase in the two cell lines, it is apparent that electrophysiological changes and depression of cyclic AMP can occur independently of one another. Thus a neurotransmitter that elicits a membrane conductance change (i.e. 5-hydroxytryptamine) does not necessarily exert an inhibitory action on $PGE_1$-stimulated adenylyl cyclase. Similarly a neurotransmitter that inhibits the effect of $PGE_1$ does not elicit an electrophysiological response (i.e. carbachol) (see Table 1).

The question remains if (1) separate receptors mediate conductance changes and modulation of $PGE_1$ effects or if (2) the same receptor "mediates" both phenomena. Electrophysiologically, dopamine and noradrenaline appear to act at a common receptor. Biochemically, dopamine and noradrenaline alter the effects of $PGE_1$. Noradrenaline may be acting at an $\alpha$-receptor to inhibit $PGE_1$ stimulation of adenylyl cyclase that does not elicit an electrophysiological response. The effect of noradrenaline electrophysiologically may be mediated by the dopamine receptor. It is tempting to speculate that dopamine is acting at a receptor which elicits both a membrane conductance change and a change in $PGE_1$-stimulated cyclic AMP production. Drugs that block the electrophysiological dopamine responses or mimic dopamine also block or mimic the effect of dopamine on $PGE_1$-stimulated cyclic AMP in differentiated cells. Although these data may not provide conclusive evidence for a clear choice between the two possibilities stated above, they do provide insight as to the complex possibilities available to cellular systems for integration and modulation of function.

Therefore, significant differences may exist between cells in exponential growth phase and cells in a differentiated state with respect to the receptor properties discussed here. It may be of importance to consider such differences in other diverse studies of cells in culture. Based upon our electrophysiological and biochemical results, we suggest that (1) receptors may change their pharmacology during differentiation and (2) the receptor for a selected neurotransmitter may be capable of effecting more than one cellular response. These observations in turn offer possibilities for interactions related to aspects of cellular function.

## REFERENCES


1. P. Hedquist, in *The Prostaglandins* (Ed. P. Ramwell), pp. 101–31. Plenum, New York (1973).
2. M. Brady and P. Kadowitz, *Fedn Proc.* **33**, 48 (1974).
3. J. Traber, K. Fischer, S. Latzin and B. Hamprecht, *Nature, Lond.* **253**, 120 (1975).
4. J. Traber, K. Fischer, S. Latzin and B. Hamprecht, *Nature, Lond.* **253**, 120 (1975).
5. J. Traber, G. Reiser, K. Fischer and B. Hamprecht, *Fedn Eur. Biochem. Soc. Lett.* **52**, 327 (1975).
6. J. C. Blosser, P. R. Myers and W. Shain, *Biochem. Pharmac.* **27**, 1167 (1978).
7. P. Myers, D. Livengood and W. Shain, *J. Cell. Physiol.* **91**, 103 (1977).
8. L. Greene, W. Shain, A. Chalazonitis, X. Breakfield, J. Minna, H. Coon and M. Nirenberg, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4923 (1975).
9. B. L. Brown, R. P. Ekins and J. D. Albano, Jr., in *Advances in Cyclic Nucleotide Research* (Eds. P. Greengard and G. A. Robinson), 2, pp. 25–40. Raven Press, New York (1972).
10. K. Prasad and A. Hsie, *Nature, New Biol* **233**, 141 (1971).
11. J. Waymire, N. Weiner and K. Prasad, *Proc. natn. Acad. Sci. U.S.A.* **69**, 2241 (1972).
12. A. Mackay and L. Iversen, *Brain Res.* **48**, 424 (1972).
13. E. Richelson, *Nature New Biol.* **242**, 175 (1973).
14. A. Chalazonitis and L. Greene, *Brain Res.* **72**, 340 (1974).