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Prostaglandin E_1 Effects on Adenosine 3′:5′-Cyclic Monophosphate Concentration and Phosphodiesterase Activity in Fibroblasts

(mouse L cells/tissue culture/enzyme kinetics/prostaglandin homologues)

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ABSTRACT **Incubation of L-929 and L-2071 fibroblasts with prostaglandin E_1 (PGE_1) caused a rapid increase in the cyclic AMP content of these cells. A maximal effect was produced with 0.2 μg PGE_1 per ml. At a concentration of 4 μg/ml, PGE_2 was almost equally effective, but $PGF_{2\alpha}$ and PGA_2 were much less so. 2.6 μM epinephrine, 0.4 mM serotonin, and 0.2% ethanol were without effect. In L-929 cells, cyclic AMP concentrations remained elevated for 2–5 hr, and then declined, although even after a 24-hr incubation the medium contained PGE_1 in a concentration sufficient to increase maximally the cyclic AMP content of cells not previously exposed to this compound. A second addition of PGE_1 after 5 or 24 hr did not produce another increase in the concentration of cyclic AMP. After incubation with PGE_1 for 24 hr, cyclic AMP phosphodiesterase activity, assayed with 0.56 μM substrate, was increased 30–100%; the activity rose further between 24 and 48 hr. It is suggested that the increase in phosphodiesterase activity that appears to be a consequence of prolonged elevation of cyclic AMP concentration may account at least in part for the apparent "refractoriness" to PGE_1 that develops after incubation for several hours with this compound.**

Effects of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and its derivatives on growth, morphology, and density-dependent inhibition of growth of several lines of mammalian cells in culture have recently been reported (1–4). Certain prostaglandins that increase the cyclic AMP concentration in cultured neuroblastoma cells (5) can influence growth and morphology and stimulate adenylate cyclase activity in fibroblasts (6). We have found that prostaglandin E_1 (PGE_1), in addition to producing a rapid rise in the cyclic AMP concentration of mouse L-cell fibroblasts, causes an increase in cyclic AMP phosphodiesterase activity assayed after 24 hr of incubation with this compound.

MATERIALS AND METHODS

L-929 and L-2071 cells were purchased from the American Type Culture Collection and maintained as monolayer cultures in 250-ml Falcon flasks (plastic) in Eagle's basal medium (Grand Island Biological Co.), supplemented with Earle's salts, 10% fetal-calf serum (Grand Island Biological Co.), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, at 36°C in a constant-temperature incubator. Cells were detached from Falcon flasks by treatment with trypsin, and 4–9 × 10^5 cells were transferred to Optilux Tissue Culture dishes (100 × 20 mm, Falcon Plastics) that contained 10 ml of growth medium. After equilibration with a humidified atmosphere containing 5% CO_2, the dishes were incubated in an airtight box at 37°C, usually for 3 or 4 days before use in experiments.

For most studies in which cyclic AMP was measured, the medium containing serum was removed and the cells were washed with serum-free medium and incubated again for 1 hr with 10 ml of serum-free growth medium. Compounds to be tested were then added and the incubation was continued at 36°C in a humidified atmosphere containing 5% CO_2. To terminate the incubation, the medium was quickly removed by aspiration and 1.5 ml of cold 5% trichloroacetic acid was added to the tissue culture dishes. The cells were removed with a rubber policeman, the dishes were washed with an additional 1.2 ml of water, and the cells plus wash were frozen in a dry ice–ethanol bath. After the addition of about 0.25 pmol of [^{3}H]cyclic AMP (16.3 Ci/mmol) and 0.3 ml of 1 N HCl, the mixture was thawed and centrifuged. The precipitate was dissolved in 1 N NaOH for measurement of protein (7).

The supernatant fluid was added to a 0.5 × 8 cm column of Dowex-50 (100–200 mesh) equilibrated with 0.1 N HCl. After elution with 4 ml of 0.1 N HCl and 1 ml of water, which removed all the Cl_3CCOOH from the column [as evidenced by a spot test sensitive to 0.5 μg of Cl_3CCOOH (8)], cyclic AMP was eluted with 10 ml of water. This fraction was lyophilized and the residue was suspended in 2 ml of water and taken to dryness by rotary evaporation under reduced pressure. Samples were dissolved in water, and aliquots were assayed at two dilutions for cyclic AMP, by the method of Gilman (9), and for recovery of [^{3}H]cyclic AMP.

For measurement of cyclic AMP phosphodiesterase activity, cells were rinsed twice with 5 ml of a solution containing 1 mM $MgSO_4$, 0.1 mM dithiothreitol, and 2 mM glycylglycine (pH 7.4). They were scraped from the culture dish with a rubber policeman, and homogenized in a tight-fitting glass Dounce homogenizer in 3–6 ml of the same medium. Cell disruption was monitored by light microscopy. Enzymatic activity was assayed at 30°C as described for fat-cell homogenates (10). The assay was done in a total volume of 0.3 ml containing 25 μmol glycine buffer (pH 8.5) and 3.0 μmol $MgCl_2$. Under these conditions, the amount of cyclic AMP hydrolyzed by L-cell homogenates was proportional to time and to enzyme concentration.

Prostaglandins (PGE_1, PGE_2, $PGF_{2\alpha}$, and PGA_2) were generously supplied by Dr. John E. Pike of the Upjohn Co., Kalamazoo, Mich. With the largest volumes of these compounds tested, the final concentration of ethanol in the experimental medium was 0.2%.

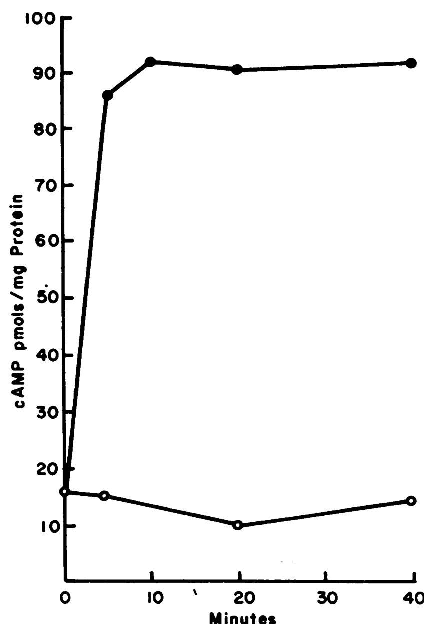

FIG. 1. L-929 cells were incubated in serum-free medium with (●) or without (○) 3 μg/ml of PGE_1. Each point represents the mean of duplicate incubations.

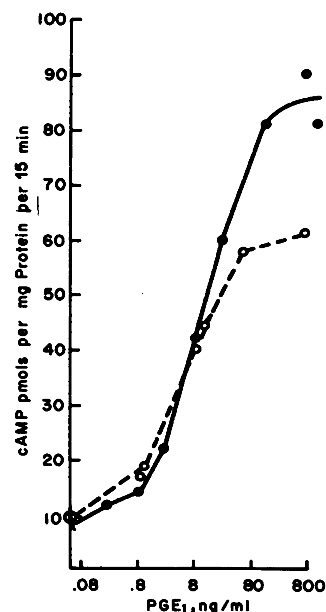

FIG. 2. L-929 and L-2071 cells were incubated in serum-free medium with (●, ○) or without (×, ⊗) PGE_1 at the indicated concentration. Each point represents the cyclic AMP content of a single tissue-culture dish, or the mean of duplicate incubations. ●——●, L-2071; ○- - -○, L-929.

RESULTS

Within 5–10 min after addition of 3 μg/ml of PGE_1 to monolayers of L-929 fibroblasts, cyclic AMP concentrations were increased at least 8-fold, and remained essentially constant for 30–50 min thereafter (Fig. 1). The response of L-2071 cells was qualitatively similar, although maximal cyclic AMP concentrations observed in these cells tended to be somewhat higher than those in the L-929 strain. As shown in Fig. 2, about 0.15 μg/ml of PGE_1 (0.42 μM) produced a maximal elevation of cyclic AMP concentration (measured at 15 min) in both cell lines. PGE_2 was perhaps somewhat less effective than PGE_1, whereas PGA_2 and $PGF_{2\alpha}$ at the same concentrations produced much smaller increases in cyclic AMP concentrations (Table 1). 1 mM Theophylline had little or no effect on basal cyclic AMP concentrations and produced relatively small increases (less than 50%) in the presence of the several prostaglandins (at concentrations that were maximally effective). Neither 2.6 μM epinephrine, 0.4 mM serotonin, or 0.2% ethanol increased cyclic AMP concentrations in either cell line (data not shown).

When, after incubation for 15 min with PGE_1, cells were washed and incubated in fresh medium without PGE_1, cyclic AMP concentration fell within 5 min to 2–3 times the basal concentration, and remained there for the next 45 min (Fig. 3). A second addition of PGE_1 30 min after the L-929 cells were washed (Expt. 2) raised the cyclic AMP concentration in 15 min to the same amount found in cells incubated for the entire hour with this compound. A similar cyclic AMP concentration was observed in cells to which PGE_1 was added for the final 15 min of incubation. A second addition of PGE_1 to cells incubated for 45 min with this compound produced no further increases in cyclic AMP concentrations.

As shown in Fig. 4*A*, cyclic AMP concentrations in L-929 cells remained at 8- to 9-times the basal value for at least 5 hr of incubation with PGE_1, but by 12 hr of incubation had declined about 30%, and remained at a similar concentration after 24 hr. For this experiment, as for all of those mentioned above in which the incubation period was 1 hr or less, medium

TABLE 1. *Effect of prostaglandins and theophylline on cyclic AMP concentration in L-929 and L-2071 fibroblasts*

Addition (μg/ml)	L-929 cells: No theophylline	L-929 cells: Plus theophylline	L-2071 cells: No theophylline	L-2071 cells: Plus theophylline
	(nmol cAMP/mg of protein)			
None	6.5 (±0.1)	8.0 (±0.6)	6.4 (±1.8)	6.5 (±0.7)
PGE_1, 3	83.1 (±0.7)	99.6	102	102
PGE_2, 4	59 (±1)	79 (±9)	78.3 (±4.1)	111 (±7)
PGA_2, 4	20.3 (±0.3)	23.3 (±0.1)	14.0 (±0.5)	22.8 (±2.8)
$PGF_{2\alpha}$, 4	14.0 (±0.4)	15.7 (±1.9)	36.2 (±0.2)	47.0 (±0.7)

Cells were incubated with or without 1.5 mM theophylline for 15 min. Values are given as means of observed data, with range of duplicate values indicated.

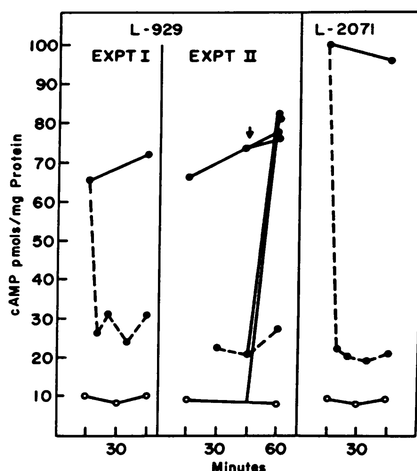

FIG. 3. L-929 and L-2071 cells were incubated with (●) or without (O) 2 μg/ml of PGE_1. After 15 min, the medium containing PGE_1 was removed from some dishes and the dishes were then washed and incubated with fresh medium without PGE_1 (- -●- -). In Expt. II, PGE_1 was added again (*arrow*) to some dishes at 45 min. Each point represents the cyclic AMP content of a single tissue-culture dish or the mean of duplicate incubations.

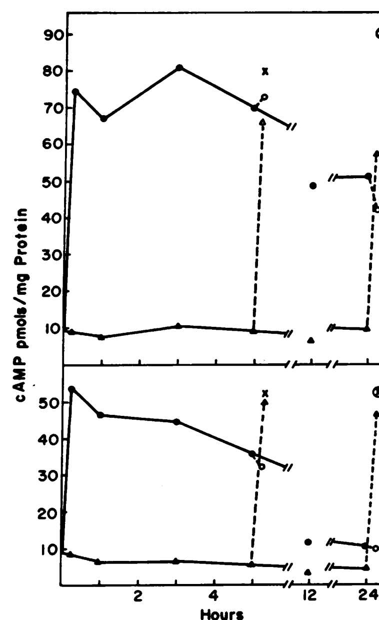

FIG. 4. L-929 cells were incubated with (●) or without (▲) 2 μg/ml of PGE_1. At 5 and 24 hr, fresh PGE_1 was added to control cells (△) and to cells incubated with PGE_1 (O). Medium that had originally contained 3 μg/ml of PGE_1 and had been incubated for 5 (×) or 24 (⊗) hr with other L-929 cells was added to some control dishes. Incubations were terminated 15 min after these additions. Each point represents the cyclic AMP content of a single tissue-culture dish or the mean of duplicate incubations. *Top*, without serum; *bottom*, with serum.

without serum was used. The somewhat different time course observed when serum was present in the medium (Fig. 4*B*) may be related at least in part to the fact that those cells were washed before fixation to remove medium protein, whereas cells incubated without serum were fixed immediately after removal of the medium. Whether or not the serum was present, addition of fresh PGE_1 to cells incubated for 5 or 24 hr with this compound produced no increase in cyclic AMP concentration. Even after incubation for 24 hr, there was sufficient PGE_1 remaining in the medium (with or without serum) to produce an increase in cyclic AMP concentration of control cells equal to that observed when fresh PGE_1 was added. Incubation of cells for 24 hr with 0.2% ethanol did not alter basal concentrations of cyclic AMP, or the effect of PGE_1 on control cells.

Phosphodiesterase activity

Homogenates of L-cells contained phosphodiesterase activity with at least two apparent Michaelis constants for cyclic AMP, one 0.5–3.0 μM and the other 100–300 μM. As shown in Table 2, with concentrations of cyclic AMP below about 30 μM, activity was enhanced by 0.5 μM guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Higher concentrations of cyclic GMP were inhibitory. Similar dual effects of cyclic GMP have been observed in several mammalian tissues (11) and in lymphocytes (12).

After incubation of L-929 cells with PGE_1 for 24 hr, phosphodiesterase activity assayed at a substrate concentration of 0.56 μM was markedly increased (Table 3). In several experiments, activity assayed at higher substrate concentrations (>100 μM) appeared to be slightly decreased. The effect of PGE_1 on phosphodiesterase activity was not influenced by the presence of serum. As shown in Fig. 5, PGE appeared to increase the *Vm* at low substrate concentrations, but the significance of the apparent changes in *Km* remains to be evaluated. Addition of PGE_1 to homogenates did not stimulate diesterase activity. In assays of mixtures of homogenates of control and treated cells, the activities were strictly additive. As shown in Table 4, phosphodiesterase activity (assayed with 0.56 μM substrate) continued to increase during the second 24 hr of incubation with PGE_1. After 48 hr, concentrations were in some cases more than twice the basal values, but increased little more between 48 and 72 hr. Under these conditions, 0.2% ethanol did not produce any increase in diesterase activity. When PGE_1 was removed after 24 hr of incubation, phosphodiesterase activity fell little, if at all, during the subsequent 24 or 48 hr. After 48 hr, the cells responded to a second addition of PGE_1 with

TABLE 2. *Effect of cyclic GMP on cyclic AMP phosphodiesterase activity in L-929 fibroblasts*

Substrate concentration Cyclic AMP (μM)	Phosphodiesterase activity (nmol per mg of protein per 10 min)	
	No cyclic GMP	0.5 μM Cyclic GMP
55	40.4	41
28	25.5	30.3
5.5	5.0	11.6
2.8	2.5	6.4
1.1	0.98	2.9
0.56	0.51	1.4
0.28	0.28	0.71

Samples of a homogenate of L-929 cells were incubated with different concentrations of cyclic AMP in the presence and absence of 0.5 μM cyclic GMP. Each value represents the mean of duplicate incubations, which agreed within ±5%.

TABLE 3. *Effect of PGE_1 on phosphodiesterase activity in L-929 cells*

Expt. no.	Additions		Phosphodiesterase activity*	
	Serum†	PGE_1‡	0.55 mM cAMP (nmol per mg protein per 10 min)	0.56 μM cAMP (nmol per mg protein per 10 min)
1	−	−	73.2 (±14.2)	0.55 (±0.14)
	+	−	68.6 (±3.9)	0.55 (±0.05)
	−	+	67.6 (±10.4)	1.18 (±0.02)
	+	+	63.7 (±0.2)	0.90 (±0.03)
2	−	−	88.2 (±2.2)	0.79 (±0.02)
	+	−	79.0 (±7)	0.63 (±0)
	−	+	67.5 (±0.5)	1.12 (±0.02)
	+	+	69.6 (±0.4)	0.91 (±0.03)

Cells were incubated for 24 hr. Data as in Table 1.
* Assayed with 0.55 mM and 0.56 μM cAMP as substrate.
† 10% v/v.
‡ 2 μg/ml.

a percentage increase in cyclic AMP concentration equal to that observed in control cells. The response 24 hr after removal of PGE_1 was clearly diminished.

DISCUSSION

The rapid rise in cyclic AMP concentration in fibroblasts produced by the addition of PGE_1 and the rapid fall after its removal are completely explicable in terms of the demonstrated (6) stimulatory effect of PGE_1 on fibroblast adenylate cyclase activity. (The failure of cyclic AMP concentrations to return completely to basal values was probably due to incomplete removal of PGE_1 by the washing procedure used, but this conjecture remains to be proven.) When incubation with PGE_1 was prolonged, cyclic AMP concentration in fibroblasts after the initial rise remained elevated for some time (more than 5 hr in medium without serum) and then began to decline. A second addition of PGE_1 at 5 or 24 hr did not produce another rise in cyclic AMP concentration and, in fact, the medium after 24 hr of incubation still contained PGE_1 at a concentration sufficient to maximally elevate cyclic AMP concentrations in fresh cells. We had previously observed similar behavior on a somewhat different time scale in fat cells incubated with epinephrine or adrenal corticotrophic hormone (ACTH), and had unsuccessfully attempted to demonstrate altered phosphodiesterase activity during the period when cyclic AMP concentrations were declining despite the continued presence of the hormone (13). In the fibroblasts, however, phosphodiesterase activity assayed with 0.56 μM substrate was invariably increased (30–100%) by incubation for 24 hr with PGE_1, and continued to rise between 24 and 48 hr. After incubation of cells with PGE_1 for 24 hr, the phosphodiesterase activity fell little, if at all, during a subsequent 24- or 48-hr incubation without this compound, while the ability of the cells to increase their cyclic AMP concentration in response to PGE_1 was partially restored. Phosphodiesterase activity as we have measured it obviously did not correlate precisely with the magnitude of the effect of PGE_1 on cyclic AMP concentrations. It seems, nevertheless, likely that when the cells become "refractory" after prolonged incubation with PGE_1, this is at least in part due to the change in phosphodiesterase activity that develops during the same period.

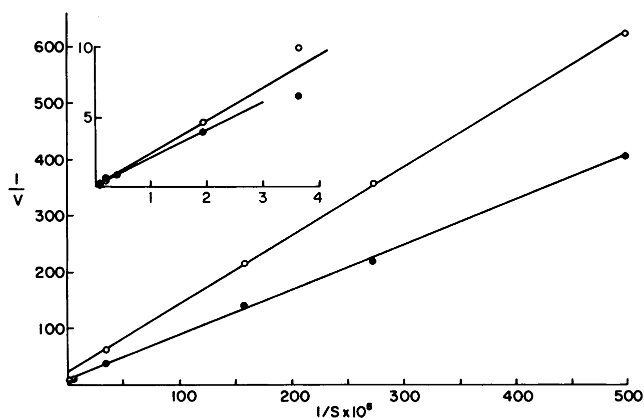

FIG. 5. L-929 cells were incubated for 24 hr with or without 2 μg/ml of PGE_1. Samples of the cell extracts were immediately assayed for phosphodiesterase activity and frozen in dry ice–ethanol. These data represent the mean of duplicate incubations of extracts that had been frozen for 24 hr at −60°C without loss of the stimulatory effect of PGE_1 on phosphodiesterase activity. Incubation mixtures contained either 29 μg of protein from control cells or 28 μg from PGE_1-treated cells. The Michaelis constants for cyclic AMP in homogenates from PGE_1-treated cells were 0.7 μM and 0.2 mM, as compared to 0.5 μM and 0.3 mM in control-cell homogenates. ○—○, basal; ●—●, PGE_1.

TABLE 4. *Effect of PGE_1 on cyclic AMP concentration and phosphodiesterase activity in L-929 cells*

Expt. no.	Conditions*	Phosphodiesterase activity† (nmol per mg protein per 10 min)	cAMP concentration pmol/mg protein: No PGE_1	cAMP concentration pmol/mg protein: Plus PGE_1
1	Basal (72)	0.80	6.4	65.5
	PGE_1 (24)	1.04	19.8	17.5
	PGE_1 (48)	1.38	ND	ND
	PGE_1 (72)	1.49	18.6	17.7
	PGE_1 (24 then basal, 48)	1.03	4.9	45.2
2	Basal (48)	0.55	4.9	49.3
	PGE_1 (24)	0.96	10.4	11.8
	PGE_1 (48)	1.44	10.2	11.7
	PGE_1 (24 then basal, 24)	0.84	4.7	27.9

Cells were incubated in medium containing serum, with or without 2 μg/ml of PGE_1, for the indicated times. Medium was changed daily. For each experimental condition, two dishes of cells were used for the determination of protein content and phosphodiesterase activity, and four for measurement of cyclic AMP content. 2 μg/ml of PGE_1 was added to two of the latter dishes for the last 15 min of incubation. Cells used for cyclic AMP measurements were fixed by addition of 5% Cl_3CCOOH immediately after aspiration of the incubation medium. All cells were harvested at the same time. Values presented are the means of duplicate determinations, which agreed within ±15%. ND = not done.
* Hours indicated in parentheses.
† Substrate concentration: 0.56 μm.

Whether the increase observed in phosphodiesterase activity only when assays are performed in the μmolar range of substrate concentrations represents a change in the amount of enzyme or in its properties (e.g., in the amount of a regulatory protein) remains to be determined. In preliminary experiments, we have found that incubation of L-929 cells with 0.5 mM dibutyryl cyclic AMP produces increases in phosphodiesterase activity at least as great as those seen in cells incubated with PGE_1 for the same period.* Thus, the alteration in phosphodiesterase activity apparently is not the result of a specific action of PGE_1, but could be secondary to a sustained elevation of intracellular cyclic AMP concentration induced in various ways. A similar regulatory mechanism may be operative in many types of cells. We are attempting to learn whether and how the increased diesterase activity may be related to the changes in growth and morphology produced in L-929 cells by PGE_1 and dibutyryl cyclic AMP (3).

NOTE ADDED IN PROOF

In an article to be published in the February issue of the Proceedings, Drs. Massimo d'Armiento, George S. Johnson, and Ira Pastan report that phosphodiesterase activity is increased in 3T3 and SV40/3T3 fibroblasts after incubation with dibutyryl cyclic AMP and theophylline and in L-929 cells after incubation with PGE_1.

* Effects of both these compounds have been observed as early as 5 hr, the shortest time yet investigated, and can be prevented by cycloheximide or actinomycin D.

We thank Mrs. Betty Hom and Miss Sally Stanley for their excellent technical assistance, and Dr. Florence K. Millar for computer programming of the cyclic AMP assay.

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