The Effects of Epinephrine and Prostaglandin E₁ on Cyclic Adenosine 3':5'-Monophosphate Levels in WI-38 Fibroblasts

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SUMMARY
1. Intact WI-38 human diploid lung fibroblasts were shown to respond to epinephrine (3 × 10⁻⁷ M) and prostaglandin E₁ (PGE₁) (0.57 × 10⁻⁷ M) with increased cyclic adenosine 3':5'-monophosphate (cyclic AMP) levels. Corticotropin (1.25 units per ml), insulin (0.02 to 200 milliunits per ml), and glucagon (5 × 10⁻⁷ M) were ineffective. Although the response to PGE₁ was not clearly maximal at concentrations up to 2.8 × 10⁻⁷ M, the response to 5.7 × 10⁻⁸ M PGE₁ was greater than the maximal response to epinephrine (10 × 10⁻⁶ M) under all conditions studied.

2. Theophylline (2 mM) potentiated the action of PGE₁ on cyclic AMP levels by at least 2-fold in all experiments, but was less effective with epinephrine; in several experiments theophylline did not potentiate the effects of the catecholamine.

3. Propranolol (0.1 mM) completely inhibited the effect of epinephrine but only slightly diminished that of PGE₁.

4. Cells grown to high population density responded poorly or not at all to epinephrine while cells at low population densities responded very well. A converse relationship was observed between PGE₁ action and population density, although it was not as dramatic. The effect of population density on the epinephrine stimulation did not appear to be due to a difference in sensitivity of the populations to epinephrine.

5. Cyclic AMP appeared in the incubation medium of WI-38 cells treated with epinephrine or PGE₁. This appearance reached maximal levels more slowly than did intracellular cyclic AMP but represented a considerable amount of cyclic nucleotide.

A number of recent studies in cell culture systems have established their value as models for investigations of the control of cyclic AMP metabolism in mammalian cells (1-3). Of particular advantage is that the problems of cellular heterogeneity and limited diffusion encountered in more complex mammalian preparations are largely eliminated in cell culture systems.

A possible objection to many of the previous studies with cultured cells has been that most of the established cell lines used, while derived from normal tissues, have become adapted to the conditions of long term culture and have taken on many of the characteristics of transformed cells (4). It is because of this limitation that we have studied the human diploid lung fibroblast, WI-38, derived by Hayflick (5). WI-38 cells maintain their diploid karyotype throughout a definite number of generations (40 to 60 population doublings), after which they cease to divide. Hence, this system has been used as a model for the study of aging (6). Further, WI-38 cells show a high degree of density-dependent growth inhibition ("contact" inhibition), and retain the ability to produce collagen as a differentiated function (7).

Several recent studies have shown that prostaglandins increased cyclic AMP levels in other fibroblast cell lines (3, 8, 9) and that the adenylate cyclase in some of these cell lines was activated by epinephrine (10-12) or PGE₁ (2, 13). This background led us to an investigation of the properties of WI-38 fibroblasts as a model for studies on the role of cyclic AMP in regulation of the normal mammalian cell.

EXPERIMENTAL PROCEDURE

Methods
Propagation of Cell Cultures—WI-38 cell cultures were obtained from Dr. L. Hayflick, Stanford University, Stanford, California. These cells were propagated from frozen stock in Eagle's minimal essential medium supplemented with penicillin-streptomycin (100 units per ml), kanamycin (100 µg per ml), and 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.). Nutrient medium was renewed every 3 to 4 days and the cells were maintained at 37°C in a humidified 95% air-5% CO₂ atmosphere. Seed cultures were maintained in T-25 (Falcon) flasks. When confluent, the cells were dispersed by treatment with 0.25% trypsin (1:250) and distributed over a total surface area four times the area of the harvest vessel; this allowed the population to double two times between subcultivations. Large populations were grown for experimental use by successive 1:4 splits in 90-mm glass Petri dishes. The experiments reported here were carried out in a humidified atmosphere; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
with cell populations which were approximately 2 to 25 doublings away from the terminal growth phase. No obvious effects of "aging" were observed throughout the work described in this communication.

**Experimental Incubation Procedure.**—Two days prior to an experiment, cells were seeded from 90-mm Petri dishes into experimental vessels (25-mm glass vials, 90-mm glass Petri dishes, or 90-mm glass Petri dishes, depending on the experiment) containing fresh growth medium. Duplicate vessels were prepared for each experimental point to be determined. On the day of the experiment, each attached cell sheet was rinsed two times in serum-free medium. Fresh serum-free medium was added at 37°C and hormones or other additions made. The vessels were replaced with their incubation atmosphere at 37°C for the duration of the experiment. The experiments were terminated by removing the incubation media, adding approximately 20,000 dpm of cyclic [3H]AMP (4.2 nCi per pmole) to each incubation vessel and immediately fixing the attached cell sheets by the addition of cold (0°C) 5% trichloroacetic acid. The fixed cells were placed on ice, a second extraction with trichloroacetic acid was carried out, and the extracts were pooled and lyophilized prior to purification. Cell residues were solubilized in 0.3 M KOH and samples were taken for protein analysis according to the method of Lowry et al. (14). Samples of incubation media were taken for analysis as incubations were terminated and immediately transferred to tubes containing HCl (to yield a final concentration of 0.1 M) and approximately 20,000 dpm of cyclic [3H]AMP. The acidified samples were lyophilized and used for purification.

**Purification of Cyclic AMP from Experimental Samples—**Lyophilized samples were redissolved in a small amount of water, 10 μl of 1 M Tris-HCl (pH 7.4) were added, the samples were neutralized with NaOH, and the volume was adjusted to 1.2 ml. The neutralized samples were applied to small columns (0.4 x 6 cm) of 100 to 200 mesh Dowex AG50W-X8 resin (Bio-Rad) which had been equilibrated with 0.05 M HCl. When a sample had passed through a column, 1.0 ml of distilled water was added and both eluates were discarded. A receiving vessel was then placed under each column, 3.0 ml of water were added, and this entire eluate was collected for cyclic AMP analysis. The 3.0-ml samples were lyophilized, the residue was taken up in a small volume of 0.25 M acetic acid (pH 4.0), and 40-μl aliquots were assayed by the method of Gilman (15). Cyclic AMP recoveries after purification were generally between 60 and 70%.

To test the validity of the cyclic AMP binding assay, samples of cell extracts and incubation media were treated with purified beef heart cyclic 3',5'-phosphodiesterase for 3 hours at pH 7.4. The apparent cyclic AMP activity was reduced by more than 98% after this treatment.

Each experimental point was determined from duplicate incubations and two samples from each duplicate were assayed for cyclic AMP; samples containing large amounts of cyclic nucleotide were assayed at two dilutions. The experimental results are expressed as picomoles of cyclic AMP accumulated per mg of cell protein ± one-half the difference between duplicate determinations. The duplicate difference is represented in figures by the vertical notation ±.

As a special note it should be emphasized that experimental controls generally reflect the limit of detection of cyclic AMP in the assay system. The control values are therefore viewed as experimental blanks and are not intended to represent true basal cyclic AMP levels in the cells.

**Materials**

Prostaglandins E1, F3, and F12 were kindly provided by Dr. John Pike of the Upjohn Company. The prostaglandins were prepared for use in these studies as follows: a small portion of known weight was solubilized in 0.2 ml per mg of absolute ethanol. The sample was then neutralized with 0.1 ml per mg of 1% NaOH and taken to a final stock concentration of 200 μg per ml by the addition of 4.7 ml per mg of balanced salt solution (Eagle's MEM). A control vehicle of identical composition but lacking PGE1 was added to incubations not containing PGE1. Epinephrine bitartrate was obtained from K & K Laboratories. Concentrated stock solutions were made up in distilled water. Stock solutions were neutralized by at least a 200-fold dilution in incubation medium.

For estimations of cyclic AMP recovery after purification, cyclic [3H]AMP (4.2 nCi per pmole) was obtained from New England Nuclear, purified on Dowex AG50W-X8 (Bio-Rad) before use, and made up to the desired concentration in 0.01 M Tris-HCl, pH 7.4. A higher specific activity cyclic [3H]AMP (20.6 mCi per pmole) was obtained and treated similarly for use in the cyclic AMP binding assay.

**Results**

Epinephrine and PGE1 caused substantial increases in cyclic AMP levels in WI-38 cells (Table I). The response to PGE1 was more dramatic than the response to epinephrine under all conditions studied. Other hormones, including ACTH (125 units per ml), insulin (at concentrations from 0.02 to 200 milliunits per ml), and glucagon (5 x 10^(-5) M) were ineffective.

Theophylline acted synergistically with epinephrine and PGE1 to increase the cyclic nucleotide levels in the WI-38 cells (Table II, Experiment 1). This synergistic effect was apparent whether theophylline was added 60 min before or at the same time as epinephrine or PGE1. However, the degree of potentiation of the epinephrine effect (relative to that of PGE1) varied considerably from experiment to experiment. In some cases theophylline potentiated the effect of epinephrine nearly as well as that of PGE1 but in others theophylline was almost without effect on the epinephrine stimulation (Table II, Experiment 2). Most commonly, however, we observed results similar to that in Experiment 1, Table II, where the epinephrine and PGE1 effects were potentiated by factors of 2.2 and 2.7, respectively.

The effects of varied concentrations of epinephrine and PGE1 on cyclic AMP levels in the WI-38 cells are summarized in Fig. 1. A significant stimulation by epinephrine was evident at a concentration of approximately 3 x 10^(-7) M and an equivalent stimulation by PGE1 occurred at a concentration of 0.57 x 10^(-5) M. The apparent maximal response to epinephrine occurred at approximately 5 x 10^(-4) M; however, the effect of PGE1 was not clearly maximal at any concentration tested (up to 2.8 x 10^(-5) M in some experiments).

The effect of epinephrine on cyclic AMP levels was completely antagonized by 0.1 mM propranolol, whereas the effect of PGE1 was only slightly decreasced by propranolol (Table III). The α-adrenergic antagonist phentolamine (4 x 10^(-4) M) caused only a small (10 to 20%) inhibition of the epinephrine effect.

The cell density of WI-38 populations markedly and specifically influenced the responses of the cells to epinephrine (Fig. 2).
3100

TABLE II

**Effects of theophylline on cyclic AMP levels in WI-38 cells**

WI-38 cells were prepared in 25-mm glass vials as described under "Methods." Incubation medium (2 ml) with or without 2 mM theophylline was added to each vial, epinephrine or PGE,

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Cyclic AMP accumulated per cell protein (pmoles/mg)</th>
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<tbody>
<tr>
<td></td>
<td>Theophylline</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>138 ± 1</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>467 ± 87</td>
</tr>
<tr>
<td>Epinephrine + PGE1 (5.7 μM)</td>
<td>1062 ± 105</td>
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<tr>
<td>Control</td>
<td>&lt;125*</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>538 ± 138</td>
</tr>
<tr>
<td>PGE1 (5.7 μM)</td>
<td>1472 ± 20</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;125*</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>538 ± 138</td>
</tr>
<tr>
<td>PGE1 (5.7 μM)</td>
<td>1472 ± 20</td>
</tr>
</tbody>
</table>

* Calculated on the basis of the limit of sensitivity of the assay system.

TABLE III

**Effects of propranolol on cyclic AMP levels in WI-38 cells exposed to epinephrine and PGE1**

WI-38 cells were prepared in 25-mm glass vials as described under "Methods." Medium (2 ml) containing 2 mM theophylline was added to each vial and the vials were preincubated 60 min before the experiment. In final incubations containing propranolol, the propranolol was added approximately 5 min prior to the addition of epinephrine or PGE1. Prostaglandin solubilization vehicle was added to vials not containing PGE1. The incubation time was 10 min. Extracts of the cells were analyzed and the results expressed as indicated under "Methods."

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Cyclic AMP accumulated per cell protein (pmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;125*</td>
</tr>
<tr>
<td>Propranolol (0.1 mM)</td>
<td>&lt;125*</td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>725.1 ± 55</td>
</tr>
<tr>
<td>Epinephrine + propranolol</td>
<td>725 ± 55</td>
</tr>
<tr>
<td>PGE1 (5.7 μM)</td>
<td>2713 ± 209</td>
</tr>
<tr>
<td>PGE1 + propranolol</td>
<td>2128.3 ± 19</td>
</tr>
</tbody>
</table>

* Calculated on the basis of the limit of sensitivity of the assay system.

Fig. 2. The effects of propranolol on cyclic AMP levels in populations of WI-38 cells grown to varied densities. A concentrated suspension of WI-38 cells was seeded directly and at two dilutions into separate sets of 90-mm glass Petri dishes; the three sets of cells were prepared for the experiment as described under "Methods." Ten milliliters of medium containing 2 mM theophylline were added to each dish, additions were made as indicated, and incubations were carried out for 10 min. In this experiment separate controls were run with (V) and without (C) prostaglandin solubilization vehicle; vehicle was not added to vessels treated with epinephrine. The concentration of epinephrine (EPI) was 10 μM and the PGE1 concentration was 5.7 μM. Cell extracts were analyzed and the results expressed as described under "Methods."

![Graph](http://www.jbc.org/)

That is, at low growth density responded to a given concentration of epinephrine with higher intracellular cyclic AMP levels than did cells grown to higher densities or to confluence. In contrast, cyclic AMP levels in cells exposed to PGE1 were approximately the same at all densities.

Further experiments were carried out to determine cyclic AMP concentrations in the media under these conditions, since it seemed possible that a stimulation of high population density cells by epinephrine might be obscured by a concomitant release of cyclic AMP into the incubation medium. Contrary to these expectations, we found exaggerated effects of cell density, in that cyclic AMP levels in response to epinephrine in both the cells and media were greater in sparsely grown cells (Table IV). Conversely, the responses to PGE1 were greater in cells from higher density populations, although these differences were not as marked.

*Two additional methods were used to obtain identical results in populations of equally varied densities. The two methods were as follows: (a) A dilute cell suspension was seeded (equal aliquots) into vessels having diameters of 90, 60, and 25 mm, and the experiment was carried out as above; and (b) a dilute cell suspension was seeded (equal aliquots) into a number of 90-mm vessels and separate sets of vessels were incubated in the presence of epinephrine or PGE1 at intervals of 2, 8, and 14 days (with intermittent medium renewal as necessary).
WI-38 cells were prepared and incubated exactly as described in Fig. 2, except for the inclusion of prostaglandin solubilization vehicle in the control and epinephrine-treated vessels. Values reported for "Cells plus media" (right-hand column) are the sums of the averaged values of the "Cells" and "Media" determinations.

### TABLE IV

Comparison of cyclic AMP levels in WI-38 cells and in incubation media after exposure of high and low density populations to epinephrine and PGE$_2$

<table>
<thead>
<tr>
<th>Cell density (pg/cm$^2$)</th>
<th>Incubation conditions</th>
<th>Cyclic AMP accumulated per cell protein</th>
<th>pmoles/mg</th>
<th>Cells</th>
<th>Media</th>
<th>Cells plus media</th>
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</thead>
<tbody>
<tr>
<td>7.94</td>
<td>Control</td>
<td>59 ± 15</td>
<td>&lt;40*</td>
<td>&lt;99*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>657 ± 48</td>
<td>904 ± 102</td>
<td>1061</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$ (5.7 μM)</td>
<td>3028 ± 207</td>
<td>967 ± 54</td>
<td>3395</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.1</td>
<td>Control</td>
<td>44 ± 9</td>
<td>&lt;25*</td>
<td>&lt;60*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>142 ± 20</td>
<td>150 ± 5</td>
<td>292</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$ (5.7 μM)</td>
<td>3508 ± 521</td>
<td>1474 ± 110</td>
<td>5042</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.5</td>
<td>Control</td>
<td>53 ± 16</td>
<td>&lt;20*</td>
<td>&lt;79*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (10 μM)</td>
<td>36 ± 17</td>
<td>33 ± 3</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$ (5.7 μM)</td>
<td>3089 ± 291</td>
<td>1761 ± 63</td>
<td>5450</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated on the basis of the limit of sensitivity of the assay system.

The media of PGE$_2$-treated cells contained approximately 1 μM cyclic AMP, which was the concentration used in the previous cell density experiments.

A comparison of cyclic AMP levels in cells and in the incubation media after various periods of incubation with PGE$_2$ is shown in Fig. 4. Intracellular levels reached an apparent maximum by 5 min and fell to about 50% of this maximum level by 10 min. From 10 to 50 min the levels were essentially unchanged, but remained well above the levels in untreated cells.

If the cyclic AMP levels at 60 min are expressed in terms of concentrations rather than as picomoles per mg of cell protein, the media of PGE$_2$-treated cells contained approximately 1 μM.

### Fig. 3

The effects of varied concentrations of epinephrine on cyclic AMP levels in high and low population density WI-38 cells. A concentrated suspension of WI-38 cells was seeded directly into one set of 90-mm glass Petri dishes, then diluted 6-fold and seeded into a second set of dishes. After further preparation as described under "Methods," medium (10 ml) containing 2 mM theophylline and 20 mM HEPES buffer, pH 7.4, was added to each dish. Epinephrine was then added at the concentrations indicated and incubations were allowed to proceed for 10 min. Incubation media were removed, the cells were fixed in trichloroacetic acid, and the extracts were analyzed and the results expressed as indicated under "Methods."

### Fig. 4

Cyclic AMP levels in WI-38 cells and in the incubation medium after various periods of exposure of the cells to PGE$_2$. Cells were seeded into 60-mm glass Petri dishes and prepared for the experiment as described under "Methods." Medium (3 ml) containing 20 mM HEPES buffer, pH 7.4, was added to each dish, the cells were then treated with either PGE$_2$ (5.7 μM) or vehicle (V) and individual vessels were incubated for the intervals indicated. Incubation media and attached cells were fixed separately and samples analyzed as described under "Methods." Results are expressed as described under "Methods."

*The use of the buffer, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid was initiated during the course of these studies as a means of supporting the bicarbonate buffer system in the maintenance of pH under widely varied conditions. HEPES (pK$_a$ 7.55) is one of a group of nonvolatile organic buffers developed by Good et al. (16) for potential use in biological systems. Two reports (17, 18) have suggested that HEPES is nontoxic to the growth of several cell lines, including WI-38, at concentrations of 20 mM. Pilot studies showed that 20 mM HEPES had no apparent influence on the cyclic AMP response of WI-38 cells to epinephrine or PGE$_2$. This concentration of HEPES considerably enhanced the pH stability of the medium.
cyclic AMP after 60 min and the corresponding cells contained approximately 20 μM cyclic AMP.

**Discussion**

The results presented here demonstrate that cyclic AMP levels in WI-38 cells are markedly but distinctively affected by epinephrine and PGE₁. The divergent effects of cell density on the responses of WI-38 cells to epinephrine and PGE₁ are reminiscent of those observed by Clark and Perkins in astrocytoma cells (19). In this latter system, cyclic AMP levels were dramatically increased by epinephrine and norepinephrine only in cells at low density, while increases caused by histamine appeared to be independent of the cell density.

The factors responsible for the divergent cellular responses of WI-38 cells to different agents at differing population densities are unknown. It seems possible that the hormonal responsiveness of cells in active growth may differ from those in stationary populations. In such were the case, the characteristic density-dependent (contact) inhibition of growth in higher density populations might explain our results. Also, the lower probability of intracellular communication among the cells of low density populations might likewise affect cyclic AMP responses in some unknown fashion. However, these are only two of many possible speculations.

Regardless of the mechanism involved, these data re-emphasize the importance of experimental conditions to the results obtained, and we offer this example as a caveat for future studies. WI-38 cells also share with astrocytoma cells (19) a relative insensitivity to theophylline when stimulated by catecholamines (as compared to the effects of theophylline in combination with other stimulatory agents). A similar situation obtains in adipocytes isolated from hamster epididymal fat pads. These cells are relatively unresponsive to methylxanthines when stimulated by norepinephrine. Conversely, the usual sort of potentiation is fully expressed with isoproterenol, epinephrine, or ACTII (20).

Theophylline potentiates the actions of most hormones which increase cyclic AMP levels by inhibiting the cyclic 3′:5′-AMP phosphodiesterase and it seems that a simple inhibition of the enzyme should yield the same degree of potentiation for all agents which increase adenylate cyclase activity in a given cell. However, in most tissues (at least experimentally) at least two phosphodiesterase activities, one with a high Kₘ and another with a lower Kₘ, are apparent (3, 21). Thus, one might speculate that a preferential inhibition of the high Kₘ activity would result in greater potentiation of the action of that agent which caused the greater increase in cyclic AMP levels (i.e., PGE₁ in WI-38 cells). An alternative explanation is that theophylline might in some way antagonize the catecholamine (but not the PGE₁) activation of adenylate cyclase.

The importance of cyclic AMP release from cells in regulation is unclear. The considerable amount of cyclic AMP which appears in the incubation medium of WI-38 cells upon prolonged stimulation by PGE₁ might suggest but by no means establish that this is a method of disposal of cyclic AMP. In any event, measurements of cyclic AMP levels in the medium may be particularly helpful to our understanding of those systems where only small apparent changes in intracellular cyclic AMP levels are associated with maximal physiological responses (22). For example, Exton et al. (23) were able to show a better correlation between increased cyclic AMP levels and increased glucose output in response to glucagon in the perfused liver when the perfusion medium was analyzed in addition to liver extracts.

**Acknowledgments**—We are indebted to Dr. L. Hayflick for providing the WI-38 cells and we thank Stephen Parker and M. Natalie Seney for excellent technical assistance.

**Addendum**—Franklin and Foster have published a report describing the appearance of cyclic AMP in the medium of human diploid lung fibroblasts incubated with PGE₁ or isoprenaline (24).

**References**

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