Adenyl Cyclase in Normal and Transformed Fibroblasts in Tissue Culture

ACTIVATION BY PROSTAGLANDINS

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SUMMARY

The activity of the enzyme adenyl cyclase has been measured in various lines of mouse, hamster, and rat fibroblasts maintained in tissue culture. All cell lines have detectable adenyl cyclase activity.

With respect to the activity of the enzyme in the presence of fluoride ions: (a) mouse embryo fibroblasts and L cells have relatively high levels of enzyme, whereas 3T3 cells derived from mouse embryo fibroblasts have low levels; (b) 3T3 cells transformed by SV40 and MSV/MuLV viruses have elevated enzyme levels, but 3T3 cells transformed by polyoma virus have slightly decreased levels; (c) hamster embryo fibroblasts and the established BHK line have relatively high levels and transformation of BHK cells by polyoma virus only slightly decreases enzyme activity; (d) rat embryo fibroblasts have the highest enzyme activity found.

With respect to stimulation of the enzyme by prostaglandins, prostaglandin E1 is the most effective when tested on L cells, followed in effectiveness by prostaglandin E2, prostaglandin F2α, and prostaglandin B1. Prostaglandin A2 produces only a small response.

Two cell lines derived from 3T3 cells by transformation with polyoma or SV40 virus are unresponsive to all prostaglandins tested. With most lines, adenyl cyclase activity remained constant during culture for 2 to 3 months. However, in rat embryo fibroblasts and SV40 (3T3) cells, the fluoride-stimulated enzyme levels (but not basal levels) fell with successive passages.

MATERIALS AND METHODS

The prostaglandins were a gift of Dr. John Pike, Upjohn Company, Kalamazoo, Michigan. Bovine-porcine glucagon was obtained from Lilly; Dowex AG 50X8, 100 to 200 mesh, from Bio-Rad; crystalline theophylline and epinephrine bitartrate from Mann; bovine plasma albumin, Fraction V, lipase and protease free, from Armour; crystalline disodium ATP from Sigma; crystalline cyclic AMP from Schwarz BioResearch; dithiothreitol, phosphoenolpyruvate, and pyruvate kinase (10 mg per ml) from Calbiochem; cyclic [8-αH]AMP (16.3 Ci per mmole) from Schwarz; [α-32P]ATP (6 Ci per mmole, pH 7.4) from International Chemical and Nuclear Corporation; 9, 10-dihydroxy-stearic acid from K and K Laboratories; Eagle's minimal essential medium and Dulbecco's modified Eagle's medium from the National Institutes of Health (NIH) media room or from Flow Labs, Inc., Rockville, Maryland; fetal bovine serum, calf serum, penicillin G, and streptomycin from Flow Labs; 100-mm plastic tissue culture dishes from Falcon Plastics Division of Becton Dickinson, Oxnard, California; polymyxin B sulfate from Grand Island Biological Company, Chagrin Falls, Ohio.

Growth of Cells—The cells lines used are shown in Table I. L-929 cells, XC cells, rat embryo fibroblasts, mouse embryo fibroblasts, and hamster embryo fibroblasts were grown in Eagle's minimal essential medium with 10% fetal bovine serum; all other cell lines were grown in Dulbecco's modified Eagle's medium with 10% calf serum. Both media also contained 7785

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The resulting homogenate was used directly as the enzyme in a glass Dounce homogenizer (tight pestle; 10 strokes). 0.5 to 2.0 ml of homogenizing medium (protein 2.5 to 10 mg per dish with a clean rubber stopper, and then homogenized in otherwise specified. The cells were scraped from the culture transfer of the cells except as noted in the text.

Preparation of Enzyme—The medium was removed, and the cells were washed four times with an ice-cold solution containing 0.15 m NaCl, 0.01 m sodium phosphate (pH 7.4) followed by four washes with ice-cold homogenizing medium (sucrose, 0.33 m; dithiothreitol, 1 mM; Tris-HCl buffer, pH 7.8, 50 mM; MgCl₂, 1 mM). All subsequent steps were carried out at 0-4°C unless otherwise specified. The cells were scraped from the culture dish with a clean rubber stopper, and then homogenized in 0.5 to 2.0 ml of homogenizing medium (protein 2.5 to 10 mg per ml) in a glass Dounce homogenizer (tight pestle; 10 strokes). The resulting homogenate was used directly as the enzyme component in the assay procedure.

Enzyme Assay—Adenyl cyclase activity was assayed by measuring the conversion of [α-³²P]ATP to cyclic [³²P]AMP (25). Each 0.06 ml of reaction mixture contained 2.7 mM ATP, 2.6 mm MgCl₂, 8 mM theophylline, 0.88 mM dithiothreitol, 0.8 mg per ml of bovine plasma albumin (Fraction V), 3.0 mM P-enolpyruvate, 4 μg per ml of pyruvate kinase (rabbit muscle), and 40 mM Tris-HCl, pH 8.3. When fluoride was added, its concentration was 8 mM. Other substances tested for effect on enzyme activity were present as specified. The reaction was started by the addition of [α-³²P]ATP (2 to 3 × 10⁵ cpm), and incubation was carried out for 10 min at 37°C with shaking. The reaction was terminated by the addition of 0.5 ml of an ice-cold solution containing a large excess of ATP, cyclic AMP, and cyclic [³H]AMP (26). Cyclic AMP was isolated from other ³²P labeled components by chromatography on Dowex AG 50W-X8 (100 to 200 mesh), followed by two BaSO₄ precipitations (recoveries averaged 30 to 40% in all assays). The supernatants were mixed with Bray’s solution and their content of radioactivity measured in a liquid scintillation spectrometer. The blank for each experiment was prepared by terminating the incubation at 0 before the addition of [α-³²P]-ATP. The blank values were subtracted from each experimental value. Protein measurements were made by the method of Lowry et al. (27) with bovine serum albumin as the standard.

The rate of cyclic AMP production was linear for at least 10 min and proportional to protein concentration over a range of 0.4 to 2.5 μg per ml. In all the five cell lines studied, the fluoride-stimulated enzyme has a pH optimum at approximately pH 8.3, as measured before the reaction was begun (Fig. 1); the final pH following the 10-min incubation was approximately pH 8.1. We chose a Mg²⁺ concentration equal to that of ATP since this value was optimal in one other adenyl cyclase system.
Adenyl cyclase activity in several mouse cell lines

Cell lines were cultured, harvested, and adenyl cyclase activity measured as described under "Materials and Methods." The enzyme was assayed in the absence and presence of fluoride and PGE1 (5 \( \mu \)g per ml except as indicated).

| Cell line                  | \(-F\) | \(+F\) | GPE1, 5 \( \mu \)g per ml |
|----------------------------|--------|--------|---------------------------|
| Mouse embryo fibroblasts   | 320    | 1,465  | 1,050                     |
| 3T3 (Swiss)                | 70     | 330    | 270                       |
| 3T6 (Swiss)                | 185    | 960    | 445                       |
| SV-T2 (Balb/c)             | 80     | 315    | 335, a                    |
| SV-40 (3T3)                | 220    | 1,330  | 650                       |
| SV-40 (3T3)                | 50     | 750    | 40, b                     |

* Twenty-five micrograms per ml.
# Measured on 12/1/70.
* Measured on 2/4/71.
# Fifty micrograms per ml.

We also assayed the enzyme levels of the parent 3T3 fibroblast, a 750 percentage decrease in adenyl cyclase was found in 3T3 cells derived from either NIH-Swiss or Balb/c mouse embryo fibroblasts. The enzyme levels in NIH-Swiss mouse embryo fibroblasts (measured after 2 to 3 transfers) are shown in Table II. The basal level is somewhat higher than L-929 cells, but the activity in the presence of fluoride is comparable. However, in 3T3 cells derived from either NIH-Swiss or Balb/c mouse embryo fibroblasts, a 75% decrease in adenyl cyclase was found.

Untransformed Mouse Cells—Mouse embryo fibroblasts and their derivatives are cells that are also commonly used for tissue culture work. Enzyme levels in NIH-Swiss mouse embryo fibroblasts (measured after 2 to 3 transfers) are shown in Table II. The basal level is somewhat higher than L-929 cells, but the activity in the presence of fluoride is comparable. However, in 3T3 cells derived from either NIH-Swiss or Balb/c mouse embryo fibroblasts, a 75% decrease in adenyl cyclase was found.

SV-40 and MSV/MuLV Transformation—Cell lines derived from 3T3 cells by transformation with SV-40 or MSV/MuLV had fluoride-stimulated adenyl cyclase levels much higher than the 3T3 cells and close to that of the mouse embryo fibroblasts (Table II). The MSV/MuLV-transformed line and one of the SV-40-transformed lines (SV-T2) also had elevated basal adenyl cyclase levels and the enzyme responded to PGE1 (5 \( \mu \)g per ml). However, the second SV-40-transformed line had lower basal enzyme levels similar to the parent 3T3 cell, and the enzyme

*s 20/4/71.
+b 2/4/71.
+c 50 micrograms per ml.

**) 50 to 100 \( \mu \)g per ml produced only a 5-fold stimulation. PGE1 was the least effective of all.

Fig. 2 shows the response of L-929 cell adenyl cyclase to varying concentrations of several prostaglandins. The enzyme responded to as little as 0.1 \( \mu \)g per ml of PGE1 and a 20-fold rise was observed at 5 \( \mu \)g per ml. PGE1 was much less effective and large concentrations of PGB1 and PGF2a (50 to 100 \( \mu \)g per ml) produced only a 5-fold stimulation. PGE1 was the least effective of all.

In the presence of sodium fluoride (5 \( \mu \)m), PGE1 (5 \( \mu \)g per ml) produced no additional increase in enzyme activity. In a control experiment, dihydroxystearic acid (5 and 50 \( \mu \)g per ml) did not alter enzyme activity.

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L-929 Cells—L-929 cells are derived from mouse cells transformed in vitro by 3-methyl cholangthrene (18). These cells have been maintained in culture for many years and have been extensively employed to study various parameters involved in the control of cell growth and metabolism. We initially studied two lines of L-929 cells. One, a monolayer adapted line (line A), we obtained from the American Type Culture Collection. The other line (line B) had been growing in spinner culture and was a gift of Dr. S. Baron. The adenyl cyclase from both lines, when grown in monolayer, had low basal activity (75 and 65 picomoles per mg of protein per 10 min) and responded in a similar fashion to fluoride and prostaglandin E1. We used line B in the remainder of our studies. Cells taken directly from spinner culture and cells which were allowed to grow to high densities in monolayer also had similar adenyl cyclase activities. Neither glucagon (10\(^{-8}\) M) nor epinephrine (10\(^{-7}\) M) produced significant stimulation of the enzyme in vitro.

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Effect of polyoma transformation on adenyl cyclase activity

Cell lines were cultured, harvested, and adenyl cyclase activity measured as described under "Materials and Methods." The enzyme was assayed in the absence and presence of fluoride and PGE1 (5 \( \mu \)g per ml except as indicated).

| Cell line                  | \(-F\) | \(+F\) | GPE1, 5 \( \mu \)g per ml |
|----------------------------|--------|--------|---------------------------|
| 3T3 (Swiss)                | 70     | 330    | 270                       |
| Py-11 (3T3)                | 50     | 240    | 50                        |
| SV-Py11 (3T3)              | 160    | 1720   | 950                       |
| Hamster embryo fibroblasts | 90     | 1080   | 640                       |
| BHK                        | 170    | 1300   | 1550, a                   |
| BHK-Py                      | 70     | 1160   | 1140, b                   |

* Twenty-five micrograms per ml.
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Effect of various concentrations of prostaglandin E1 on adenyl cyclase activity in several mouse cell lines. Cell lines were cultured and harvested; adenyl cyclase activity was measured as described under "Materials and Methods." The enzyme was assayed in the absence and presence of fluoride and varying concentrations of PGE1. Results are expressed as picomoles of cyclic AMP formed per mg of protein per 10 min. MEF, mouse embryo fibroblasts.

TABLE IV

Effect of other prostaglandins on adenyl cyclase activity

Cell lines were cultured, harvested, and adenyl cyclase activity measured as described under "Materials and Methods." The enzyme was assayed in the absence and presence of the various prostaglandins (50 μg per ml except as indicated). Each value is the mean ± the standard error of the mean of four or more samples.

| Cell line            | -F | +PGE1 | +PGE2 | +PGE2A |
|----------------------|----|-------|-------|--------|
| Mouse embryo fibroblasts | 223 ± 55 | 305 ± 57 | 386 ± 37 | 280 ± 53 |
| 3T3                  | 29 ± 8    | 53 ± 11 | 103 ± 20 | 47 ± 22 |
| SV-T2 (3T3)          | 118 ± 38  | 122 ± 51 | 213 ± 64  | 164 ± 43 |
| SV-40 (3T3)          | 32 ± 7    | 47 ± 17 | 25 ± 9   | 36 ± 15 |
| MSV/MuLV (3T3)       | 465 ± 26  | 512 ± 23 | 454 ± 22 | 427 ± 13 |
| Py-11 (3T3)          | 34 ± 15   | 19 ± 6  | 26 ± 9   | 26 ± 13 |

* p < 0.05. ° p > 0.10. ° 0.05 < p < 0.1. ° Assayed 2/4/71.

was also unresponsive to PGE1. During the 2-month period that these cells were maintained in our laboratory, their level of fluoride-stimulated adenyl cyclase decreased, but basal levels remained unchanged.

Polyoma Transformation—The effect of polyoma transformation on enzyme levels in mouse and hamster cells is shown in Table III. In line Py-11 (3T3), polyoma transformation results in a rather small decrease in fluoride-stimulated adenyl cyclase activity, and a loss of responsiveness to PGE1. However, when line Py-11 (3T3) was subsequently transformed by SV-40 virus (SV-Py-11 (3T3)), the enzymatic activity measured in the presence of fluoride was elevated and the enzyme became very responsive to PGE1. Bar (28) has previously reported that BHK cells transformed by polyoma virus had about a 50% decrease in basal adenyl cyclase activity; however, he did not assay the enzyme in the presence of fluoride.

Prostaglandins—The response of the adenyl cyclases from several mouse cell lines to increasing concentrations of PGE1 is shown in Fig. 3. Relative to fluoride-stimulated levels, good responses are seen in mouse embryo fibroblasts and 3T3 cells with a lessened response to PGE1 noted in the MSV/MuLV-transformed line. No significant response was found in either Py-11 (3T3) or SV-40 (3T3) cells. It is apparent that transformation can result in a decreased or absent sensitivity of the enzyme to PGE1.

The response of various mouse cell lines to prostaglandin other than PGE1 is shown in Table IV. A small response to PGE2 is observed with mouse embryo fibroblasts and 3T3 cells, and a small response to PGB2 with mouse embryo fibroblasts. No response to PGA2 is found when tested on mouse embryo fibroblasts, SV-40 (3T3), MSV/MuLV (3T3), or Py-11 cells (data not shown).

Rat Cells—We measured adenyl cyclase activity in XC cells, a transformed rat cell line originally isolated from a rat inoculated with Rous sarcoma virus (17), and in rat embryo fibroblasts which were carried in culture for as long as 11 transfers (Table V). The adenyl cyclase activity of the rat embryo fibroblasts measured either in the presence of fluoride or PGE1 decreased over this period, but a substantial response to both substances remained. The rather high basal activity was unchanged.

TABLE V

Adenyl cyclase activity in rat cell lines

XC cells and rat embryo fibroblasts (after varying numbers of passages) were cultured and harvested; adenyl cyclase activity was measured as described under "Materials and Methods." The enzyme was assayed in the absence and presence of fluoride and several prostaglandins (5 μg per ml except as indicated).

| Cell line      | -F | +F | +PGE1 | +PGE2 | +PGE2A |
|----------------|----|----|-------|-------|--------|
| REF, 2°        | 460| 485| 2020  | 850   | 650    |
| REF, 3°        | 445| 3460| 2440 | 850   | 650    |
| REF, 5°        | 615| 2870| 2330 | 400   | 450    |
| REF, 6°        | 445| 2340| 1000 | 400   | 450    |
| REF, 11°       | 380| 2240| 960  | 405   | 430    |
| XC             | 420| 850| 695  | 530*  | 640*   |

* Rat embryo fibroblasts.
* Twenty-five micrograms per ml.
XC cells also had a high basal adenyl cyclase activity but the enzyme responded poorly to fluoride and less well to PGE₁.

**DISCUSSION**

When various transformed and normal fibroblasts are treated with cyclic AMP, N⁶-O²'-dibutyl cyclic AMP, or theophylline, their growth is slowed, and the cells become more elongated and adhere more tightly to the substratum (3, 29). Similar cell elongation was found with treatment of Chinese hamster cells (30). These findings suggest that cyclic AMP normally regulates cell growth and morphology. Since intracellular cyclic AMP levels are determined, in part, by the levels of adenyl cyclase, we measured the activity of adenyl cyclase in various normal and transformed cell lines.

The simplest model of the adenyl cyclase enzyme complex divides the enzyme into a catalytic subunit which is exposed to the interior of the cell and one or more regulatory subunits which are exposed to the cell exterior (1, 2). Presumably, fluoride, which activates the enzyme only in broken cell preparations, acts on the catalytic subunit, whereas prostaglandins act on one or more regulatory subunits. The effects of transformation on the activity of the enzyme will be considered in terms of this model.

The 3T3 and 3T6 cell lines are stable lines derived from mouse embryo fibroblasts (23). 3T3 cells were selected for their tendency not to overgrow, whereas the 3T6 cells and the parent mouse embryo fibroblasts do overgrow. We found that mouse embryo fibroblasts had high adenyl cyclase activity when measured with or without fluoride, or with PGE₁. In contrast, two different lines of 3T3 cells had low basal, fluoride, and PGE₁-stimulated adenyl cyclase activity. The 3T6 cells had intermediate levels of enzyme activity. Makman (31) has previously reported somewhat higher fluoride-activated levels in 3T6 cells than those found in this study. Thus, the selection for contact-inhibited cells appears to result in cells with low adenyl cyclase activity. The low adenyl cyclase in 3T3 cells is probably due to a low level of catalytic subunit activity, since both basal and fluoride-dependent activities are diminished. However, this decrease in adenyl cyclase activity does not appear to be simply a property of established cell lines since the established BHK-21 hamster cell line has high adenyl cyclase activity (Table III).

**Virus Transformation**—Transformation of 3T3 cells by various onecogenic viruses leads not only to cells with altered growth properties (19), but also to changes in cell membrane proteins and glycoproteins (32, 33). Therefore, it seemed possible that transformed cells might also have alterations in their adenyl cyclase levels. We found that 3T3 cells transformed by SV-40 and MSV/MuLV viruses did have an elevation in fluoride-stimulated activity, whereas both 3T3 and BHK cells transformed by polyoma virus had a slight decrease in activity.

The response of transformed cells to prostaglandins is more complex. The enzyme from L-929 cells, which have been in culture for about 30 years, retains its responsiveness to all prostaglandins. PGE₁ and PGE₂ are by far the most effective; the others are much less active. The enzyme from two virally transformed lines, SV-40 (3T3) and Py-11 (3T3), has lost its responsiveness to PGE₁, whereas in other lines, SV-T2 (3T3), SV-Py11 (3T3) and BHKPy, it has retained its responsiveness.

Apparent, the loss of PGE₁ responsiveness by Py-11 (3T3) was not due to a loss of genetic material, since transformation of these cells by SV-40 restored prostaglandin responsiveness. The detection of cell lines unresponsive to PGE₁ suggests that the prostaglandin receptor is not present or that it has been structurally altered. A similar model has been utilized by Pennington et al. (34) to explain the divergence in epinephrine responsiveness of adenyl cyclase in liver and Morris hepatoma cells. The isolation of cell lines with altered responses to prostaglandins promises to be very useful for biochemical studies on the mechanism of prostaglandin action, and these lines also offer another marker for use in cell fusion studies. Further, a number of SV-40 and polyoma transformed lines need to be studied to ascertain how frequently this loss of responsiveness to PGE₁ occurs, and the responsive lines need to be cloned to determine whether there is a mixture of responsive and unresponsive cells.

Since prostaglandin E₁ activates adenyl cyclase in homogenates of L-929 cells, this agent would be expected to elevate cyclic AMP levels in intact cells, and the elevated cyclic AMP levels, in turn, would be expected to restore morphology towards normal and to inhibit growth. In recent studies, we have confirmed both of these predictions. ³, ⁴

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