Regulation of Aryl Hydrocarbon (Benzo(a)pyrene) Hydroxylase Activity in Mammalian Cells

INDUCTION OF HYDROXYLASE ACTIVITY BY N\textsuperscript{6},O\textsuperscript{2}-DIBUTYRYL ADENOSINE 3':5'-MONOPHOSPHATE AND AMINOPHYLLINE*

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Treatment of hamster BHK cells with N\textsuperscript{6},O\textsuperscript{2}-dibutyryl adenosine 3':5'-monophosphate (Bt\textsubscript{2}cAMP), aminophylline, theophylline, or papaverine increased the level of aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity. The highest increase, 100-fold, was obtained with Bt\textsubscript{2}cAMP plus aminophylline or theophylline. N\textsuperscript{2},O\textsuperscript{2}-Dibutyryl guanosine 3':5'-monophosphate gave a lower induction than Bt\textsubscript{2}cAMP. The level of hydroxylase activity started to decrease 6 hours after treatment with the inducer and was reduced to almost the uninduced level after 24 hours. Repeated addition of Bt\textsubscript{2}cAMP and aminophylline did not prevent this decrease. The hydroxylase can also be induced by treating cells with benz(a)anthracene, and the level of this induced activity was maintained for 24 hours. Aminophylline gave a 2- to 8-fold stimulation of the induction by benz(a)anthracene.

The enzyme activity induced by Bt\textsubscript{2}cAMP, aminophylline, and benz(a)anthracene converted benzo(a)pyrene to similar alkali-extractable metabolites with a fluorescence spectra similar to that of 3-hydroxybenzo(a)pyrene. These induced enzyme activities also showed a similar heat stability. Induction by Bt\textsubscript{2}cAMP and aminophylline, like induction by benz(a)anthracene, required continued protein synthesis and only an initial period of RNA synthesis. Compared to the benz(a)anthracene-induced hydroxylase with a $K_{m}$ of 4.3 $\mu$M, the hydroxylase induced by Bt\textsubscript{2}cAMP and aminophylline showed a $K_{m}$ of 0.14 $\mu$M, and was 100-fold more sensitive to inhibition by 7,8-benzoflavone. Increasing the serum concentration in the culture medium stimulated the induction by aminophylline but did not stimulate induction by benz(a)anthracene.

The results indicate that aryl hydrocarbon (benzo(a)pyrene) hydroxylase can be induced by compounds that increase the level of adenosine 3':5'-monophosphate and that this induction and induced enzyme activity differs from that caused by benz(a)anthracene.

Aryl hydrocarbon (benzo(a)pyrene) hydroxylase belongs to a group of mixed function oxidases that metabolize drugs, steroids, and carcinogens (1). These enzymes are important in chemical carcinogenesis, since they metabolically activate chemically inert carcinogens into metabolites that can be cytotoxic, mutagenic, and carcinogenic (2-12). These enzymes are inducible by a variety of compounds including polycyclic hydrocarbons (13), and this induction is genetically controlled (14-16). Although it has been reported that induction of the hydroxylase is not mediated by adenosine 3':5'-monophosphate (17, 18), we have shown that treatment of cultured mammalian cells with Bt\textsubscript{2}cAMP and other compounds that increase the level of cAMP, increased the metabolism of polycyclic hydrocarbons to water-soluble products (19). In view of these results, we have now studied whether the induction of aryl hydrocarbon hydroxylase can be mediated by cAMP. The present experiments describe the induction of the hydroxylase after treatment of cultured mammalian cells with Bt\textsubscript{2}cAMP and with inhibitors of cyclic nucleotide phosphodiesterase and compare this induction with that obtained with benz(a)anthracene. We have used in these experiments the cultured hamster cell line BHK2 which has a low basal level of aryl hydrocarbon hydroxylase activity.

EXPERIMENTAL PROCEDURE

Materials

Benzo(a)anthracene was obtained from K & K Laboratories, Plainview, New York, benzo(a)pyrene, Bt\textsubscript{2}cAMP, cAMP, Bt\textsubscript{2}GMP, aminophylline, theophylline, papaverine, and NADPH were from Sigma.

*BHK is a clone isolated from the baby hamster kidney BHK21 cell line (20).
Chemical Co., St. Louis, Mo., AMP, adenosine, GMP, guanine, guanosine, and actinomycin D were from Calbiochem, Los Angeles, Calif., 7,8-benzoflavone was from Eastman Organic Chemicals, Rochester, N. Y., and cycloheximide was from Nutritional Biochemicals Co., Cleveland, Ohio. [3H]Leucine and [3H]uridine were kindly supplied by Drs. H. V. Gelboin and J. A. Selkirk, National Institutes of Health, Bethesda, Maryland. The benz(a)anthracene was dissolved in acetone and then diluted in culture medium to give a final concentration of 0.5% acetone. This concentration of acetone showed no effect on the hydroxylase activity induction.

Methods

Cell Cultures—Cells from a clone isolated from the hamster cell line BHK21 were grown in Eagle’s medium with a 4-fold concentration of amino acids and vitamins supplemented with 10% fetal calf serum. The cells were used for experiments 2 days after seeding 2 × 10^6 cells per 100-mm tissue culture plastic Petri dish (NUNC Co., or Falcon Co.) in 8 ml of culture medium.

Enzyme Assay— Aryl hydrocarbon hydroxylase activity in cell homogenates was assayed as described (9). The cells were washed with 20 ml of cold phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 0.2 g of KH₂PO₄, 1.15 g of Na₂HPO₄, 0.1 g of CaCl₂·2H₂O, and 0.1 g of MgCl₂·6H₂O per liter) and removed from the petri dish with a rubber policeman in 3 ml of the same buffered saline. After centrifugation at 1000 × g, cells were washed with 5 ml of cold 0.25 M Tris-HCl buffer, pH 7.5, and then homogenized in 0.35 to 0.50 ml of the same buffer in a small tight fitting glass homogenizer. The reaction mixture for the standard assay of the hydroxylase activity contained 50 μmol of Tris-HCl buffer, pH 7.5, 0.36 μmol of NADPH, 3 μmol of MgCl₂, 0.1 ml of cell homogenates (containing 0.3 to 0.5 mg of protein), and 62.5 nmol of benz(a)pyrene (added in 50 μl of ethanol just before starting the reaction). The total volume was 1.0 ml. After 30 min of incubation with shaking in air at 37°, the reaction was stopped with 1 ml of acetone, and the hydrocarbons were extracted with 5 ml of 1-hexane. After storing the mixture overnight at 4°, 4 ml of the hexane layer were transferred to another tube, and the hydroxy derivatives of benz(a)pyrene were extracted with 2 ml of 1 N NaOH. To the control tube, 1 ml of acetic acid was added before the addition of benz(a)pyrene. The hydroxylase activity was linear up to 30 min with 0.3 to 2.0 mg of protein. Fluorescence of the base-extractable metabolites was measured by an Aminco fluorometer, with excitation at 396 nm and emission at 425 nm. A unit of aryl hydrocarbon hydroxylase activity was defined as that amount catalyzing the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of Zs-hydroxybenzo(a)pyrene. The protein was measured according to Lowry et al. (21). This decrease was not prevented by repeated treatment of the cell cultures every 3 hours with the inducing agents. Aryl hydrocarbon hydroxylase activity can also be induced by treating the BHK cells with benz(a)anthracene. In this case there was a lag period of less than 1 hour, the level of the

Table I

| Compound added | Specific activity of hydroxylase units/mg protein |
|----------------|-----------------------------------------------|
| None           | 1-4                                           |
| cAMP, 10 mM    | 8-13                                          |
| Bt,cAMP, 1 mM  | 25-35                                         |
| Aminophylline, 0.7 mM | 70-100                                    |
| Aminophylline, 0.7 mM + Bt,cAMP, 1 mM | 150-250                                  |
| Theophylline, 0.3 mM | 70-90                                      |
| Theophylline, 0.3 mM + Bt,cAMP, 1 mM | 150-250                                  |
| Papaverine, 17 μM | 20-35                                       |
| Papaverine, 17 μM + Bt,cAMP, 1 mM | 40-70                                     |
| Bt,cGMP, 1 mM   | 10-25                                         |
| Bt,cGMP, 1 mM + aminophylline, 0.7 mM | 100-150                                   |

Results

Induction of Aryl Hydrocarbon Hydroxylase Activity by cAMP, Bt,cAMP, Bt,cGMP, and Inhibitors of Cyclic Nucleotide Phosphodiesterase

The compounds were added to cultures of BHK cells for 6 hours. The cells were harvested, homogenized, and the hydroxylase activity was assayed as described under "Methods." The enzyme activities given are a range of values from three to five experiments.

FIG. 1. Induction of aryl hydrocarbon hydroxylase activity in BHK cells by cAMP, Bt,cAMP, Bt,cGMP, and inhibitors of cyclic phosphodiesterase

(a) with different concentrations of Bt,cAMP, aminophylline, and papaverine. Cells were incubated (a) with different concentrations of Bt,cAMP in the presence (0) or absence (0) of 0.7 mM aminophylline: (b) with different amounts of aminophylline; and (c) with different amounts of papaverine. Papaverine was first dissolved in 0.1 N HCl and then diluted in the culture medium. The enzyme activity was measured in cell homogenates 6 hours after addition of the compounds.
hydroxylase activity increased for about 6 hours, and this increased level was maintained for an additional 18 hours (Fig. 2b). Optimal induction was obtained with 1.3 μM or higher concentrations of benz(a)anthracene. The addition of aminophylline gave a further 2- to 8-fold increase in enzyme induction (Fig. 3).

Effect of Actinomycin D and Cycloheximide—Treatment of BHK cells with 0.5 μg/ml of actinomycin D inhibited 85% of RNA synthesis. This concentration of actinomycin D inhibited enzyme induction when added together with Bt,cAMP and aminophylline. Addition of actinomycin D at 1 and 2 hours after Bt,cAMP plus aminophylline inhibited induction by 90% and 50%, respectively, whereas addition of actinomycin D at 3 hours showed no inhibition of enzyme induction (Fig. 4). Cycloheximide (1.5 μg/ml) blocked enzyme induction when added together with or up to 3 hours after treatment with the inducer.

These results indicate that, as with induction by benz(a)anthracene (24), induction by Bt,cAMP and aminophylline requires continued protein synthesis and only an initial period of RNA synthesis.

Properties of Induced Enzyme—The fluorescence spectrum of the alkali-extractable metabolites produced by the hydroxylase activity was determined for the uninduced and induced enzyme activities (Fig. 5). The results indicate that the hydroxylase in control cells and in cells induced by Bt,cAMP plus aminophylline or by benz(a)anthracene, metabolize benzo(a)pyrene to alkali-extractable metabolites with a fluorescence spectrum similar to that of 3-hydroxybenzo(a)pyrene.

The heat stability of Bt,cAMP plus aminophylline-induced and benz(a)anthracene-induced hydroxylase activity was determined by incubating cell homogenates for 5 min at 0-60°. Both cell homogenates showed the same pattern of heat inactivation, with 50% inactivation of enzyme activity at about 47°.

7,8-Benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase activity (25, 26), was tested for its effect on the enzyme activity induced by Bt,cAMP plus aminophylline and by benz(a)anthracene. Although both were inhibited, the Bt,cAMP plus aminophylline-induced enzyme was about 100 times more sensitive than the benz(a)anthracene-induced enzyme (Fig. 6). Since 7,8-benzoflavone is also a polycyclic hydrocarbon, this difference in the inhibition may be due to a difference in the affinity of the enzyme to the substrate. The Michaelis constant (Km value) obtained from the double
reciprocal plots of benzo(a)pyrene concentration and initial reaction velocity has indeed shown that the Bt,cAMP plus aminophylline-induced enzyme had a 30-fold higher affinity to benzo(a)pyrene ($K_m = 0.14 \mu M$) than the benz(a)anthracene-induced enzyme ($K_m = 4.2 \mu M$) (Fig. 7). The addition of Bt,cAMP plus aminophylline to homogenates of the benz(a)anthracene-induced enzyme did not alter either the $K_m$ of the hydroxylase for the substrate or the sensitivity of the hydroxylase to 7,8-benzoflavone. The $K_m$ of the uninduced hydroxylase activity could not be determined because of its low activity in untreated BHK cells.

**Induction of Enzyme Activity by Fetal Calf Serum—** Induction by Bt,cAMP and aminophylline was tested in the presence of various concentrations of fetal calf serum (0 to 50%) in the culture medium. The addition of serum, without these compounds, induced aryl hydrocarbon hydroxylase activity, and there was a 30-fold increase with 50% serum (Fig. 8). Induction by Bt,cAMP and aminophylline can occur in the absence of serum. However, the degree of induction was increased in the presence of serum, and the highest induction was also with 50% serum (Fig. 8). The kinetics of enzyme induction with 10% serum showed that the increased enzyme activity declined to the basal level within 12 hours after addition of fresh serum. Three hourly treatments with fresh 10% serum prevented this decline in contrast to the effect of 3 hourly treatments with aminophylline. Although Bt,cAMP plus aminophylline-induced and basal enzyme levels were, therefore, dependent on concentration of serum, there was no effect of serum concentration on enzyme inducibility with benz(a)anthracene.

**Discussion**

We have previously shown that the treatment of a variety of mammalian cultured cells with Bt,cAMP and aminophylline increased the metabolism of benzo(a)pyrene to water-soluble products (19). The present results showed that this increase in benzo(a)pyrene metabolism is due to the induction by these compounds of aryl hydrocarbon hydroxylase activity. These results suggest that cAMP is a regulator of aryl hydrocarbon hydroxylase in mammalian cells. The kinetics of the hydroxylase induction has shown a maximal enzyme activity at 6 hours, followed by a decline to the basal enzyme level 24 hours after the addition of Bt,cAMP and aminophylline. This can explain the lack of detectable induction in measurements made 24 hours after addition of Bt,cAMP (17, 18).

The results have also shown that the enzyme activities induced by Bt,cAMP plus aminophylline or by benz(a)anthracene converted a substrate, benzo(a)pyrene, to similar alkali-extractable metabolites with a fluorescence spectrum similar to that of 3-hydroxybenzo(a)pyrene and had a similar heat stability. However, the 30-fold difference in $K_m$ values and 100-fold difference in sensitivity to the enzyme inhibitor 7,8-benzoflavone suggest that the cAMP-induced enzyme is different from that induced by benz(a)anthracene. Since cytochrome P450 is supposed to be rate-limiting in aryl hydrocarbon hydroxylase activity (27–29), cAMP may induce a new type of cytochrome P450 which has a higher affinity for benzo(a)pyrene, or cAMP may stimulate enzyme activity by affecting the active site of a pre-existing cytochrome P450.

As with induction by benz(a)anthracene (24), induction by Bt,cAMP and aminophylline requires RNA synthesis only for an initial period. However, there was a different time course and maintenance of induction by these different compounds, and fresh serum stimulated induction of the cAMP-inducible enzyme but not of the benz(a)anthracene-inducible enzyme. This suggests a different mechanism for induction of the hydroxylase by these different compounds.

The differences in the $K_m$ values have shown that the cAMP-induced aryl hydrocarbon hydroxylase is more efficient than benz(a)anthracene-induced enzyme in metabolizing carcinogenic polycyclic hydrocarbons at a low substrate concentration. This cAMP-induced hydroxylase may, therefore, be of importance in chemical carcinogenesis, since our environment contains small amounts of chemical carcinogens.

Since Bt,GMP also induces aryl hydrocarbon hydroxylase activity in BHK cells, and aminophylline and papaverine are known to inhibit cyclic phosphodiesterase which decompose all 3'-5' cyclic nucleotides (30), other cyclic nucleotides such as cGMP may also be regulators of this hydroxylase activity.

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