Cyclic Adenosine 3′:5′-Monophosphate and Cyclic Guanosine 3′:5′-Monophosphate Phosphodiesterase Activities Are under Separate Genetic Control

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SUMMARY

Agents that raise cyclic adenosine 3′:5′-monophosphate (cyclic AMP) levels in chicken embryonic fibroblasts increase cyclic AMP phosphodiesterase activity. Cyclic guanosine 3′:5′-monophosphate (cyclic GMP) phosphodiesterase activity is not increased indicating that these enzyme activities are under separate regulation. The increase in cyclic AMP phosphodiesterase activity requires cellular protein and RNA synthesis, and the regulation of induction probably occurs at the transcriptional level. The induced cyclic AMP phosphodiesterase activity is very unstable. The enzyme activity decays from the induced level with a half-life of 70 to 80 min. Increased enzyme activity is found in both the particulate and soluble cell fractions. The particulate enzyme can be solubilized by sonic disruption. DEAE-cellulose chromatography of the solubilized enzyme yields two fractions that hydrolyze cyclic AMP. Induction causes an increase in the activity of the second fraction. Neither of the particulate enzymes hydrolyze cyclic GMP.

DEAE-cellulose chromatography of the soluble enzyme also yields two fractions. The first peak hydrolyzes both cyclic AMP and cyclic GMP and the second only cyclic AMP. Induction increases the activity of the second fraction. Chicken embryonic fibroblasts thus contain mechanisms for effectively modulating cyclic AMP phosphodiesterase activity via protein synthesis and enzyme turnover. Cyclic GMP phosphodiesterase activity is not affected by this regulatory scheme.

Cyclic AMP1 (1) plays an extremely important role in the regulation of cell growth. Cyclic AMP levels are low during logarithmic growth but rise when contact-inhibited cells reach confluency and stop growing (1, 2). Cyclic AMP levels do not rise in cells that do not display contact inhibition of growth (1–3). The levels of cyclic AMP are controlled by synthesis via adenylate cyclase and degradation by cyclic AMP phosphodiesterases. Regulation of cyclic AMP levels by cyclic AMP phosphodiesterases is quite complex. There are at least two and perhaps more forms of the enzyme in many types of cells; these forms can be partially resolved by column chromatography. The activity of one of these forms is affected by the concentration of certain cations and a protein activator (4, 5). Another form displays negatively cooperative kinetics (6–8). Cyclic AMP phosphodiesterase activity is found both in the soluble and plasma membrane portions of the cell (9). The relationship of the plasma membrane forms of the enzyme to the soluble forms is still obscure.

Total phosphodiesterase activity is increased when cyclic AMP levels are elevated (10–12). In the present study we have begun to investigate the nature of the enzyme induced by cyclic AMP in chicken embryonic fibroblasts. Since cyclic GMP phosphodiesterases also are present in many cells (9, 13–17) and since the potent inducer SC-2964 raises both cyclic AMP and cyclic GMP levels (18), the activity of cyclic GMP phosphodiesterase was also determined.

We find that (a) cyclic AMP phosphodiesterase activity is increased by treatment of chicken embryonic fibroblasts with a potent cyclic AMP phosphodiesterase inhibitor (SC-2964) or analogues of cyclic AMP, (b) the induction requires RNA and protein synthesis and regulation of induction probably occurs at the level of transcription, (c) the induced enzyme is unstable and decays with a half-life of 70 to 80 min, (d) the induced enzyme is located in both the soluble and membrane fractions and is readily separated from the form that hydrolyzes cyclic GMP. The level of cyclic GMP phosphodiesterase is unchanged in cells treated with cyclic GMP, analogues of cyclic AMP, or the phosphodiesterase inhibitor SC-2964.

We conclude the induced cyclic AMP phosphodiesterase and the cyclic GMP phosphodiesterase are controlled separately, probably at the genetic level.

MATERIALS AND METHODS

The secondary chicken embryonic fibroblasts were grown in Eagle’s modified essential medium supplemented with fetal bu-
vine serum, sodium glutamate (2 mM), pyruvate (1 mM), dextrose (2 g per liter), tylosine (1%), penicillin (1%), streptomycin (1%), and trypotide phosphate broth (10%). The cells were plated in medium containing 10% fetal bovine serum and the medium was then changed daily with 5% serum. Secondary chicken embryonic fibroblasts (1.5 to 2 mg of protein per 50-cm² dish) were used in all experiments. The cells were rinsed with cold phosphate-buffered 0.9% NaCl solution four times. Cells were homogenized (0.8 ml of buffer per dish) with a Dounce homogenizer ("B" pestle, 15 strokes). Homogenization buffer contained 50 mM Tris-acetate, pH 0.0-0.975 mM mercaptoethanol. All steps were carried out at 4°C. Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as standard.

**Materials**—3H-Labeled cyclic AMP (22.1 Ci per mM) and 3H-labeled cyclic GMP (4.4 Ci per mM) were obtained from New England Nuclear, cyclic AMP from Schwarz-Mann, cyclic GMP and actinomycin D from Calbiochem, and anion exchange resin (AG 1-X2, 200 to 400 mesh) and Cellex D from Bio-Rad. Both compounds had any effect on total homogenate cyclic GMP phosphodiesterase; 1.0 mM SC-2964. O—O—, cyclic AMP phosphodiesterase; 2.0 mM SC-2964. O—O—, cyclic GMP phosphodiesterase; 1.0 mM SC-2964.

**RESULTS**

**Inducibility of Cyclic AMP and Cyclic GMP Phosphodiesterase**—In the current study we mainly employed the potent phosphodiesterase inhibitor, SC-2964, to measure enzyme induction. SC-2964 raises cyclic AMP levels in chicken embryonic fibroblasts 6-fold 15 min after addition at a concentration of 1.0 mM. The cyclic AMP levels fall to a 2-fold elevation 24 hours after its addition. Half-maximal induction of total cyclic AMP phosphodiesterase activity occurred between 0.1 and 0.2 mM SC-2964 (Fig. 1A) when cells were treated for 4 hours. A time course (Fig. 1B) showed that with 0.2 mM SC-2964, maximal induction occurred by 4 hours, whereas with 1.0 mM SC-2964 cyclic AMP phosphodiesterase activity was still increasing at 8 hours.

SC-2964 is also a potent inhibitor of cyclic GMP phosphodiesterase activity in extracts of chicken embryonic fibroblasts and raises cyclic GMP levels in cells (18). However, treatment of cells with 1.0 mM SC-2964 did not affect total homogenate cyclic GMP phosphodiesterase activity during an 8-hour time course (Fig. 1B). After 18 hours cyclic GMP phosphodiesterase activity was still unaffected.

In an attempt to determine whether other agents that either raise the concentration of cyclic AMP or cyclic GMP or mimic their action might increase the activity of either enzyme, cells were treated with 1.0 mM concentrations of cyclic AMP, cyclic GMP, 8-bromo cyclic AMP, dibutyryl cyclic AMP, or theophylline for 4 hours. None of the compounds had any effect on total homogenate cyclic GMP phosphodiesterase activity (data not shown). Of the compounds tested, theophylline, dibutyryl cyclic AMP, and 8-bromo-cyclic AMP all increased total homogenate cyclic AMP phosphodiesterase 30 to 50%. Under these conditions SC-2964 increased cyclic AMP phosphodiesterase activity at least 2-fold.

**Stability of Induced Cyclic AMP Phosphodiesterase**—To determine the stability of the increased cyclic AMP phosphodiesterase activity, cells were treated overnight with 0.9 mM SC-2964 and then the cells were rinsed and new medium without SC-2964 was added. Total cyclic AMP phosphodiesterase activity was then measured as a function of time. The results from experiments performed on chicken embryonic fibroblasts obtained from two different embryos revealed that the induced cyclic AMP phosphodiesterase activity is extremely unstable. The inactivation of cyclic AMP phosphodiesterase follows a first order exponential decay (Fig. 2) with a half-life of 70 to 80 min. A similar half-life is obtained if cells are treated with cycloheximide following the removal of the inducer (data not shown).

**Subcellular Localization and Characterization of Induced Phos-
Induction of cyclic AMP phosphodiesterase

| Effector | Concentration | Cell fraction   | Cyclic AMP phosphodiesterase | Cyclic GMP phosphodiesterase |
|----------|---------------|----------------|-------------------------------|------------------------------|
| None     |               | Homogenate     | 1.0                          | 1.0                          |
| SC-29644 | 1             | Homogenate     | 2.5                          | 1.13                         |
| SC-2964  | 1             | 800 X g pellet | 3.1                          | N.D.                         |
| SC-2964  | 1             | 30,000 X g supernatant | 2.7                          | 0.96                         |

* Cyclic AMP concentration is 2 µM.
* Cyclic GMP concentration is 0.75 µM.
* Data are listed relative to the enzyme activity of untreated cells.
* Cells were treated for 4 hours with the indicated inducer and then the cells were homogenized and assayed for enzyme activity.
* Not detectable.

Chicken embryonic fibroblasts contain particulate and soluble cyclic AMP phosphodiesterase activity. The particulate cyclic AMP phosphodiesterase activity is located in the plasma membrane of chicken embryonic fibroblasts (9). Treatment of these cells with SC-2964 causes an increase in the specific activity of both the particulate and soluble enzymes (Table I). Kinetic analysis of the crude particulate fraction before and after treatment with SC-2964 shows that induction causes an increase in \( V_{\max} \) and a change in the shape of the velocity curve (Fig. 3A). To investigate further the kinetic properties of the particulate enzyme, the membrane was disrupted sonically and the solubilized cyclic AMP phosphodiesterase (50 to 60% yield) was chromatographed on DEAE-cellulose (Fig. 4A). Two peaks of cyclic AMP phosphodiesterase activity are apparent. Cyclic GMP phosphodiesterase activity is not present in the particulate fraction using standard assay conditions. Fractions from the second half of the second peak (to eliminate contamination due to the first peak) were pooled, concentrated, and the kinetics studied. The results suggest that this enzyme is negatively cooperative (data not shown). Whether or not this enzyme is truly negatively cooperative must await its further purification. When cells were treated with SC-2964, the induced membrane cyclic AMP phosphodiesterase co-chromatographed with the second peak (Fig. 4B). A kinetic analysis of the induced enzyme showed that the new enzyme also exhibited negatively cooperative kinetics. No evidence for an increase in the activity of the enzyme in the first peak was found. Thus induction increases the activity of only one of the membrane forms of cyclic AMP phosphodiesterase.

Kinetic analysis of the supernatant fraction confirmed the initial observation that cyclic GMP phosphodiesterase activity is not inducible as neither the \( K_m \) nor the \( V_{\max} \) is altered after treatment of chicken embryonic fibroblasts with 1.0 mM SC-2964 (Fig. 3B). Since SC-2964 is a potent inhibitor of cyclic GMP phosphodiesterase (1 mM SC-2964 inhibits cyclic GMP phosphodiesterase 80%) supernatant fractions were dialyzed and reassayed. No detectable difference in cyclic GMP phosphodiesterase activity was evident (32.5 units before dialysis versus 36.5 units after dialysis). DEAE-cellulose chromatography of the supernatant fraction revealed two peaks of hydrolytic activity, the first peak hydrolyzed both cyclic AMP and cyclic GMP. Whether both these activities reside in the same molecule or not is unclear. The second peak hydrolyzed only cyclic AMP (Fig. 4, C and D). Induction increased the cyclic AMP phosphodiesterase activity (Fig. 4D). Cyclic GMP phosphodiesterase was unchanged (Fig. 4 C and D). In both the particulate and soluble fractions the induced enzyme
for each experiment and equal to 0.08 PM. The cyclic GMP recovery was 54% for cyclic AMP hydrolysis and 38% for cyclic activities chromatographed at the same salt concentration as that of the uninduced control enzyme (Fig. 4, A to D).

**Regulation of Induction Occurs at Transcriptional Level**—It has been shown previously that the increase in cyclic AMP phosphodiesterase activity caused by agents that raise intracellular levels of cyclic AMP is blocked by drugs that inhibit both RNA and protein synthesis (10-12). However, whether or not control of induction occurs at the transcriptional level for cyclic AMP phosphodiesterase and other inducible eukaryotic enzymes has not been well established. The induction of total homogenate cyclic AMP phosphodiesterase in chicken embryonic fibroblasts is also blocked by actinomycin D and cycloheximide (Table II). If the regulation of induction occurs at the transcriptional level, i.e. at the level of mRNA synthesis, then it should be possible to treat chicken embryonic fibroblasts with inducer in the presence of cycloheximide and form the putative cyclic AMP phosphodiesterase mRNA which due to the presence of cycloheximide could not be translated into new protein. However, the putative mRNA should be translated into new phosphodiesterase activity upon removal of cycloheximide. Addition of actinomycin D after removal of inducer and cycloheximide would prevent any induction due to residual inducer that was not washed out of cells by the rinse procedure. Chicken embryonic fibroblasts treated for 2 hours with SC-2964 and cycloheximide, washed, and incubated with actinomycin D (Fig. 5) show a significant increase in enzyme activity an hour after inducer was washed out of the cells. The 3-fold increase seen in an hour (Fig. 5) indicates that pretreatment with inducer caused a large increase in the putative mRNA synthesis for a 3-fold increase is not seen for 3 to 4 hours in a standard induction experiment (Fig. 1B). Since the induced cyclic AMP phosphodiesterase activity has a short half-life (Fig. 2), enzyme activity begins to fall after an hour (Fig. 5) due to the short half-life of cyclic AMP phosphodiesterase and perhaps to the turnover of the putative phosphodiesterase mRNA. Increased inducibility (superinduction) caused by the addition of actinomycin D to a system already containing inducer (21) was ruled out as follows. Cells were treated as in Fig. 5 and cyclic AMP phosphodiesterase activities measured at times 0, 60, and 90 min. The specific activities were 34, 92, and 81, respectively, when the rinse medium contained actinomycin D, and 34, 108, and 92 when it did not.

**Discussion**

Since cyclic AMP and cyclic GMP are regulatory molecules in numerous biological systems (1-3, 22, 23), it is necessary to
study the enzymes that synthesize and degrade these cyclic nucleotides to understand better the intracellular regulation of these compounds. The studies reported here show that cyclic AMP phosphodiesterase activity is increased by cyclic AMP analogues and agents that raise intracellular cyclic AMP concentrations in chicken embryonic fibroblasts and that the regulation of induction occurs at the transcriptional level. However, none of the compounds tested had any affect on cyclic GMP phosphodiesterase activity. We conclude that the two enzyme activities are under separate genetic regulation.

To ascertain which of the several cyclic AMP-hydrolyzing enzymes were being synthesized, the cell extracts were separated into a soluble and a particulate portion. Increased activity was found in both fractions. Then the particulate enzyme (solubilized by sonication) and the soluble enzyme were purified by DEAE-cellulose chromatography. Both preparations were resolved into two peaks. Induction increased the activity of Peak II prepared from both the particulate and soluble fractions; no change in Peak I was observed. Since Peak II chromatographed at the same salt concentration and had the same anomolous kinetic properties whether prepared from the soluble fraction or the membrane fraction, we believe they are the same enzyme. In many cells this enzyme appears to be almost completely membrane-bound (15, 16).

In eukaryotic organisms it has not been well established whether induction of protein synthesis is controlled at either or both the transcriptional or translational level. Evidence reported here strongly supports the concept that the regulation of the induction of cyclic AMP phosphodiesterase in chicken embryonic fibroblasts occurs at the transcriptional level. First, actinomycin D which blocks RNA synthesis blocked the induction of cyclic AMP phosphodiesterase when cells were pretreated with the drug. Second, when cells were treated with inducer in the presence of cycloheximide, and then the inducer and cycloheximide were washed out of the cultures and actinomycin D added, a 3-fold increase in enzyme activity occurred over the ensuing hour. These experiments support the concept that the inducer turns on the synthesis of a putative cyclic AMP phosphodiesterase mRNA which cannot be translated into protein due to the presence of cycloheximide. Removal of the cycloheximide then allows for the translation of the mRNA into new phosphodiesterase activity. A significant amount of mRNA must be formed in the presence of SC-2964 and cycloheximide since at least 3 hours is required to form this much phosphodiesterase activity by treating cells with inducer in a standard induction experiment. The induced cyclic AMP phosphodiesterase activity is relatively unstable as it rapidly disappears upon removal of inducer. The enzyme turnover follows a first order exponential decay with a half-life of 70 to 80 min. An identical half-life of the induced enzyme is also seen in the presence of cycloheximide. Basal enzyme activity turnover (Table II) in the presence of cycloheximide is much slower than that of the induced enzyme.

An inducible enzyme should reach a new steady state level at a time equal to 3 to 4 times the half-life of the enzyme (24). In the case of the induction of cyclic AMP phosphodiesterase which has a half-life of 70 to 80 min a new steady state level should be reached around 4 hours. When cells are treated with 0.2 mM SC-2964 a new steady state level is obtained by 4 hours. However, when cells are treated with 1 mM SC-2964 enzyme activity is still increasing at 8 hours suggesting (24) that higher inducer concentrations are not only turning on the synthesis of new phosphodiesterase activity but also probably effecting the degradation rate of the induced enzyme.

Increased enzyme activity could be due to synthesis of new catalytic units or synthesis of a specific activator for the enzyme. The activation of phosphodiesterase by a protein activator that has been described in many other cell types affects an enzyme with properties resembling Peak I. No evidence for an activator of Peak II was found. Determination of whether or not induction involves the synthesis of new phosphodiesterase catalytic units must await purification of the enzyme and the formation of antibodies to the purified enzyme.

Of a variety of compounds tested for induction of cyclic AMP phosphodiesterase, SC-2964 was the most potent. This compound which is structurally related to cyclic AMP is a potent inhibitor of cyclic AMP phosphodiesterase raising cyclic AMP to high levels. Therefore it seems likely that cyclic AMP is the true inducer. But it is also possible that SC-2964 itself is the inducer. This point probably will not be settled until enzyme induction in a cell-free system can be achieved.

Intracellular concentrations of cyclic AMP correlate well with the doubling time of normal and transformed fibroblasts grown in tissue culture and probably have an important role in regulating cell growth (1-3). The other naturally occurring cyclic nucleotide, cyclic GMP, may also be involved in the regulation of cell growth (22). Changes in cyclic AMP phosphodiesterase activity are involved in determining whether or not cyclic AMP levels rise when cells reach confluency (3). Cyclic AMP phosphodiesterase activity is increased when cyclic AMP levels rise, through induction of new enzyme activity and is decreased upon removal of the inducer; cyclic GMP phosphodiesterase is not affected by this regulatory scheme. Therefore, cyclic GMP phosphodiesterase and cyclic AMP phosphodiesterase activities are probably regulated by separate genes.

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