Cyclic AMP-Mediated Induction of the Cyclic AMP Phosphodiesterase of C-6 Glioma Cells

(dibutyryl cyclic AMP/norepinephrine/norepinephrine refractoriness)

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ABSTRACT Long-term regulation of the cyclic nucleotide phosphodiesterase of the C-6 rat glioma cell line has been studied. Both the low $K_m$ and high $K_m$ activities can be induced by elevation of intracellular cyclic AMP levels following either dibutyryl cyclic AMP or norepinephrine treatment of the cells. The enzymes are maximally induced by 3-4 hr. The presence of either cycloheximide or actinomycin D prevents induction by either dibutyryl cyclic AMP or norepinephrine. Evidence is presented that the norepinephrine effect is mediated by the $\beta$-catecholamine receptor. The increased phosphodiesterase activity causes a partial refractoriness to a second challenge with norepinephrine, which can be overcome by blockade of the induction with cycloheximide. The results suggest that just as short-term regulation of cyclic AMP levels occurs via changes in the rates of synthesis or degradation, long-term alterations of the system may also involve either the adenylate cyclase or the phosphodiesterase.

Intracellular levels of cyclic adenosine-3':5'-monophosphate (cAMP) can be regulated in several ways: by alterations in the rate of synthesis, of degradation, or of excretion. The C-6 rat glioma cell line has been shown to respond to norepinephrine (NE) or other $\beta$-agonists with adenylate cyclase activation (1) and a resultant elevation of intracellular cAMP levels up to 250-fold (2). Both low $K_m$ (1.5 $\mu$M) and high $K_m$ (200 $\mu$M) cyclic AMP phosphodiesterase activities are present in the cells (1) and both activities are inducible by treatment of the cells with dibutyryl (Bt2cAMP) (1). A similar induction of phosphodiesterase by Bt2cAMP has been demonstrated in other cell lines (3-6). This suggests that cAMP levels may be subject not only to short-term regulation by hormone stimulation of the adenylate cyclase but also to long-term regulation by increased activity of the degradative enzyme phosphodiesterase.

Recent evidence suggests that elevated phosphodiesterase activity may be at least partially responsible for the refractoriness to a second addition of NE observed in the C-6 glioma cells. Thus the smaller rise in cAMP levels following a second application of NE could be partially overcome by concomitant addition of a phosphodiesterase inhibitor, either papaverine or isobutylyxanthine (7, 8). In this paper, we present evidence that either Bt2cAMP or NE treatment of the C-6 glioma cells results in an induction of both low and high $K_m$ phosphodiesterases which is detectable within 30 min and maximal by 3-4 hr. The induction requires both protein and RNA synthesis. The NE effect is prevented by compounds that block $\beta$-catecholamine receptors. The addition of cycloheximide with the first NE treatment is shown to prevent not only the rise in phosphodiesterase activity but also the refractoriness to a second NE treatment. A preliminary report of these results has been presented (9).

MATERIALS AND METHODS

Cell Culture. The C-6 glioma cell line was obtained from the American Type Culture Collection. The cells were grown in Falcon plastic flasks (25 cm$^2$) with Ham's F-10 medium containing 10% fetal calf serum (Grand Island Biological Co.) in a humidified atmosphere of 95% air-5% CO$_2$ at 37°. All subcultures contained 33 units/ml of penicillin G and 50 $\mu$g/ml of streptomycin. Other details were as previously described (1).

Phosphodiesterase Assay. 3',5'-Cyclic AMP phosphodiesterase (EC 3.1.4.17) activity was determined according to the method of Thompson and Appleman (10), using cells suspended by incubation with 0.5 mM ethylenediaminetetraacetate (EDTA) in balanced salts solution and resuspended in 50 mM imidazole, pH 7.6, containing 3 mM MgCl$_2$ and 25 $\mu$g/ml of alkaline phosphatase. The low $K_m$ activity was assayed at 1.4 $\mu$M cAMP and the high at 1 mM cAMP, using $[\text{H}]$cAMP.

$[\text{H}]$Leucine Incorporation. Cells were incubated in fresh Ham's F-10 containing 1.25 $\mu$Ci (0.42 $\mu$Ci/ml) of $[\text{H}]$leucine. At the end of the incubation, the cells were suspended as described above and pelleted at 1000 x g, and the pellet was taken up in 1 ml of 5% trichloroacetic acid. The precipitate was washed three times with 2-ml portions of trichloroacetic acid and finally dissolved in 500 $\mu$l of 88% formic acid. One aliquot was assayed for protein by the method of Lowry et al. (11), while radioactivity was determined in another, by means of a Packard Tri-Carb liquid scintillation spectrometer.

Cyclic AMP Assay. Cyclic AMP was assayed by a modification (12) of the Gilman binding assay (13).

Materials. Materials were purchased as follows: Sigma Chemical Co.: alkaline phosphatase (type III), dl-propranolol-HCl, cycloheximide, l-arterenol bitartrate, Bt2cAMP (monosodium salt), actinomycin D; Boehringer Mannheim Corp.: cyclic AMP; New England Nuclear Corp.: $[\text{H}]$cyclic AMP (24.1 Ci/m mole), $[\text{H}]$leucine (5.0 Ci/-m mole); Aldrich Chemical Co.: dichloroisoproterenol-HCl. We thank Smith, Kline and French Co. for the donation of phenoxybenzamine-HCl.

Abbreviations: NE, norepinephrine; Bt2cAMP, $N^\alpha, O^\alpha$-dibutyryl adenosine-3':5'-monophosphate.
RESULTS

Previous experiments had shown that growth of the C-6 glioma cells in the presence of 1 mM Bt\textsubscript{2}cAMP plus 1 mM theophylline for 40 hr resulted in elevated cyclic AMP phosphodiesterase activity (1). Fig. 1 illustrates that within 4 hr of incubation with 1 mM Bt\textsubscript{2}cAMP plus 1 mM theophylline, both the low and high \( K_m \) activities have doubled. Sodium butyrate, at 1 mM, had no effect on enzyme activity (data not shown). The results shown in Table 1 indicate that although there is a partial increase in activity when the cells are treated with 0.3 mM Bt\textsubscript{2}cAMP in the presence of 1 mM theophylline, 1.0 mM \( K_m \) has a greater effect. Larger doses of Bt\textsubscript{2}cAMP were not used because they adversely affected cell growth. Also shown in Table 1 are the effects of cycloheximide, a drug that blocks protein synthesis, and actinomycin D, a drug that blocks RNA synthesis. When either drug was included in the 4-hr incubation with Bt\textsubscript{2}cAMP, the effects of Bt\textsubscript{2}cAMP were completely inhibited. However, when cycloheximide or actinomycin D was added without Bt\textsubscript{2}cAMP, there was no change in enzyme activity. The dose of cycloheximide utilized was that dose which blocked overall protein synthesis approx. 95\% (data not shown). These results demonstrate that the increase in phosphodiesterase activity produced by Bt\textsubscript{2}cAMP requires both new RNA and protein synthesis.

Since norepinephrine can elevate the C-6 glioma cell cAMP content (2), we next asked whether a long-term effect of this rise in cAMP might be phosphodiesterase induction. Incubation of the cells with 0.1 mM NE resulted in increasing phosphodiesterase activity with time (Fig. 2). By 4 hr, both the low and high \( K_m \) activities are maximally elevated. Half-maximal induction was achieved with 5–10 \( \mu \)M NE (results not shown). The results are very similar to those obtained for Bt\textsubscript{2}cAMP treatment (Fig. 1), although NE caused a faster rate of increase in activity. In addition, enzyme activity starts to fall off after 4–6 hr with NE, whereas it remains elevated as long as the Bt\textsubscript{2}cAMP concentration is maintained. That this increase also requires new protein and RNA synthesis is shown in Table 2. Again, the presence of either cycloheximide or actinomycin D during the incubation with NE prevented the rise in enzyme activity. Also shown in this table are cpm of \(^{[3]}H\)leucine incorporated per \( \mu \)g of total cellular protein during the course of the incubation. While NE has no effect on the relative incorporation, cycloheximide inhibited it approx. 95\%. Actinomycin D had no effect on \(^{[3]}H\)leucine incorporation.

<table>
<thead>
<tr>
<th>Treatment (4-hr incubation)</th>
<th>Cyclic AMP phosphodiesterase activity (pmoles/mg of protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Low ( K_m ) activity 32.9 ± 3.1; High ( K_m ) 369 ± 4</td>
</tr>
<tr>
<td>0.1 mM Bt\textsubscript{2}cAMP + 1 mM theophylline</td>
<td>30.6 ± 3.3; 488 ± 62</td>
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<tr>
<td>0.3 mM Bt\textsubscript{2}cAMP + 1 mM theophylline</td>
<td>51.3 ± 2.8; 607 ± 50</td>
</tr>
<tr>
<td>1.0 mM Bt\textsubscript{2}cAMP + 1 mM theophylline</td>
<td>75.1 ± 5.3; 708 ± 72</td>
</tr>
<tr>
<td>1.0 mM Bt\textsubscript{2}cAMP + 1 mM theophylline + 25 ( \mu )g/ml of cycloheximide</td>
<td>30.7 ± 3.3; 233 ± 23</td>
</tr>
<tr>
<td>1.0 mM Bt\textsubscript{2}cAMP + 1 mM theophylline + 5 ( \mu )g/ml of actinomycin D</td>
<td>30.6 ± 1.2; 180 ± 24</td>
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</table>

The experiment was carried out as described in the legend to Fig. 1. The results represent the mean ± SEM for \( n = 8 \).
Either had a isoproterenol, or is ... 3846 Biochemistry: the rise under Fig. by These with observed. is this cAMP experiments. SEM 0.1 mM 0.1mMNE 91.3 ± 4.1 350 ± 10 7.11 ± 0.58 0.1mMNE + 25 µg/ml of cycloheximide 91.3 ± 4.1 700 ± 30 6.95 ± 1.17 0.1 mM NE + 5 µg/ml of actinomycin D 51.6 ± 3.2 363 ± 27 0.59 ± 0.07 43.0 ± 6.0 386 ± 69 8.18 ± 0.74

The experiment was carried out as described in the legend to Fig. 1. The [3H]leucine incorporation was done as described under Materials and Methods. The results represent the mean ± SEM for n = 4. The experiment is one representative of three experiments.

Since NE can interact with both α and β receptors, we examined which type was involved in this enzyme induction. Either dichloroisoproterenol or propranolol, both β-receptor antagonists, was found to block the induction of phosphodiesterase by NE, whereas the α-blocker phenoxybenzamine had almost no effect (Table 3). Both β-blockers also prevent the rise in cAMP, while the α-blocker does not (data not shown). These results suggest that NE is acting via the β-receptor. This concept is further supported by the finding that isoproterenol, a pure β-agonist, causes a similar enzyme induction (data not shown).

The above results suggest that an elevation of intracellular cAMP levels in the C-6 cells, whether produced by incubation with Bt2cAMP or NE, mediates the increase in cyclic AMP phosphodiesterase observed. Further evidence for this suggestion is shown in Fig. 3. In this experiment, cells were incubated with NE for zero to 5 min. Some cells were fixed after this incubation in order to determine cAMP levels, while others were incubated an additional 3 hr without NE, following which phosphodiesterase activity was measured. The results demonstrate that with increasing time of exposure to NE, cAMP levels are increasingly elevated. The relative increase of low K_m phosphodiesterase activity corresponded closely with the relative elevation of cAMP levels, although minimal induction of the enzyme apparently occurred with cAMP levels above 1000 pmoles/mg of protein. Similar results were obtained for the high K_m enzyme.

Readdition of NE to cells 4 hr after an initial incubation with the drug results in a much smaller elevation of cAMP levels (8), relative to the rise seen with the first addition of NE. In order to determine whether this refractoriness might be partially explained by the increased phosphodiesterase activities measurable at this time, we carried out the experiment shown in Table 4. Cells were incubated for an initial 4-hr period followed by a second 10-min incubation. The presence of NE during the first 4-hr incubation resulted in the expected rise of phosphodiesterase activity. This rise was blocked by the additional presence of cycloheximide during this first incubation. NE addition during the second (10-min) incubation caused cAMP levels to rise to 1711 pmoles/mg of protein, versus 12.2 in control cells. If the NE were present during only the first incubation, the cAMP level was still approx. 5-fold.

### Table 4. Correlation between phosphodiesterase activity and refractoriness of the cells to a second norepinephrine treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4-hr incubation</th>
<th>10-min incubation</th>
<th>Low K_m activity (pmoles/mg of protein per min)</th>
<th>High K_m activity (pmoles/mg of protein per min)</th>
<th>cyclic AMP (pmoles/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Low K_m activity</td>
<td>High K_m activity</td>
<td>12.2 ± 1.0</td>
<td>1711 ± 203</td>
<td>1486 ± 141</td>
</tr>
<tr>
<td></td>
<td>(pmoles/mg of protein per min)</td>
<td>(pmoles/mg of protein)</td>
<td>(pmoles/mg of protein)</td>
<td>(pmoles/mg of protein)</td>
<td>(pmoles/mg of protein)</td>
</tr>
<tr>
<td>NE</td>
<td>32.8 ± 1.6</td>
<td>360 ± 30</td>
<td>57.2 ± 7.1</td>
<td>285 ± 12</td>
<td>57.4 ± 10.9</td>
</tr>
<tr>
<td>NE + CH</td>
<td>78.1 ± 6.6</td>
<td>704 ± 49</td>
<td>57.4 ± 10.9</td>
<td>265 ± 12</td>
<td>57.4 ± 10.9</td>
</tr>
<tr>
<td>CH</td>
<td>42.0 ± 1.7</td>
<td>416 ± 49</td>
<td>1486 ± 141</td>
<td>1594 ± 115</td>
<td>1486 ± 141</td>
</tr>
</tbody>
</table>

After removal of old medium, the cells were washed twice with Hanks' balanced salts solution and incubated in 3 ml of Ham's F-10 at 37°C. Norepinephrine (NE) was present at 0.1 mM and cycloheximide (CH) at 25 µg/ml. At the end of the first 4-hr incubation, the medium was removed, the cells were washed once, and 3 ml of medium containing the indicated drug were added for a second 10-min incubation. Cyclic AMP and phosphodiesterase were determined as described under Materials and Methods. The results represent the mean ± SEM (n = 4 for phosphodiesterase; n = 3 for cyclic AMP). The experiment was repeated three times.
higher than control at the end of the 4 hr. A second addition of NE caused only a 4.6-fold rise of cAMP, rather than the 140-fold elevation seen with the first NE treatment. The presence of cycloheximide during the first 4 hr had no effect on the cAMP concentration when added either in the presence or absence of NE. However, when cycloheximide had been present with NE during the first incubation, a second addition of NE now produced levels of cAMP comparable to those seen with a single NE treatment. Since phosphodiesterase activity is at control level under these conditions, the results suggest that cAMP phosphodiesterase induction may cause at least partial refractoriness to a second NE treatment.

**DISCUSSION**

The intracellular level of cAMP is determined by at least three factors: the rate of synthesis, the rate of degradation, and the rate of excretion. Much work has shown that the rate of synthesis is affected by hormonal activation of the adenylate cyclase system. The extra cAMP produced is then degraded by the cyclic nucleotide phosphodiesterase, or partially excreted in certain situations. This rise and fall of cAMP usually occurs within minutes, and is considered to be a short-term response. Whereas the catecholamines and polypeptide hormones cause these short-term effects, other hormones, notably the steroids, can have more long-term effects on the basal level and/or responsiveness of the adenylate cyclase (14, 15).

The results presented in this paper suggest that long-term hormonal regulation of the phosphodiesterase also exists. Treatment of the C-6 glioma cells with either Bt2cAMP or NE resulted in increased phosphodiesterase activity. Since this increase was blocked by either cycloheximide or actinomycin D, it is the result of new RNA and protein synthesis. The simplest interpretation would be that additional molecules of the phosphodiesterase have been synthesized. However, it is also possible that the rate of synthesis of some effector protein, for example, the heat-stable activator first described by Cheung (16), was increased. Further work will be required to distinguish between these possibilities.

Elevated cAMP levels mediate phosphodiesterase induction in other cell lines. Dibutyryl-cAMP treatment increases phosphodiesterase activity, presumably by elevating intracellular cAMP levels, in several cell lines (3–6). Usunov et al. (17) reported that norepinephrine increased one of two forms of phosphodiesterase in the C-2A astrocytoma cells. That cAMP exerts this effect via activation of the protein kinase is suggested by the work of Bourne et al. (6). They have isolated a lymphoma cell line deficient in protein kinase, in which the phosphodiesterase is no longer inducible by treatment with cAMP. Exposure to insulin has also been shown to result in increased phosphodiesterase activity in adipose tissue (18, 19), liver (20), and muscle (21), but these effects cannot be mediated by elevated cAMP levels since insulin in general lowers cAMP (22–24). Thus, there must exist at least two separate mechanisms whereby phosphodiesterase activity can be elevated.

In the brain, increased neuronal activity may result in locally elevated concentrations of NE. The short-term response of glia in the region might be elevation of cAMP levels, while the long-term response would be induction of phosphodiesterase activity. The phenomenon of refractoriness, whereby cells fail to respond to a second application of hor-

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**FIG. 3.** Correlation between intracellular cyclic AMP levels and low $K_v$ phosphodiesterase (PDE) induction. The cells were grown as described under Materials and Methods. On the day of the experiment, the cells were washed two times with 3 ml aliquots of Hanks’ balanced salts solution and incubated with 3 ml of Ham’s F-10 containing 0.1 mM norepinephrine. For measurement of cyclic AMP, the medium was removed and the cells were fixed in 1 ml of 5% trichloroacetic acid at the times indicated on the figure. Cyclic AMP was determined on the neutralized extracts as described under Materials and Methods. For phosphodiesterase activity, the medium was removed at the times indicated and the cells were washed once with 3 ml of medium and incubated with 3 ml of medium for 3 hr at 37°. Phosphodiesterase activity was then determined on the suspended cells as described under Materials and Methods. Each point represents the mean of three dishes for cyclic AMP and four dishes for phosphodiesterase. The experiment was repeated twice.

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