Glucocorticoid Receptors and Regulation of Phenylethanolamine-*N*-methyltransferase Activity in Cultured Chromaffin Cells¹

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Abstract

Glucocorticoids are known to regulate the enzyme phenylethanolamine-*N*-methyltransferase (PNMT) in the adrenal medulla of the rat and are thereby thought to control the synthesis of epinephrine. We have examined the details of this relationship in a simplified system, chromaffin cell primary cultures derived from bovine adrenal medulla. Cultured chromaffin cells were found to have a cytosolic, high affinity, saturable glucocorticoid-binding protein with the steroid specificity of a classical glucocorticoid receptor and a K_d of ~ 1 nM. Treatment of cultured cells with dexamethasone or hydrocortisone at any time up to 21 days in culture increased PNMT activity in the soluble fraction of the cell. The concentration of hormone required to produce a half-maximal response was 10 nM dexamethasone when cells were cultured in the presence of 5% fetal calf serum, or 1 nM in a defined serum-free medium. These dose-response relationships are consistent with mediation of this effect by the glucocorticoid receptor. Unexpectedly, however, the glucocorticoid-induced increment in PNMT activity was not inhibited by cycloheximide at concentrations up to 50 μ M, and an acceleration of protein synthesis by insulin treatment did not augment the glucocorticoid effect on PNMT. Treatment of the cells with dexamethasone (100 μ M) prevented the decline in the epinephrine-to-norepinephrine ratio seen over time in culture, an effect consistent with increased PNMT activity. However, there was no effect of dexamethasone on the ability of the cells to secrete catecholamines in response to stimulation with high KCl or 30 μ M nicotine. We interpret these data to suggest that PNMT activity and, thus, epinephrine biosynthesis can be controlled in cultured chromaffin cells by a mechanism which is probably tied to the glucocorticoid receptor detected in these cultures. Furthermore, these primary cultures provide a model for detailed biochemical analysis of steroid regulation of catecholamine metabolism.

Phenylethanolamine-*N*-methyltransferase (E.C. 2.1.1.28; PNMT), the terminal enzyme of the epinephrine biosynthetic pathway, is an ideal marker for the differentiated adrenal chromaffin cell, since it is

central to one of the cell's primary functions—epinephrine biosynthesis and secretion. In the rat, PNMT is subject to dual regulation, by both glucocorticoid hormones and direct innervation via the splanchnic nerve (Ciaranello, 1977). Glucocorticoid regulation of PNMT was demonstrated by Wurtman and Axelrod (1965, 1966) when they reported that withdrawal of endogenous glucocorticoids by hypophysectomy in the rat caused a decrease in adrenal PNMT activity. This could be reversed only by administration of high doses of replacement dexamethasone, a synthetic glucocorticoid, or by administration of ACTH. The importance of splanchnic innervation is illustrated by the fact that activation of neural input to the adrenal with 6-hydroxydopamine (Ciaranello and Black, 1971) or reserpine (Thoenen et al., 1970; Ciaranello, 1977) increases PNMT activity in rat adrenal.

Berenbeim et al. (1979) have proposed that these two types of regulation, hormonal and neural, occur via distinct biochemical pathways. They suggest that glucocorticoids decrease the rate of PNMT degradation and, therefore, cause an increase in PNMT activity, whereas splanchnic nerve stimulation increases PNMT synthesis directly. Alternatively, Bohn (1983) has suggested that, although PNMT appearance during development does not depend on glucocorticoids, per se, the continued expression of the enzyme depends on maintenance of mRNA levels by glucocorticoids. It seemed possible to us that we could resolve these apparently conflicting ideas by analysis of bovine adrenal medullary chromaffin cell primary cultures. In such an *in vitro* system of neural crest-derived cells which are less heterogeneous than those of the central nervous system, the external milieu can be controlled and biochemical processes can be easily analyzed.

In this paper, we report our studies on the influence of glucocorticoids on PNMT activity in cultures of bovine chromaffin cells. Not only were we able to replicate the *in vivo* finding of PNMT regulation by glucocorticoids in these cultures, but we were also able to detect an endogenous glucocorticoid receptor with an affinity in the range of physiological action of glucocorticoids on PNMT activity. These studies also suggest the potential utility of these primary cultures for investigating steroid regulation of other aspects of catecholamine metabolism.

Materials and Methods

Cell culture. Adrenal medullary cells were isolated from 10 to 20 bovine adrenal glands that were obtained from the local slaughterhouse and kept on ice for 1 to 2 hr. To remove blood, the glands were perfused five times by retrograde injection into the adrenal vein, using a sterile isotonic salt solution kept at 37°C. This salt solution contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 2.2 mM CaCl₂, 10 mM glucose, and 25 mM HEPES (pH 7.35). The glands were then perfused three times with a sterile solution of 0.095% collagenase (Sigma) which was dissolved in isotonic salt solution and kept at 37°C. The glands were incubated at 37°C for 15 min between perfusions. The medullae were dissected from the surrounding cortex, drained in 180- μ m nylon mesh to remove red blood cells, and minced with scissors in 10 ml of the 0.095% collagenase solution. The minced tissue was added to 80

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Figure 1. Time course of PNMT activity, cell number, and soluble protein in cultured chromaffin cells. Chromaffin cells were maintained in culture for 25 days. At various times, PNMT activity, soluble protein levels, and cell number per well were determined as described under "Materials and Methods." Each point represents the mean $\pm$ SD of the values obtained from three wells. Where no error bars are shown, the standard deviation is smaller than the symbol. Within the following groups of data, there were no significant differences ($p < 0.01$): A, days 1 to 5, days 5 and 6, days 11 to 25; B, days 2 to 5, days 11 to 25; C, days 1 to 5, days 7 to 25; D, days 2 to 5, days 12 to 25.

ml of 0.095% collagenase and was shaken for 30 min at 37°C. Single cells and cell clusters were separated from the undigested tissue by filtration through 180- μ m nylon mesh and pelleted at 800 $\times$ g. The collagenase was removed from the cells by three resuspension and centrifugation cycles in the same sterile isotonic salt solution. After a final filtration through 180- μ m nylon mesh, the cells were resuspended in 10 to 20 ml of sterile isotonic salt containing 1% bovine serum albumin. Cells were plated in 24-well cluster tissue culture plates which had been previously coated with 15 μ g of vitronectin (Grand Island Biological Co. (GIBCO)) well in Dulbecco's modified Eagle's minimal essential medium (DMEM) (GIBCO) supplemented with 5% fetal calf serum stripped of endogenous steroids. Serum was stripped by heating serum at 56°C for 30 min with 2 gm/100 ml of activated charcoal (Sigma). The medium also contained 100 μ g/ml of penicillin, 100 μ g/ml of streptomycin, 5 μ g/ml of gentamicin, and 10 μ g/ml of arabinosylcytosine to prevent division of non-chromaffin cells. The plating density was 1×10^6 cells/well. Approximately 50% of these cells adhered to the plate after the first medium change. This procedure yields a population of cells and cell clusters which are 99% viable by trypan blue exclusion and >95% positive by neutral red staining. The contamination by cortical cells, estimated morphologically, is <5% (R. Ornberg, personal communication). Medium was changed every 3 to 4 days.

In some experiments, we used a modification of the defined medium described by Bottenstein and Sato (1979) and Wilson and Viveros (1981). This medium contained the same additions as described above, with the following exceptions. Standard DMEM was replaced by 50% Ham's F12 Nutrient Mix (GIBCO) and 50% DMEM in 1.5 mM HEPES (pH 7.4) containing 1.2 gm of Na_2CO_3 /liter, and serum was omitted.

Steroid treatment. Steroids were obtained from Sigma. The appropriate aliquot of a 10 mM stock solution of each steroid in 95% ethanol was added to medium to yield the desired final concentration. The medium was sterilized

by filtration through a 0.22- μ m filter and 1.5 ml were added to each culture well in place of a regular medium change. The concentration of ethanol was less than 1.0% in all cases. Control wells contained an equivalent amount of ethanol. In experiments where the amount of ethanol varied, the control wells contained alcohol concentrations equivalent to that used for the highest concentration of steroid.

Enzyme assays. PNMT activity was measured by a modification of the method of Axelrod (1961), as described by Pollard et al. (1979). Cell lysate was prepared by subjecting chromaffin cells to water lysis, a freeze/thaw cycle, and centrifugation (Beckman Microfuge) to remove particulate matter. Fifty-microliter aliquots of lysate were assayed for PNMT activity. A parallel set of samples containing lysate, but no substrate, was used to control for endogenous substrate.

Dopamine β -hydroxylase (DBH) activity was measured by a modification of the method of Nagatsu and Udenfriend (1972). The assay mixture contained 200 μ M sodium acetate, pH 5.5, 10 mM disodium fumarate, 100 μ g of catalase, 20 mM *N*-ethylmaleimide, 10 mM ascorbic acid, 10 mM tyramine, and 200 μ l of sample in a final volume of 500 μ l. The mixture was incubated at 37°C for 20 min and the reaction was stopped with 500 μ l of 1.0 N perchloric acid. After removing catecholamines by aluminum hydroxide-gel adsorption, the samples were transferred to 0.2-ml ion-exchange columns (AG 50Wx8, 200 to 400 mesh, Bio-Rad). The columns were washed twice with water and adsorbed amines were eluted with 1.0 ml of 4 N ammonium hydroxide. Octopamine was converted to *p*-hydroxybenzaldehyde by the periodate-oxidation method and measured at 330 nm.

Lactate dehydrogenase (LDH) was measured in water lysates of chromaffin cells by monitoring the formation of NADH from NAD. Aliquots (100 μ l) of cell lysate were incubated with 60 mM lactate, 100 mM Tris, pH 9.0, and 1 mM NAD at room temperature for 30 min. The increase in absorbance at 340 nm was taken as an indication of LDH activity. The molar quantity of substrate converted was calculated, taking into account the temperature and the absorption of NADH.

Protein amounts were quantitated by the method of Lowry et al. (1951) in aliquots of cell lysate which had been precipitated with 10% trichloroacetic acid (TCA).

Protein synthesis studies. Protein synthesis was monitored in cultured cells by the addition of 2 μ Ci of ^3H -amino acid mixture (New England Nuclear) to the standard culture medium and subsequent determination of amino acid incorporation into acid-precipitable protein. At selected times, the cells were lysed by a freeze/thaw cycle in water, and particulate material was removed by centrifugation in a Beckman Microfuge 12 at 12,000 $\times$ g for 5 min. The supernatant was acidified by the addition of 100% TCA (final concentration, 10%), the acid-precipitable material was pelleted by centrifugation, and the amount of radioactivity in the pellet was determined by scintillation spectrometry. Arabinosylcytosine was omitted from the cell culture medium in all protein synthesis experiments. Using this method, ^3H -amino acid incorporation into protein was linear for 48 hr. Therefore, 36 hr was chosen as the standard time point for examining the effects of insulin, cycloheximide, and glucocorticoids on protein synthesis.

Glucocorticoid receptor binding assay. Glucocorticoid binding was measured in a high speed supernatant of cultured chromaffin cells by a modification of the sucrose pad/exchange assay, originally used for cytoplasmic estrogen receptor (Kelner et al., 1981). Chromaffin cells which had been maintained in culture for 3 days were washed three times with isotonic salt solution and homogenized with 10 strokes of a tight pestle in a Dounce homogenizer in 4 ml of ice-cold buffer containing 10 mM Tris, 1 mM EDTA, 10 mM sodium molybdate, 1 mM dithiothreitol, and 10% glycerol (pH 7.4). The homogenate was centrifuged at 100,000 $\times$ g for 1 hr at 4°C. Various concentrations of (6,7- ^3H (N)-dexamethasone (38.0 Ci/mmol) and (6,7- ^3H (N)-triamcinolone acetonide (31.3 Ci/mmol) (New England Nuclear) were incubated with aliquots of the supernatant at 0°C for 18 to 24 hr, conditions which were found to be optimal for ligand binding to the glucocorticoid receptor. Protamine sulfate precipitation was used to separate bound from free steroid. Unlabeled steroids used as competitors were purchased from Sigma. The data were analyzed by a modification of the Rosenthal resolution as described by Kelner et al. (1981).

Catecholamine measurement. Epinephrine and norepinephrine levels were determined by one of two methods. The first was a modification of the trihydroxyindole fluorescence method of Anton and Sayre (1962). Oxidation of aliquots of each sample at pH 7.0, a condition under which both norepinephrine and epinephrine are converted to their trihydroxyindole products, or at pH 2.0, a pH at which only epinephrine is significantly oxidized, allowed determination of the relative levels of these two catecholamines. In some experiments, catecholamines were measured using a high performance liquid chromatography reverse phase separation (3 μ m, 100 $\times$ 4.6 mm, ODS column, Chromanetics) coupled with electrochemical detection (ESA model

Figure 2. Dose response relationships between dexamethasone and PNMT activity in medium containing 5% fetal calf serum (left) and in serum-free defined medium (right). On day 7 of culture, plated chromaffin cells were treated with various concentrations of dexamethasone for 3 days in medium containing 5% fetal calf serum or in defined serum-free medium, described in detail under "Materials and Methods." PNMT activity and soluble protein levels were measured in water lysates of these cells, and PNMT activity per milligram of soluble protein was calculated. Data were expressed as percentage of untreated control. Each bar represents the mean $\pm$ SD of the values obtained from four wells.

5100 A dual electrode detector). The mobile phase (10 mM phosphate, 10 mM TCA, 0.02% sodium dodecyl sulfate, pH 2.80) was pumped at 1.0 ml/min using a Gilson model 303 pump. The area under the peaks was integrated with a Gilson Datamaster and a model 620 Apple II E. This assay has been completely described and compared to the trihydroxyindole oxidation method previously (Kelner et al., 1985).

Statistical analyses. In experiments with two groups, the statistical significance of the data was calculated by the Student's *t* test. In multiple group experiments with one variable, the presence of significant differences among groups was tested by analysis of variance and, when indicated, the significance of differences between each possible pair of points was determined by Tukey's *t* test using the honestly significant difference.

Results

PNMT activity in acute and cultured cells. PNMT activity in lysates of acutely isolated chromaffin cells was 22.6 ± 1.6 (SEM) nmol/30 min/mg of protein ($n = 3$) or 1.04 ± 0.02 nmol/30 min/ 10^6 cells ($n = 3$). After 2 days in culture, the activity declined to 10.8 ± 0.7 nmol/30 min/mg of protein ($47.9 \pm 8.6\%$ of acute cell level, $n = 4$) or 0.58 ± 0.03 nmol/30 min/ 10^6 cells ($55.5 \pm 2.0\%$ of acute cell level, $n = 4$). This drop was not due to selective plating of norepinephrine-containing cells which did not contain PNMT since the epinephrine-to-norepinephrine ratio was the same in both acute and day 1 to 3 cultured cells.

With time in culture, the specific activity of PNMT continued to fall over the course of 25 days. This is shown in figure 1, which indicates PNMT specific activity expressed as both nanomoles per 30 min per milligram of protein (Fig. 1A) and as nanomoles per 30 min per 10^6 cells (Fig. 1B), and the corresponding cell number (Fig. 1C) and soluble protein content (Fig. 1D). For the first 5 days after plating, PNMT levels expressed per milligram of protein remained constant. However, when expressed per cell, they showed a significant increase on days 2 to 5 over the levels seen on day 1 ($p < 0.01$). Cell number per well was also quite stable on days 1 to 5, but there was a significant rise in soluble protein on day 2 which paralleled the increase in PNMT activity per cell ($p < 0.01$), perhaps reflecting cell repair after isolation. After this initial period, there was a fairly rapid decline of PNMT activity to a low, but measurable value by day 25. This was accompanied by a decrease of approximately 25% in the cell number and soluble protein levels. Nonetheless, the

total soluble protein per cell remained constant over the 25 days in culture (6.13 ± 0.08 (SEM) μ g of soluble protein/ 10^5 cells, $n = 5$).

Since cell density has been shown to affect the levels of catecholamine biosynthetic enzymes in plated chromaffin cells (Acheson and Thoenen, 1983), we examined the effect of cell number per well on PNMT levels in our system. As the plating density was increased, the PNMT activity per cell also increased dramatically, reaching a plateau at approximately 150,000 cells/cm² (300,000 cells/well) (data not shown). All experiments reported in this paper were performed on cells plated above a density of 150,000 cells/cm².

Glucocorticoids and PNMT activity. We tested the effect of glucocorticoids on PNMT activity in chromaffin cells cultured for various times. Addition of dexamethasone (100 μ M) to the culture medium at 3, 7, or 21 days after initial plating caused an increase in PNMT specific activity, measured in the soluble fraction, of 28%, 46%, and 66% over control values, respectively. These data indicate that the cells retained responsiveness to glucocorticoids *in vitro* for at least 3 weeks in culture (data not shown).

Using cells cultured for 3 days, dose-response curves (1.0 nM to 100 μ M) were generated with the endogenous bovine glucocorticoid, hydrocortisone, and the synthetic glucocorticoid, dexamethasone. Two types of culture media were used: standard culture medium containing 5% stripped fetal calf serum and a defined medium without serum (see "Materials and Methods"). Figures 2 and 3 illustrate the dose-response curves obtained after 2 days of incubation. In the presence of serum, hydrocortisone and dexamethasone produced broad dose-response patterns of PNMT activation, with maxima at high concentrations of hormone (100 μ M for hydrocortisone and 10 μ M for dexamethasone). However, in a serum-free medium, a narrower dose-response curve was seen, with increases of PNMT activity occurring at lower concentrations of hormone, approximately 1 nM, and reaching maxima at 10 to 100 nM.

The activation of PNMT by glucocorticoids required 2 days for appearance, but additional time in culture did not increase the size of the effect further (see Fig. 4). The magnitude of the effect was constant regardless of whether treatment was initiated on day 3 or on day 8 and remained the same at least until day 14 of culture (11 days or 6 days of treatment, respectively). The falling control value

Figure 3. Dose-response relationships between hydrocortisone and PNMT activity in medium containing 5% fetal calf serum (left) and in serum-free defined medium (right). Plated chromaffin cells were treated exactly as described for Figure 2, except that hydrocortisone was substituted for dexamethasone. Data were calculated and expressed as for Figure 2.

Figure 4. Time course of the hydrocortisone-induced increase in PNMT activity. Plated chromaffin cells were maintained in culture for 14 days. On days 3 and 8, some wells were treated with 10 μ M hydrocortisone (arrows). At time points indicated, the cells were lysed with water and the levels of PNMT activity and soluble protein were measured. Data were expressed as nanomoles per 30 min per milligram of protein. Each point represents the mean $\pm$ SD of the values obtained from three wells. Where no error bars are indicated, the standard deviation is smaller than the symbol. Statistical analyses indicated that there were no significant differences among points in the following groups ($p < 0.05$): control, days 2 and 3, days 3 to 6, days 6 and 7; treated, days 3 to 6, day 10, day 14. Treated points were different from controls ($p < 0.05$) on day 6 and day 10.

caused an apparent increase in the percentage of stimulation over time.

To test the specificity of the effect of glucocorticoids on PNMT, the lysate from cells incubated for 3 days with dexamethasone was assayed for increases in the specific activity of two other enzymes, DBH and LDH. Under conditions in which PNMT activity increased significantly, neither enzyme showed any change in activity (Table I, $p < 0.01$).

The role of protein synthesis in mediating the increase in PNMT activity seen with glucocorticoids was investigated using the protein synthesis inhibitor, cycloheximide. At concentrations up to 50 μ M, there was no effect of protein synthesis inhibition by cycloheximide on dexamethasone's ability to stimulate PNMT activity (see Fig. 5, top). Dexamethasone treatment alone did not affect the rate of protein synthesis (data not shown). In addition, acceleration of

protein synthesis by 37% by treatment of the cells with 5 μ g/ml of insulin did not affect the magnitude of the increase in PNMT activity produced by dexamethasone (data not shown). Figure 5, bottom, documents that, under the conditions used for this study, cycloheximide inhibited amino acid incorporation into acid-precipitable protein in chromaffin cells. At 50 μ M cycloheximide, the highest concentration tested, amino acid incorporation was reduced to 2.3% of the control level.

Epinephrine synthesis in glucocorticoid-treated cells. If PNMT activity in cell lysates reflected epinephrine synthesis in the cell, then under conditions in which PNMT activity is altered one would expect to see changes in the levels of cellular norepinephrine and epinephrine, the substrate and product of the enzyme, respectively. To test this, we measured the effect of constant exposure of chromaffin cells to dexamethasone on the cellular content of epinephrine and

TABLE 1

Dexamethasone effects on chromaffin cell enzymes

PNMT, DBH, and LDH activities were measured in water lysates of 3-day chromaffin cells that had been maintained in the presence or absence of 100 μ M dexamethasone for 3 days as described under "Materials and Methods." The significance of the difference between groups was tested by the Student's *t* test.

	PNMT (nmol/30 min/mg of protein)	DBH (μ mol/30 min/mg of protein)	LDH (nmol/30 min/ 10^6 cells)
Control	3.16 $\pm$ 0.13	3.54 $\pm$ 0.11	102.9 $\pm$ 7.6
Dexamethasone treated	39.4 $\pm$ 0.04 ^a	3.21 $\pm$ 0.28 ^b	101.0 $\pm$ 15.2 ^b

^a $p < 0.01$.^b Not significantly different from control.

norepinephrine. The levels of the two catecholamines in acutely isolated cells were 30.9 ± 2.2 nmol (SEM) of norepinephrine/ 10^6 cells ($n = 6$) and 63.4 ± 4.0 nmol of epinephrine/ 10^6 cells ($n = 6$). On day 1 of culture, these levels dropped to 56% and 48% of these values, respectively. However, during the first few days in culture, the ratio of the two catecholamines remained the same as in the acutely isolated cells ($29.8\% \pm 0.72$ (SEM) norepinephrine and $70.2\% \pm 3.08$ epinephrine) (see Fig. 6). At these times, dexamethasone had no effect on either the absolute amount of epinephrine and norepinephrine in the cells (cf. Fig. 6, *left and right upper panels*) or on the relative amounts of these catecholamines (cf. Fig. 6, *left and right lower panels*). With longer times in culture (more than 7 days), there was a significant drop in epinephrine levels per well, whereas norepinephrine levels remained constant ($p < 0.01$), resulting in a ratio of norepinephrine to epinephrine of $52\% \pm 3.0$ (SEM); $48\% \pm 1.8$ at day 17 (Fig. 6, *left panels*). When the cells were maintained in the presence of dexamethasone (Fig. 6, *right panels*), there was a smaller but significant drop in epinephrine levels, as well as a drop in norepinephrine levels ($p < 0.01$). The ratio of epinephrine to norepinephrine remained constant ($29.5\% \pm 0.5$ (SEM) norepinephrine: $70.5\% \pm 0.5$ epinephrine on day 17). Thus, dexamethasone treatment allowed the cells to maintain their initial proportions of norepinephrine and epinephrine.

We tested whether pretreatment with dexamethasone had any effect on the ability of cultured chromaffin cells to secrete catecholamines in response to secretagogues. Cells were treated with 10 μ M dexamethasone acutely or for 1, 3, or 6 days. Subsequently, 30 μ M nicotine or 65 mM KCl was applied to the cells for 15 min, and the norepinephrine and epinephrine released into the medium during that time period was measured by the trihydroxyindole method. For both catecholamines, basal release was less than 10% and was not affected by any dexamethasone pretreatment. Similarly, dexamethasone pretreatment of any duration had no consistent effect on the fraction of either catecholamine released into the medium with nicotine or KCl (data not shown).

Glucocorticoid receptor in chromaffin cells. Since the cultured chromaffin cells were able to respond to glucocorticoids, we examined them for the presence of a glucocorticoid receptor. Saturation analyses of high speed supernatants of cultured chromaffin cells revealed the presence of a specific saturable binding site when either 3 H-dexamethasone or 3 H-triamcinolone acetonide was used as ligand. The saturation plots and their corresponding Scatchard analyses, shown in Figures 7 and 8, illustrate that, with both ligands, the dissociation constant of ligand binding was approximately 1×10^{-9} M. The number of binding sites ranged from 200 to 350 fmol/mg of soluble protein in all experiments.

A comparison of the ability of various steroids to compete for 3 H-dexamethasone binding showed an order of potency of: testosterone $\approx$ estradiol $\ll$ progesterone $\ll$ corticosterone $<$ triamcinolone acetonide $\approx$ hydrocortisone $\approx$ dexamethasone (data not shown).

Figure 5. The effect of cycloheximide on the dexamethasone-induced increase in PNMT activity (*upper panel*) and protein synthesis (*lower panel*). *Upper panel.* On day 5 of culture, plated chromaffin cells were treated with various concentrations of cycloheximide in the presence or absence of 100 μ M dexamethasone. After 3 days, the cells were lysed and the PNMT activity and soluble protein levels were determined as described in the text. Each point represents the mean $\pm$ SEM of the values obtained from 12 wells in two separate experiments. There was no significant difference among the points within the control group or the treated group. The treated value was significantly different from control at 0, 1, and 50 μ M cycloheximide. The difference at 10 μ M was not significant (in all cases $p < 0.01$). *Lower panel.* On day 5 of culture, plated chromaffin cells were treated with various concentrations of cycloheximide in the presence of 2 μ Ci of 3 H-amino acids/well. After 36 hr, the amount of radioactivity in acid-precipitable material was determined, as described in the text. Each point is the mean of the values obtained from three wells.

Discussion

Glucocorticoid regulation of PNMT activity *in vitro*. Maintenance of the levels of adrenal PNMT, the methyltransferase responsible for epinephrine synthesis, is known to depend on glucocorticoids in the rat. It was therefore reasonable to suspect that some or all of the drop in PNMT levels seen over time in cultured chromaffin cells (see Fig. 1) might be due to withdrawal of the cells from the endogenous source of this hormone and that replacement of glucocorticoids might restore activity. In fact, treatment of the cultured cells with the synthetic glucocorticoid, dexamethasone, or with the endogenous bovine glucocorticoid, hydrocortisone, caused a dose-dependent increase in PNMT activity when treatment was initiated at any time

Figure 6. The effect of dexamethasone treatment on maintenance of epinephrine and norepinephrine levels in cultured chromaffin cells. Plated chromaffin cells were cultured for 17 days in the absence (left panels) or presence (right panels) of 100 μ M dexamethasone. At regular intervals, wells were lysed in 10% acetic acid and the levels of norepinephrine and epinephrine were analyzed by high performance liquid chromatography, as described under "Materials and Methods." The data are expressed as nanomoles per well (upper panels) or as percentage of total catecholamine (lower panels), where total catecholamine equals the sum of the epinephrine and norepinephrine values. Each point represents the mean $\pm$ SD of the values obtained from four wells. Where no error bar is present, the error was smaller than the symbol. Statistical analysis indicates that the following values are significantly different from the day 3 values ($p < 0.01$): control, none (N); days 12 and 17 (E); dexamethasone, days 12 and 17 (N); days 12 and 17 (E).

from day 3 to day 21 in culture. The specificity of this effect is illustrated by the fact that the levels of two other enzymes involved in catecholamine metabolism, DBH and tyrosine hydroxylase, have been shown to be stable over time in culture (Waymire, 1977) and that glucocorticoids have no effect on the levels of DBH or LDH (Table I).

In vivo, the glucocorticoid-induced increase in PNMT activity requires unusually high concentrations of dexamethasone (Wurtman and Axelrod, 1966). In the cultured cells, there is also a requirement for high concentrations of dexamethasone to increase PNMT activity in the presence of serum, but this requirement is notably absent in the absence of serum (Hersey and Distefano, 1979; Figs. 2 and 3). These serum-dependent differences seen in the dose requirements for the glucocorticoid-induced increase in PNMT may reflect the presence of glucocorticoid binding activity derived from the serum which could buffer the added glucocorticoid and, thus, lower the amount of steroid available to the cell. It is not known whether a similar buffering effect of glucocorticoid-binding proteins contributes to the high steroid requirement *in vivo* or, alternatively, whether the properties of the receptor are altered in cultured cells.

The increase in PNMT activity produced by glucocorticoid treatment was characterized by a slow time course, requiring 2 days to become evident. When protein synthesis was inhibited to 2.3% of normal rates, the response was not blunted. Conversely, acceleration of protein synthesis with insulin did not enhance the magnitude of the response. These latter results are consistent with the notion that the glucocorticoid-induced increase in PNMT does not require synthesis of new protein. This is in contrast to the situation in the adult and developing rat, where PNMT increases are blocked by protein synthesis inhibitors (Wurtman and Axelrod, 1966; Bohn, 1983). However, we have not ruled out the possibility that complete inhibition (i.e., more than 97.7%) of protein synthesis would prevent the increase in PNMT in chromaffin cells. Use of higher concentrations of cycloheximide to accomplish this, however, produced toxic effects in the cells such that the results were uninterpretable.

The PNMT response to glucocorticoids in chromaffin cells is unlike the induction of many other steroid-regulated enzymes in that there is no massive increase in enzyme activity. After reaching a

maximum value of about 3 nmol/30 min/mg of protein by 2 days, the PNMT elevation above the control level was constant over time, although the control activity continued to fall. In fact, the glucocorticoid-treated cells simply showed a slower rate of PNMT loss. The levels of the enzyme were never increased by glucocorticoid treatment to those found in acutely isolated cells or even to the lower levels found in the cultures on day 1. This mimics the situation *in vivo*, where restoration of PNMT levels in hypophysectomized rats treated with replacement dexamethasone is never complete (Pohorecky and Wurtman, 1968; Ciaranello, 1977). The drop in PNMT activity in culture that occurs regardless of the presence of glucocorticoids may be due to the absence of other required regulatory influences, such as splanchnic nerve innervation, which is known to be required in the rat for maintenance of PNMT levels. The time-dependent loss of cells in culture would also be expected to cause a drop in PNMT specific activity per cell which could account for at least part of the drop in PNMT specific activity (see "Results"). In addition, the non-optimal long-term culture of these cells may require reorientation of the cell protein synthetic machinery toward proteins involved in cell maintenance and viability.

Glucocorticoid receptor in chromaffin cells. The soluble glucocorticoid-binding site which we have demonstrated to be present in chromaffin cell cultures has the characteristics of a classical glucocorticoid type II receptor. The dissociation constant (approximately 1×10^{-9} M), the receptor concentration (200 to 300 fmol/mg of soluble protein), and the steroid specificity are consistent with the notion that both the 3 H-dexamethasone binding and the 3 H-triamcinolone acetonide binding in cultured chromaffin cells represent binding to a true glucocorticoid receptor (Feldman et al., 1978; Do et al., 1979). The small variations in the steroid specificity and dissociation constant seen between this receptor and others (eg., the 1 nM K_d reported here versus 3 to 15 nM in the literature) are probably due to differing tissue compositions and assay methods and do not necessarily reflect unique properties of the chromaffin cell receptor. Although Do et al. (1979) have shown that some bovine tissues (i.e., thymus, kidney, liver, adipose tissue, and adrenal cortex) contain another glucocorticoid-binding site which is not the classical receptor, this other binding site does not appear to be present in chro-

Figure 7. Saturation analysis of ³H-dexamethasone binding to chromaffin cell cytosolic extracts. Binding isotherms of ³H-dexamethasone (³H-Dex) binding to chromaffin cell cytosolic extracts were constructed using concentrations of ³H-dexamethasone ranging from 0.1 to 10 nM with the procedure described under "Materials and Methods." The inset shows the saturation analysis, the upper line indicating total binding and the lower line representing the amount of binding obtained in the presence of 300-fold molar excess unlabeled dexamethasone, which was assumed to represent nonspecific binding. The main panel shows a Scatchard analysis of the data from the same experiment analyzed as described in the text. Each point represents a single determination. The reproducibility of the method can be seen by the replication illustrated in the inset.

maffin cell cultures in significant quantities. This is illustrated by the similarity of the saturation analyses obtained using ³H-dexamethasone and ³H-triamcinolone acetonide, since the second non-receptor binding site does not have an appreciable affinity for triamcinolone acetonide (Do et al., 1979).

The mechanism of the glucocorticoid-induced increase in PNMT activity. There are several pieces of evidence that suggest that the glucocorticoid receptor mediates the glucocorticoid-induced increase in PNMT activity. Half-maximal activation of the PNMT response occurs at approximately 1 nM dexamethasone in the absence of serum. Half-maximal saturation of the receptor also occurs at this concentration of steroid, the K_d being approximately 1 nM. Both saturation of the receptor and saturation of the response occur over a dose range of two orders of magnitude. In addition, the steroid specificity of this response is identical to the steroid specificity of the receptor (Hersey and Distefano, 1979; K. L. Kelner, unpublished observation).

The sequence of events occurring between the binding of the glucocorticoids to the receptor and the increase in PNMT activity in cultured chromaffin cells is not known, but our data may allow us to differentiate between potential mechanisms. In many systems, glucocorticoids have been shown to alter the levels of enzymes and proteins by a receptor-mediated process in which changes in specific gene transcription are caused by action of the receptor-steroid

Figure 8. Saturation analyses of ³H-triamcinolone acetonide binding to chromaffin cell cytosolic extracts. Binding isotherms of ³H-triamcinolone acetonide (³H-TA) binding to chromaffin cell cytosolic extracts were constructed as described for Figure 7. The data were analyzed and are displayed as for Figure 7.

complex on regulatory sites on the DNA. The altered levels of mRNA lead to changes in the rate of synthesis of the regulated protein. This is the mechanism proposed by Bohn (1983) for PNMT regulation by glucocorticoids. Protein synthesis inhibitors will block this type of steroid effect. For example, the hydrocortisone-induced increase in the rate of synthesis of glycerol phosphate dehydrogenase in C6 glioma cells is inhibited by 35 μ M cycloheximide, as well as by RNA synthesis inhibitors (McGinnis and de Vellis, 1978). Thus, the insensitivity of the PNMT response to 50 μ M cycloheximide reported here is inconsistent with this proposed mechanism, suggesting that increased PNMT synthesis is not the cause of the elevation in activity.

Glucocorticoids are also known to increase protein degradation in muscle (Goldberg, 1969; Tomas et al., 1979). In this regard, Ciaranello (1977) has suggested that, in the rat, the glucocorticoid-induced increase in PNMT activity is due to a slower rate of enzyme degradation. He suggests that this is caused by the protective action of increased levels of the PNMT cofactor, S-adenosylmethionine, which are a result of glucocorticoid treatment. Our data are consistent with this proposal. The amount of PNMT activity declines with time in culture, even in the presence of glucocorticoids, indicating that the rate of degradation is higher than the synthetic rate. Since protein synthesis inhibitors do not alter the glucocorticoid effect and also do not decrease the PNMT pool size (K. L. Kelner, unpublished observation), the PNMT synthetic rate must be small. Thus, the rate of degradation is the primary regulator of PNMT pool size, and a glucocorticoid-induced decrease in the rate of degradation of the enzyme would yield the results we have observed, an increase in PNMT activity. Although our data are consistent with the notion that altered protein degradation rates, rather than increased transcription and/or translation, may mediate the glucocorticoid effect on PNMT in cultured chromaffin cells, it is also possible that it occurs by an

alternative process. For example, glucocorticoids may activate a pre-existing pool of PNMT molecules. There is also increasing evidence that some effects of steroids may occur by an unknown mechanism which is operative at the level of the plasma membrane (McEwen et al., 1979).

Functional consequences of glucocorticoid treatment. The exposure of chromaffin cells to glucocorticoids caused the levels of the substrate and the product of the PNMT reaction to be altered but had no effect on the release process itself. Without steroid treatment, the cells were unable to maintain their original proportions of epinephrine and norepinephrine over extended time (i.e., weeks) in culture (see Fig. 6). The steady loss of epinephrine, perhaps due to a low level of basal secretion, was prevented by maintaining the cells in the presence of dexamethasone. Addition of glucocorticoids to the medium *in vitro* may mimic the *in vivo* situation where the adrenal medullary cells are exposed to high concentrations of glucocorticoid hormones which are released into the adrenal circulation by the adrenal cortex. This glucocorticoid-induced ability to maintain catecholamine levels is probably due to the increase in PNMT activity seen in the soluble lysates of these cells. However, these data do not rule out the possibility that other factors could contribute to these changes, such as increases in substrate or cofactor availability to PNMT.

In summary, these studies have demonstrated the presence of a classical glucocorticoid receptor in primary cultures of chromaffin cells isolated from bovine adrenal medulla. This receptor seems to mediate a slow dose-dependent increase in the activity of PNMT over control levels by a mechanism that is not easily inhibited by cycloheximide. This increase in PNMT activity may allow the cells to retain the initial content of epinephrine over extended time in culture. Thus, primary cultures of chromaffin cells provide an excellent system for further biochemical investigations of glucocorticoid regulation of catecholamine metabolism.

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