

RAPID COMMUNICATIONS

GLUCOCORTICOID INDUCTION OF TYROSINE HYDROXYLASE IN A CONTINUOUS CELL LINE OF RAT PHEOCHROMOCYTOMA

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ABSTRACT

We have established a continuous cell line (G1) in which the tyrosine hydroxylase specific activity is increased as much as 50–100-fold in response to dexamethasone. This response is specific for the glucocorticoid class of steroid hormones; it is elicited by dexamethasone, corticosterone, and triamcinolone, but not by estradiol, testosterone, progesterone, or deoxycorticosterone acetate. The increase in tyrosine hydroxylase specific activity is likely to be due to the increased synthesis of new enzyme protein rather than an activation of existing protein molecules, inasmuch as this increase is completely blocked by cycloheximide.

KEY WORDS tyrosine hydroxylase · glucocorticoids · pheochromocytoma · cell culture · enzyme induction

Continuously growing functional cell culture lines have been very useful in a number of disciplines for the elucidation of molecular mechanisms underlying the regulation of biological functions. The development of continuous neuroblastoma (1, 21) and glioblastoma (2) cell lines which retain a wide spectrum of nervous-system-specific functions has extended this cell culture approach to neurobiology (17).

A variety of experimental conditions which increase activity of preganglionic cholinergic nerves lead to a selective induction of tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH) in terminal adrenergic neurons. Other enzymes involved in the synthesis or degradation of the transmitter norepinephrine remain unchanged (22). This phenomenon has been termed "trans-synaptic induction". Several organ culture

model systems have been developed to study the molecular events involved in this provocative control mechanism (6, 15).

Both "in vivo" and organ culture systems have been used to demonstrate that glucocorticoids are intimately involved in regulation and/or modulation of the trans-synaptic induction of the adrenergic enzymes TH and DBH (23). We have now established a continuous cell culture system in which modulation of the adrenergic pathway by glucocorticoids occurs. This cell line, which we have designated the G1 cell line, is derived from the transplantable rat pheochromocytoma described by Warren and Chute (25). Under appropriate conditions, G1 cells in culture respond to the glucocorticoid dexamethasone (Dex) by a selective induction of TH. The G1 cell line should prove to be a useful model system to further elucidate the site of action and the mechanism by which steroid hormones exert an influence on the regulation of the adrenergic enzymes.

MATERIALS AND METHODS

Tissue Culture Procedures

Pheochromocytoma cell cultures were derived from solid tumors passaged subcutaneously in New England Deaconess Hospital strain albino rats (25). Breeding pairs and tumor-bearing rats were generously provided by Dr. S. Warren, New England Deaconess Hospital, Boston, Mass. Primary cultures were prepared by seeding mechanically dissociated tumor cells at relatively high densities in tissue culture dishes (Falcon Plastics, Oxnard, Calif.). No attempt was made to ensure a single-cell suspension. The cultures were grown for 6 days in RPMI 1640 medium supplemented with 15% sheep lymph (generously supplied by the Roche Institute of Immunology, Basel, Switzerland) and 2% heat-inactivated fetal calf serum. (RPMI medium and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N.Y., from Flow Laboratories, Inc., Ayshire, Scotland, or from Reheis Co., Inc., Phoenix, Ariz.). The cells were then gently pipetted off the dish, resuspended in a solution of 0.05% EDTA in phosphate-buffered saline, aspirated to break up clumps, centrifuged, and reseeded in culture dishes at a 1:2 dilution. After 45 min, the cells that had not attached were transferred with the medium to a fresh dish and allowed to grow for 7 days. No medium change was made unless the pH fell below 6.9. Cells were passaged five times by this selective attachment procedure. At the end of this period, the cultures were free of fibroblasts by morphological criteria. They were subcultured at a ratio of 1:4 in the above medium for a further 5 mo. We have designated this cell line the G1 cell line; it has now been in culture continuously for over 2 yr.

After 8 mo in culture (20 subpassages) the cells were adapted to RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. During a period of 2 mo in the latter medium, the induction of TH in response to Dex, as well as the basal TH level, slowly diminished. At the end of 2 mo, no TH was detectable. Apparently, growth in fetal calf serum alone selected for a population of cells which did not express TH. We initiated a procedure by which we hoped to enrich for a population of cells which would again respond to Dex. This protocol consisted of growing the cells in tyrosine-free RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 10^{-6} M Dex. Tyrosine-free medium has been used to isolate TH-positive neuroblastoma (3) and hybrid cell lines (7) from heterogeneous cell populations. Dex was included in the hope of reestablishing a cell population in which the TH level was modulated by steroids, as opposed to a population with a constitutively elevated TH level. After eight passages in this medium, the cells were subcultured without Dex for 1 wk, then retested for Dex modulation of TH. A cell population responsive to Dex was again present. Subsequently, G1 cells were grown in tyrosine-

free RPMI 1640 medium supplemented as described above until 1 wk before any experiment. For all experiments described in this report, the cells were first subcultured for 1 wk in tyrosine-free RPMI medium with Dex omitted, then subcultured again in the Dex-free medium at a density of 5×10^5 cells/100 mm culture dish. 24 h later, the experimental agents were added to each dish; this time-point is "0 time". Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. Dexamethasone, corticosterone, triamcinolone, β -estradiol, testosterone, and progesterone were purchased from Sigma Chemical Co., St. Louis, Mo. and were dissolved at 10^{-2} M in 95% ethanol before use in experiments.

Enzyme Analysis

At the termination of all experiments, the medium was removed and the cells were scraped from the dish with a rubber policeman into 1.0 ml of calcium and magnesium-free phosphate-buffered saline. After centrifugation in a microfuge for 30 s the cells were washed twice with 1.0 ml of the same buffer. The pellet was sonicated for 6 s in 0.3 ml of 5 mM tris buffer (pH 7.4) containing 0.1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). The TH enzyme activities were measured according to the method of Levitt et al. (11) with modifications described by Mueller et al. (14). The concentration of the substrate L-tyrosine was 13.3 μ M; the cofactor (6-methyl-5,6,7,8-tetrahydropterine HCl) was 600 μ M. Reagent mixtures containing the tris-Triton buffer instead of the enzyme preparation served as blanks. TH specific activity is defined as nanomoles of dopa formed per hour per milligram of total protein (³H₂O is measured after removal of [³H]tyrosine by ion-exchange chromatography). Blank to enzyme ratio is <3% in all TH assays on induced samples. Dialyzing the samples overnight has no effect on enzyme activity; endogenous tyrosine and catechols are not sufficient to affect enzyme measurements. 10^{-12} - 10^{-8} M Dex does not affect the enzyme assay. Assays are linear with respect to both time and protein concentration. Protein was measured according to the method of Lowry et al. (12). DBH was measured by the method of Molinoff et al. (13), dopa decarboxylase (DDC) by the method described by Håkanson and Owman (10), and choline acetyltransferase (CAT) by the method of Fonnum (4).

Materials

L-tyrosine (ring 3,5-³H, specific radioactivity 48 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. The 6-methyl-5,6,7,8-tetrahydropterine HCl, A grade, was obtained from Calbiochem, San Diego, Calif., and insulin and cycloheximide from Sigma Chemical Co. β -nerve growth factor was prepared by the method of Schenker et al. (19), and epidermal growth factor by the method of Savage and Cohen (18).

RESULTS

Increase in TH Activity in Response to Dex; Concentration Dependence

Dex showed a marked ability to increase the TH specific activity at doses above 10^{-8} M (Fig. 1). The "0 time" enzyme specific activity was 0.05 nmol of dopa/h per mg of protein. The response appears to be biphasic, with a plateau of ~ 3 nmol of dopa/h per mg of protein at 10^{-7} – 10^{-6} M Dex and a further increase to 8 nmol of dopa/h per mg of protein at 10^{-4} M Dex. In a single experiment, using 10^{-6} M Dex, the degree of induction of TH, as measured by $^3\text{H}_2\text{O}$ production from [^3H]tyrosine, [^{14}C]dopa production from [^{14}C]tyrosine, and immunotitration with antibody to TH were equivalent (Joh, Goodman, and Herschman, unpublished observations).

Although the G1 cells contain TH, the levels of the two other enzymes involved in catecholamine biosynthesis, DDC and DBH, were below the limits of detectability in both the presence and absence of Dex treatment (Table I). The cells do, however, contain the enzyme CAT, the specific activity of which is more than doubled after 4 days treatment with Dex (Table I). Preliminary experiments (in collaboration with Dr. Donald Jenden) have shown that these cells also contain acetylcholine. This transmitter is elevated in cells treated with Dex.

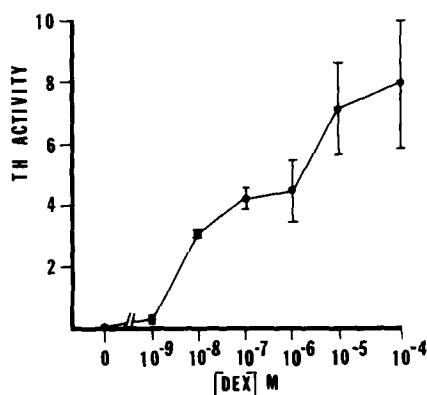


FIGURE 1 Concentration-dependence of Dex-mediated TH increase in G1 cell cultures. G1 cells were cultured for 5 days in the presence of various concentrations of Dex. The cultures were sonicated and the TH activity was determined. Values, expressed as nanomoles of Dopa formed per hour per milligram of protein, are means \pm SD of three cultures.

TABLE I
Enzyme Specific Activities of the G1 Cell Line

Enzyme	Specific activity	
	Control	After 4 days, 10^{-6} M Dex
Dopamine β -hydroxylase	<0.04	<0.04
Dopa decarboxylase	<0.07	<0.07
Choline acetyltransferase	4.4 ± 0.4	12.2 ± 0.7

After 7 days in complete RPMI medium, the cells were grown for a further 4 days in the presence or absence of 10^{-6} M Dex. The results for the CAT assay are expressed as means \pm SEM ($n = 4$). Difference between control and Dex-treated CAT specific activities are significant at $P < 0.001$.

Time-Course of TH Induction

Fig. 2 illustrates the time-course of TH induction by 10^{-6} M Dex, both in the presence and absence of exogenous tyrosine. After 24 h, TH activity was elevated nearly 20-fold over the "0

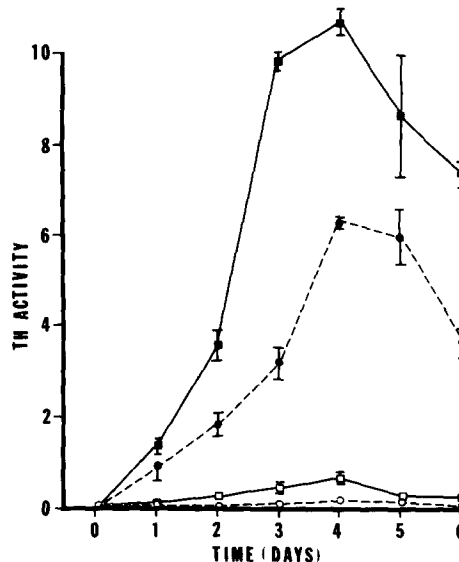


FIGURE 2 Time-course of induction of TH by Dex in the presence and absence of tyrosine. G1 cells were cultured as described in Materials and Methods in tyrosine-free RPMI (○), tyrosine-free RPMI supplemented with 10^{-6} M Dex (●), RPMI containing 1×10^{-4} M tyrosine (□), or RPMI containing 1×10^{-4} M tyrosine and supplemented with 10^{-6} M Dex (■). Data are expressed as in Fig. 1; values are means \pm SD of three cultures.

time" levels of 0.05 nmol of dopa/h per mg of protein, in both the presence and absence of tyrosine. The induction reached a maximum after 4 days of continuous exposure to Dex, increasing to 6.4 nmol of dopa/h per mg of protein in the absence of additional tyrosine and 10.8 nmol dopa/h per mg of protein in the presence of 10^{-4} M tyrosine. There were only minor differences between control cultures and those incubated in the presence of Dexamethasone either in the increase in total cell protein or in cell number during the course of this experiment. The doubling time for G1 cells (in the presence of tyrosine) is ~36-48 h, in the presence or absence of Dex; elevation of TH does not result from selection of a subpopulation of cells with a high TH level.

Cycloheximide Inhibition of TH Induction

The increase in TH specific activity after 24-h exposure to Dex could be prevented if $5 \mu\text{g/ml}$ of cycloheximide was present during the incubation period to inhibit protein synthesis (Fig. 3, groups labeled "24 h"). This concentration of cycloheximide inhibited incorporation of ^3H -amino acids into TCA-precipitable protein by >95%. To de-

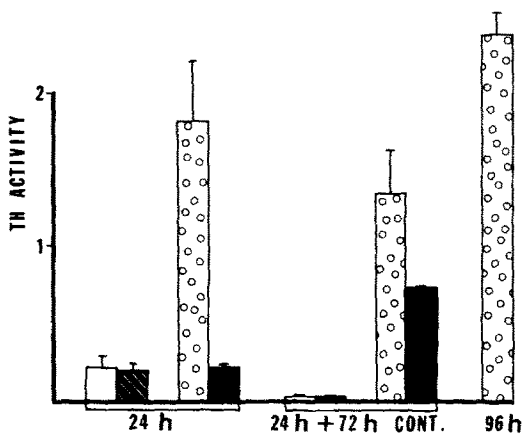


FIGURE 3 Cycloheximide inhibition of the Dex-mediated induction of TH in G1 cells. G1 cells were incubated in control medium (clear bar), medium containing $5 \mu\text{M}$ cycloheximide (hatched bar), $1 \mu\text{M}$ Dex (bar with circles), or both (black bar) for 24 h. After 24 h, half the cultures were harvested and the other half were rinsed three times with control medium and incubated for a further 72 h in control medium before being harvested. The TH specific activity was determined. Data are expressed as in Fig. 1; values are means \pm SD of three cultures.

termine whether cells (a) were still metabolically proficient and (b) had accumulated the capacity to synthesize TH in the presence of Dex and cycloheximide, we treated a second set of cultures (as described previously) for 24 h, then washed them free of cycloheximide and Dex. After 72 additional hours of incubation in medium, these cells were harvested and assayed (Fig. 3, "24 h + 72 h cont."). For comparison, a final set of cultures was exposed only to Dex for the entire 96-h period. When the Dex and cycloheximide were removed from the cultures after a 24-h incubation period and replaced with fresh control medium, and the cells were incubated a further 3 days, the TH level was significantly elevated above the controls. However, induction under such conditions was only half that observed when Dex alone had been present during the initial 24-h incubation period.

Steroid Specificity of the TH Response

The induction of TH specific activity in G1 cells was limited to the glucocorticoid class of steroid hormones. An increased level of TH activity was observed in response to corticosterone, Dex, and triamcinolone, but not in response to other steroids such as β -estradiol, testosterone, and progesterone (Fig. 4). The response to Dex was four-fold greater than the response to the natural glucocorticoid, corticosterone. This difference is most likely due to the fact that the corticosterone is metabolized whereas Dex is not. Nerve growth factor (NGF), insulin, or epidermal growth factor

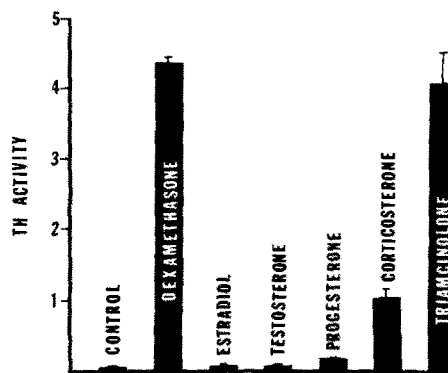


FIGURE 4 Steroid specificity of the Dex-mediated induction of TH. G1 cells were cultured for 4 days in the presence of various steroids at concentrations of 10^{-6} M. Data are expressed as in Fig. 1; values are means \pm SD of three cultures.

alone had no effect on levels of TH activity, the morphology or growth rate of these cells.

Loss of TH Activity after Removal of Dex

The loss of TH activity in G1 cells after transfer to medium without Dex appears to be a rather slow process (Fig. 5). The maximal activity of 6.5 nmol of dopa/h per mg of protein declined to 4.5 by day 3; by day 6, <10% of the initial activity remained, and by day 35 (data not shown), the levels of TH were below the limits of detectability of the assay (≤ 0.002 nmol/h per mg).

DISCUSSION

During the last few years, organ culture methods for sympathetic ganglia and adrenal medulla have continually been improved (6, 15). They have now reached a level of sophistication that allows one to follow in more detail, under defined experimental conditions, the process of selective induction of specific adrenergic enzymes initiated by cholinomimetics (reflecting trans-synaptic induction by increased preganglionic nerve activity) and by NGF (23). It has thus become possible to approach the molecular mechanisms of trans-synaptic and NGF-mediated enzyme induction, as well as their modulation, by glucocorticoids. However, a serious limitation in such investigations is the small amount of available tissue from organ culture. In general, attempts to develop a continuously cultured cell line which could be used as a model system have been hampered by the fact

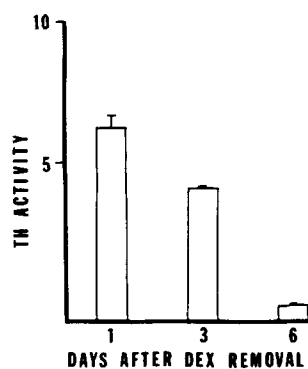


FIGURE 5 Loss of TH activity after removal of Dex. G1 cells were cultured for 8 wk in the presence of 10^{-6} M Dex. The cultures were washed three times with control medium and incubated for a further 6 days in the control medium. Cultures were harvested and assayed for TH activity on days 1, 3, and 6. Data are expressed as in Fig. 1; values are means \pm SD of three cultures.

that it is very difficult to maintain highly differentiated neuronal cell functions, such as the synthesis of enzymes involved in transmitter synthesis and their physiological regulation.

We have established a cell line derived from a rat pheochromocytoma tumor which responds to the glucocorticoid class of steroid hormones by an increase in the synthesis of the enzyme tyrosine hydroxylase, the rate-limiting step in the adrenergic pathway. Maintenance of this characteristic is highly dependent on the medium in which the cells are grown and the manner in which they are subcultured. We have not been able to derive clonal lines that have similar induction properties. The clones produced to date (over 30) from the G1 line have no detectable TH activity.

The original pheochromocytoma from which we developed the continuous G1 cell line expresses many of the differentiated functions of the sympathoadrenomedullary system, i.e., all the enzymes involved in the synthesis of the adrenergic transmitter norepinephrine (9). The G1 cell line exhibits several properties which distinguish it from cell lines of neuroblastoma with adrenergic properties. These pheochromocytoma cells in culture contain CAT (Table I), the activity of which is induced by Dex. Since the G1 line is not clonal, we have not yet determined whether the synthesis of TH and CAT occurs in the same cell. The PC12 clonal cell line derived from the same rat pheochromocytoma (9) contains both CAT and TH (20, 8). The presence of both transmitter enzymes is of particular interest in view of the recent findings of Patterson and Chun (16) that adrenergic neurons, depending on the cellular environment, may be converted into cholinergic ones, or at least can exhibit cholinergic functions in addition to their original adrenergic characteristics (5, 16). In our continuous G1 cell line, no evidence could be obtained for inhibition of growth or induction of morphological differentiation by NGF, in contrast to Tischler and Greene's results with primary cultures (24) or the PC12 clone (9) of this tumor. The G1 cell line grows as flattened cells in relatively sparse cultures. These cells pile up to form clumps as the cultures become more confluent. They contain very few chromaffin granules. In addition, we have been unable to detect DBH or dopa decarboxylase in the G1 cells.

Although not all the elements of differentiated adrenergic functions of the original tumor could be preserved in the G1 cell line, it does exhibit

properties that make it suitable as a model for studying some aspects of the regulatory processes in the synthesis of neurotransmitters. In contrast, however, to the adrenal medulla and the sympathetic ganglia (where glucocorticoids apparently serve only to modulate the trans-synaptic enzyme induction, cf reference 23), glucocorticoids alone produce a marked elevation of TH in G1 cells.

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