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 reseded 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 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 μM; the cofactor (6-methyl-5,6,7,8-tetrahydropteridine HCl) was 600 μM. 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 (H2O is measured after removal of [3H]tyrosine by ion-exchange chromatography). Blank to enzyme ratio is <5% in all TH assays on induced samples. Dializing the samples overnight has no effect on enzyme activity; endogenous tyrosine and catechols are not sufficient to affect enzyme measurements. 10-12-10^-4 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 Hakanson and Owman (10), and choline acetyltransferase (CAT) by the method of Ponnun (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-tetrahydropteridine 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 $10^{-6}$ 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 $^3$H$_2$O production from $^3$H[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](image1.png)

**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 ± SD of three cultures.

![Figure 2](image2.png)

**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 $1 \times 10^{-4}$ M tyrosine (□), or RPMI containing $1 \times 10^{-4}$ M tyrosine and supplemented with $10^{-6}$ M Dex (■). Data are expressed as in Fig. 1; values are means ± SD of three cultures.

**Table I**

| Enzyme Specific Activities of the G1 Cell Line |
|---------------------------------------------|
| **Enzyme** | **Control** | **After 4 days, $10^{-4}$ 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 $10^{-4}$ M Dex. The results for the CAT assay are expressed as means ± 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
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 μg/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 ^{3}H-amino acids into TCA-precipitable protein by >95%. To determine 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...
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 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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REFERENCES

1. August-Tocco, J., and G. H. Sato. 1969. Establishment of functional clonal lines of neurons from mouse neuroblastoma. Proc. Natl. Acad. Sci. U. S. A. 64:311-315.
2. Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differentiated rat glial cell strain in tissue culture. Science (Wash. D. C.). 161:370-371.
3. Breakfield, X. O., and M. W. Nirenberg. 1974. Selection for neuroblastoma cells that synthesize certain transmitters. Proc. Natl. Acad. Sci. U. S. A. 71:2530-2533.
4. Fonnun, F. 1975. A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. 24:407-410.
5. Furushan, E. J., P. R. MacLeish, P. H. O’Lague, and D. D. Potter. 1976. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc. Natl. Acad. Sci. U. S. A. 73:4225-4229.
6. Goodman, R., U. Otten, and H. Thoenen. 1975. Organ culture of the rat adrenal medulla: a model system for the study of trans-synaptic enzyme induction. J. Neurochem. 25:423-427.
7. Greene, L. A., W. Shain, A. Chalazonitis, X. Breakfield, J. Minna, H. G. Coon, and M. W. Nirenberg. 1975. Neuronal properties of hybrid neuroblastoma x sympathetic ganglion cells. Proc. Natl. Acad. Sci. U. S. A. 72:4923-4927.
8. Greene, L. A., and G. Rein. 1977. Synthesis, storage and release of acetylcholine by a noradrenergic pheochromocytoma cell line. Nature (Lond.). 268:349-351.
9. Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U. S. A. 73:2424-2428.
10. Håkanson, R., and C. Owman. 1965. Effect of denervation and enzyme inhibition of DOPA decarboxylase and monoamine oxidase activities of rat pineal gland. J. Neurochem. 12:417-429.
11. LeVitt, M., J. W. Gibb, J. W. Daly, M. Lipton, and S. Udenfriend. 1967. A new class of tyrosine hydroxylase inhibitors and a simple assay of inhibition in vivo. Biochem. Pharmacol. 16:1313-1321.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
13. Molinoff, P. B., R. Wenshlboum, and J. Axelrod. 1971. A sensitive enzymatic assay for dopamine beta-hydroxylase. J. Pharmacol. Exp. Ther. 178:425-431.
14. Muller, R. A., H. Thoenen, and J. Axelrod. 1969. Increase in tyrosine hydroxylase activity after reserpine administration. J. Pharmacol. Exp. Ther. 169:74-79.
15. Otten, U., and H. Thoenen. 1976. Selective induction of tyrosine hydroxylase and dopamine beta-hydroxylase in sympathetic ganglia in organ culture: role of glucocorticoids as modulators. Mol. Pharmacol. 12:353-361.
16. Patterson, P., and L. Chun. 1977. The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. Dev. Biol. 56:263-280.
17. Sato, G., editor. 1973. Tissue culture of the nervous system. In Current Topics in Neurobiology. Plenum Publishing Corp., New York. 1.
18. Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. 247:7609-7611.
19. Schemner, A., W. C. Mobley, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry. 15:5543-5551.
20. Schubert, D., S. Heinemann, and Y. Kidokoro. 1977. Cholinergic metabolism and synapse forma-
tion by a rat nerve cell line. Proc. Natl. Acad. Sci. U. S. A. 74:2579-2583.
21. SCHUBERT, D., S. HUMPHREYS, C. BARONI, and M. COHN. 1969. In vitro differentiation of a mouse neuroblastoma. Proc. Natl. Acad. Sci. U. S. A. 64:316-323.
22. THOENEN, H., and U. OTTEN. 1976. Molecular events in trans-synaptic regulation of the synthesis of macromolecules. In Essays in Neurochemistry and Neuropharmacology. John Wiley & Sons, Inc., New York. 1:73-101.
23. THOENEN, H., and U. OTTEN. 1977. Role of adrenocortical hormones in the modulation of synthesis and degradation of enzymes involved in the formation of catecholamines. In Frontiers in Neuroendocrinology. Raven Press, New York. 5. In press.
24. TISCHLER, A. S., and L. A. GREENE. 1975. Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells. Nature (Lond.). 258:341-342.
25. WARREN, S., and R. N. CHUTE. 1972. Pheochromocytoma. Cancer (Phila.). 29:327-331.