EPIDERMAL GROWTH FACTOR INDUCES TYROSINE HYDROXYLASE IN A CLONAL PHEOCHROMOCYTOMA CELL LINE, PC-G2

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ABSTRACT

We have previously described the isolation of a clonal cell line (PC-G2) in which the level of tyrosine hydroxylase (TH), the rate-limiting step in the synthesis of the catecholamine neurotransmitters, is induced by nerve growth factor (NGF). We now report that epidermal growth factor (EGF) also induces TH in the PC-G2 cell line. Although EGF has been shown to be mitogenic for many cultured cells, no neuronal function has been previously reported for this protein. The TH response to EGF is elicited in a dose-dependent fashion at concentrations as low as 0.1 ng/ml and is maximal at 10 ng/ml EGF. The maximal response is observed after 3–4 d of exposure to 10 ng/ml EGF. The induction by NGF and EGF is inhibited by their respective antisera. Dexamethasone, a synthetic glucocorticoid which we have previously shown modulates the response of PC-G2 cells to NGF, also modulates the TH induction elicited by EGF.

We have recently described the isolation, from an experimentally induced rat pheochromocytoma tumor, of a clonal cell line (PC-G2) in which the tyrosine hydroxylase (TH) levels are elevated in the presence of nerve growth factor (NGF) (13). The NGF-mediated induction of TH in the PC-G2 cell line is similar in magnitude and in concentration dependence to that seen both in vivo and in organ cultures of rat superior cervical ganglia (24, 31).

Male mouse salivary glands are by far the richest source of NGF, and are the most common starting material for the isolation of NGF. Epidermal growth factor (EGF) is also found in high concentrations in this organ. In addition, the levels of both proteins are elevated after androgen administration (7, 28). However, EGF and NGF, despite their common source and hormonal modulation, have no sequence homology or serologic cross reactivity (3, 29). Although little is known about the physiological role of EGF, its effects on the proliferation of cultured fibroblastic cells (8, 15, 27), as well as its induction of early tooth eruption and eyelid opening (10), suggest that EGF may be an important regulator of cellular proliferation and/or differentiation.

To our knowledge, EGF and NGF have never been reported to modulate a common biological function. NGF is apparently necessary for the development and maintenance of sympathetic neurons (17, 21) and affects sensory neurons at least during a limited period of the embryologic development (17). EGF has been reported to be a mitogen for a number of cultured cell lines (8, 15,

27). EGF also elicits the expression of differentiated function in several cell types in culture (6, 9, 18, 26, 30). We have now examined the effect of EGF on the neuronal enzyme TH in the PC-G2 cell line, and find that EGF, at concentrations above 0.1–1.0 ng/ml, causes a 5- to 10-fold increase in the specific activity of this enzyme.

MATERIALS AND METHODS

The PC-G2 clonal cell line was isolated from a rat pheochromocytoma tumor carried in New England Deaconess Hospital albino rats (32). We have previously described the isolation of this clone (13). PC-G2 cells are grown on complete RPMI 1640 medium containing 10% fetal calf serum (FCS). For all experiments described in this report, the cells were subcultured at a density of 5×10^5 cells/100-mm culture dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). 24 h later, the experimental agents were added to each dish; this time point is "0-time." All incubations were carried out for 4 d at 37°C in a humidified atmosphere of 5% CO_2 in air, unless otherwise specified.

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 bicarbonate-buffered saline. After centrifugation in a microfuge for 30 s, the cells were washed 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. The TH enzyme activities were determined according to the method of Levitt et al. (19), with modifications described by Mueller et al. (23). The concentration of the substrate L-tyrosine was 13.3 μM; the cofactor (6-methyl-5,6,7,8-tetrahydropterine HCl) was 600 μM. Reagent mixtures containing the Tris-Triton buffer instead of the enzyme preparation served as blanks. TH specific activities are expressed as nanomoles dopa formed per hour per milligram total protein. Protein was measured according to the method of Lowry et al. (20). Cyanogen bromide (CNBr) cleavage was carried out as previously described by Angeletti et al. (4). NGF and EGF, dissolved in 70% formic acid, were reacted with a 30-fold molar excess of CNBr for 24 h at 22°C. The reactions were stopped by dilution with water followed by lyophilization.

EGF and NGF were purified from mouse salivary glands by the methods of Savage and Cohen (28) and Mobley et al. (22), respectively. The purity of both preparations was evaluated by electrophoresis on both native and SDS acrylamide gels (27). Antisera to NGF and EGF were prepared in rabbits. Dexamethasone and 6-methyl-5,6,7,8-tetrahydropterine HCl, A grade, were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-tyrosine (ring 3,5-^{3}H, specific radioactivity, 48 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). Dexamethasone was dissolved in 95% ethanol before use.

RESULTS

Dose Response for Induction of TH by EGF in PC-G2 Cells

Exposure of PC-G2 cells to EGF results in a dose-dependent increase in the specific activity of TH. After exposure of PC-G2 cells to 10 ng/ml of EGF for 4 d, the TH level was elevated fivefold over that of control cultures (Fig. 1). In similar experiments, higher concentrations of EGF (up to 100 ng/ml) cause no further increase in the TH levels (data not shown). Although the maximal increase in TH occurred with 10 ng/ml of EGF, significant elevation of TH specific activity could be observed with as little as 0.1 ng/ml of EGF (i.e., 2×10^{-11} M EGF).

Time-Course for the Induction of TH by EGF in PC-G2 Cells

PC-G2 cells were treated with 10 ng/ml of EGF for varying lengths of time. After the time intervals indicated in Fig. 2, EGF-treated and control cultures were harvested and stored at −20°C for assay. A significant increase in the specific activity of TH was observed within 24 h of exposure of the PC-G2 cells to 10 ng/ml of EGF. Shorter times were not examined. Maximal TH stimulation (eightfold in this experiment) required 4 d of exposure to EGF.

Effect of CNBr Cleavage of NGF and EGF on the Induction of TH in PC-G2 Cells

In our previous report on the induction of TH in the PC-G2 cells by NGF (13), we observed that this induction occurred at concentrations of NGF in the μg/ml (10^{-7}–10^{-8} M) range, i.e., concentra-
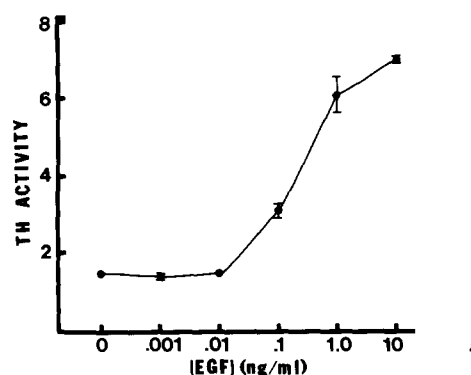

FIGURE 1 Concentration dependence of EGF-mediated induction of TH in PC-G2 cells. PC-G2 cells were exposed to varying concentrations of EGF for 4 d. Culture conditions are described in Materials and Methods. The cells were then harvested and assayed for TH activity. The TH activity is expressed as nanomoles dopa formed per hour per milligram protein. The TH activity of the control cultures was 1.44 ± 0.02 nmol dopa/h per mg protein. Values are means ±SEM of three cultures.
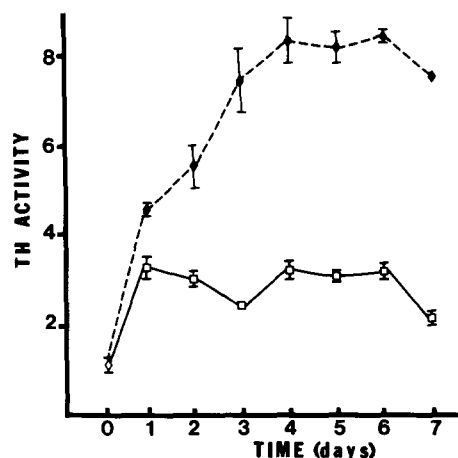
FIGURE 2 Time-course of EGF-mediated increase in TH in PC-G2 cells. PC-G2 cells were exposed to 10 ng/ml EGF for varying time intervals beginning 24 h after the cells were plated. Data are expressed as described in Fig. 1. The TH activity of the "0-time" cultures was 1.12 ± 0.12 nmol dopa/h per mg protein. Values given represent the means ±SEM of three cultures.

tions two to three orders of magnitude greater than that necessary for maximal induction of TH in PC-G2 cells by EGF (Fig. 1). Because of this large difference in the concentrations of EGF and NGF required for TH induction in PC-G2 cells, we became concerned that the NGF induction might be caused by a small contamination of our NGF preparations by EGF, as both growth factors are prepared from the same source. To determine whether there was a minor contamination of the NGF preparation by EGF, we cleaved each protein with CNBr and tested the CNBr-treated preparations for their ability to induce TH in PC-G2 cells. The results of this experiment are shown in Fig. 3. Treatment of EGF with CNBr results in the total destruction of the EGF-mediated induction of TH in the PC-G2 cells. In contrast, CNBr treatment of NGF had no effect on the NGF-mediated induction of TH in the PC-G2 cells.

Antibody Inhibition of EGF-Mediated TH Induction in PC-G2 Cells

The induction of TH observed upon treatment of PC-G2 cells with 10 ng/ml of EGF was totally inhibited by antiserum to EGF (Fig. 4). Neither normal rabbit serum nor antiserum to NGF inhibited the EGF-mediated TH induction. In addition, antiserum to EGF alone had no effect on the TH levels in the PC-G2 cells. The NGF-me-

diated induction of TH can be inhibited by anti-NGF (13), but not by anti-EGF (Fig. 5). At higher NGF concentrations (necessary to induce TH to greater levels; reference 13), our antisera to NGF were able to only partially absorb all of the inductive activity because of the large quantities of NGF required. However, EGF antiserum absorption which was able to completely block threefold elevations of TH by EGF (c.f. Fig. 4) had no effect on NGF induction of TH to a comparable level (data not shown). These serologic studies demonstrate, as do the CNBr inactivation experiments, that NGF induction of TH in PC-G2 cells is not caused by a minor EGF contamination.

Dexamethasone Effect on TH Induction by EGF

Preliminary experiments indicated that the non-metabolized glucocorticoid, dexamethasone, was maximally active at inducing TH in PC-G2 cells at 10^{-8} M. This effect was variable, ranging from zero to twofold (data not shown) stimulation of TH. This variability may result from differences in the steroid levels in the calf serum used in the medium. To investigate the interactions between glucocorticoids and EGF, we incubated PC-G2
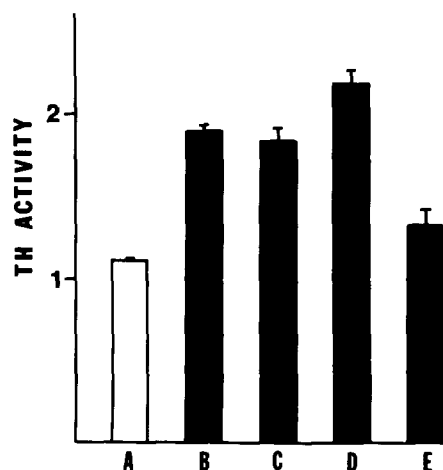

FIGURE 3 CNBr sensitivity of NGF- and EGF-mediated TH induction in PC-G2 cells. PC-G2 cells were exposed to control medium (A), 5 μg/ml of NGF (B), 5 μg ml of CNBr-treated NGF (C), 10 ng/ml of EGF (D), or 10 ng/ml of CNBr-treated EGF (E) for 3 d. The cultures were then harvested and assayed for TH as described in Materials and Methods. Data are expressed as in Fig. 1. The TH activity in control cultures was 1.10 ± 0.01 nmol dopa/h per mg protein. Values given represent the means ±SEM of three cultures.
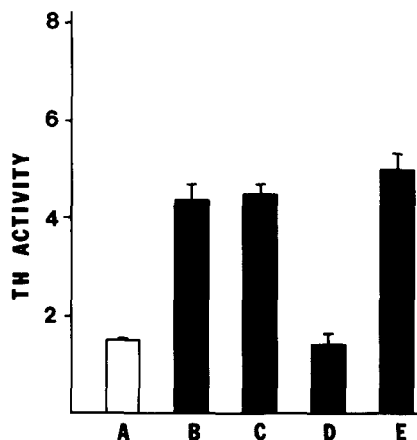
FIGURE 4 Inhibition by anti-EGF antibody of EGF-mediated TH induction. PC-G2 cells were exposed to control medium (A), 10 ng/ml EGF (B), NGF antibody-treated EGF solution (C), EGF antibody-treated EGF solution (D), or EGF incubated with normal rabbit serum (E) for 4 d. The treatment of EGF with anti-EGF, anti-NGF, and normal serum was carried out by incubating 0.5 ml of EGF at a concentration of 1 μg/ml with either 200 μl of anti-NGF antibody, 200 μl of anti-EGF antibody, or 200 μl of normal rabbit serum for 1 h at 37°C and then 16 h at 4°C. This solution was then centrifuged and the supernate was diluted 1:100 in control medium and added to the cultures. The solution of EGF alone was incubated in the same way. The cells were harvested and assayed for TH activity as described in Materials and Methods. The TH enzyme activity in control cultures was 1.52 ± 0.02 nmol dopa/h per mg protein. Values given represent the means ±SEM of three cultures.

cells with varying concentrations of EGF in the presence and absence of dexamethasone. After 4 d of incubation, the cultures were harvested and the specific activity of the TH was determined. Regardless of whether the steroid alone increased TH levels, we consistently observed an elevation in TH at all concentrations of EGF, in the presence of dexamethasone (Fig. 6).

DISCUSSION

EGF was originally discovered because of its induction of precocious eye opening and incisor eruption in neonatal mice (10). Its most studied function, however, has been as a mitogen for cells, in culture, of fibroblastic and epidermal origin (8, 15, 27). Recently, EGF has been reported to influence the differentiation of a number of cell types in cell culture. EGF causes enhanced synthesis of hyaluronic acid in human fibroblasts (16), induces

ornithine decarboxylase synthesis in mouse fibroblasts (30), causes increased prostaglandin synthesis by canine kidney cells (18), and controls the expression of large external transformation-sensitive protein in mouse fibroblast cells (9) and the secretion of human chorionic gonadotropin by cultured human choriocarcinoma cells (6).

Our laboratory has utilized EGF for a number of studies of mitogenesis and receptor-ligand interactions (1, 2, 12). We initially exposed PC-G2 cells to EGF under the assumption that EGF would be a readily accessible, highly purified polypeptide growth factor serving as a negative control for NGF induction of TH. We have previously shown that insulin, a protein whose prohormone has sequence homology to NGF, does not induce TH activity in the PC-G2 cell line (13). To our surprise, the PC-G2 cell line, which responds to NGF by the induction of TH, responds in a similar manner to EGF. Moreover, EGF was able to elicit this response at a much lower concentration than
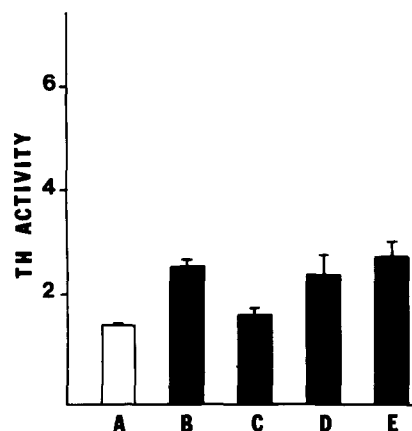
FIGURE 5 EGF antisera do not inhibit NGF-mediated TH induction. PC-G2 cells were exposed to control medium (A), 100 ng/ml NGF (B), NGF which had been treated with NGF antisera (C), EGF antisera (D), or normal rabbit sera (E) for 4 d. The treatment of NGF with antisera was carried out by incubating 1 ml of NGF at a concentration of 10 μg/ml with 200 μl of NGF antibody, 200 μl of EGF antibody, or 200 μl of normal rabbit serum for 1 h at 37°C and then 24 h at 4°C. The solution was then centrifuged and the supernate was diluted 1:100 in control medium and added to the cultures. The solution of NGF alone was incubated in the same way. The cells were harvested and assayed for TH activity as described in Materials and Methods. The enzyme activity in control cultures was 1.48 ± 0.02 nmol dopa/h per mg protein. Values given represent the means ±SEM of three cultures.
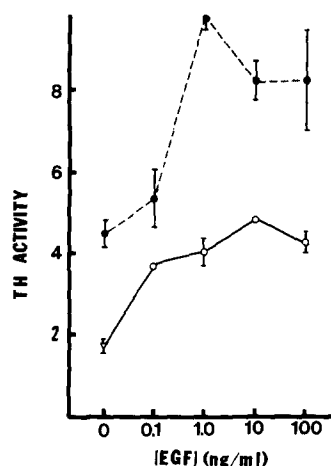
FIGURE 6 Dexamethasone augmentation of EGF-mediated TH induction. PC-G2 cells were exposed to varying concentrations of EGF (○) or to EGF plus 10^{-8} M dexamethasone (●) for 4 d. The cells were then harvested and assayed for TH activity as described in Materials and Methods. The TH enzyme activity in control cultures was 1.69 ± 0.17 nmol dopa/h per mg protein. Values shown represent means ±SEM of three cultures.

NGF. An NGF concentration of 100 ng/ml (4 × 10^{-9} M) is required to elicit significant TH induction in PC-G2 cells; maximal TH induction requires 5–10 µg/ml of NGF (13). These concentrations of NGF are similar to those needed to elicit TH induction in organ cultures of rat superior cervical ganglia (25). EGF, however, at concentrations as low as 0.1 ng/ml (2 × 10^{-11} M) (Fig. 1) elicited a significant induction of TH enzyme in PC-G2 cells. The TH activity was maximally stimulated by 10 ng/ml EGF. This concentration of EGF is the same as that required to obtain maximal mitogenic activity in cultured fibroblasts (27). Growth of PC-G2 cells in the presence of either NGF (1 µg/ml) or EGF (10 ng/ml) leads to a modest (<50%) increase in final cell density. With these concentrations, the induction of TH is much greater with EGF than with NGF (Fig. 1 and reference 13). These relatively small effects on cell number suggest that the response is inductive rather than selective. Moreover, removal of EGF from the culture medium of confluent PC-G2 cells is followed by a reduction in the specific activity of TH.

The EGF-mediated TH induction occurs at a concentration of EGF that was two to three orders of magnitude lower than the minimal NGF concentrations. As both NGF and EGF are isolated from male mouse salivary glands, we felt it nec-

essary to rule out the possibility that the TH induction in PC-G2 cells observed with NGF preparations was caused by minor contamination by EGF. Cohen and Savage (11) have reported that CNBr cleavage of EGF totally abolishes the EGF mitogenic activity in fibroblast cells in culture. In contrast, CNBr cleavage of NGF, which results in the cleavage of a nonapeptide fragment from the N-terminal end of β-NGF, has no effect on the biological activity of NGF (R. Bradshaw, personal communication). CNBr cleavage of EGF totally abolished the EGF-mediated TH induction in PC-G2 cells; CNBr cleavage of the NGF preparation did not affect its inductive capacity (Fig. 3). Thus, the induction of TH in PC-G2 cells by NGF is caused by NGF, not by a minor contamination by EGF. To further emphasize this result we treated EGF and NGF preparations with the homologous and heterologous antisera and observed that inductive effects were inhibited only by homologous antiserum (Figs. 4 and 5). TH levels in PC-G2 cells are regulated by at least two protein factors, NGF and EGF. Whether EGF acts in vivo to modulate the activity of TH in the sympathetic neurons remains to be seen. We are currently carrying out studies to determine whether the TH induction by EGF, observed in the PC-G2 cell line, also occurs in vivo.

Glucocorticoids have been shown to alter the NGF-mediated induction of TH observed both in PC-G2 cells (13) and in organ cultures of rat superior cervical ganglia (24, 25). Baker et al. (5) have recently reported that glucocorticoids affect the binding of EGF to EGF receptors in cultured fibroblast cells. The elevation of TH by EGF in PC-G2 cells is also modulated in the presence of the glucocorticoid dexamethasone (Fig. 6). As yet, we do not know whether this effect is caused by changes in the number of EGF receptors or in the affinity of the receptors for EGF, or whether some other mechanism is operative.

ADDENDUM

gesting the CNBr experiment.

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REFERENCES

1. AHARONOV, A., D. S. PASSOVOY, and H. R. HERSCHMAN. 1978. Mitogenic response to epidermal growth factor: Relationship to number, affinity, and down-regulation of EGF receptors in three murine embryo cell lines. *J. Supramol. Struct.* 9:41–45.
2. AHARONOV, A., R. M. PRUSS, and H. R. HERSCHMAN. 1978. Epidermal growth factor: Relationship between receptor regulation and mitogenesis in 3T3 cells. *J. Biol. Chem.* 253:3970–3977.
3. ANGELETTI, R. H., and R. A. BRADSHAW. 1971. Nerve growth factor from mouse submaxillary gland: Amino acid sequence. *Proc. Natl. Acad. Sci. U. S. A.* 68:2417–2420.
4. ANGELETTI, R. H., M. A. HERMODSON, and R. A. BRADSHAW. 1973. Amino acid sequences of mouse 2.5s nerve growth factor. II. Isolation and characterization of the thermolytic and peptic peptides and the complete covalent structure. *Biochemistry.* 12:100–115.
5. BAKER, J. B., G. S. BARSH, D. H. CARNEY, and D. D. CUNNINGHAM. 1978. Dexamethasone modulates binding and action of epidermal growth factor in serum-free cell culture. *Proc. Natl. Acad. Sci. U. S. A.* 75:1882–1886.
6. BENVENISTE, R., K. V. SPEEG, JR., G. CARPENTER, S. COHEN, J. LINDNER, and D. RABINOWITZ. 1978. Epidermal growth factor stimulates secretion of human chorionic gonadotropin by cultured human choriocarcinoma cells. *J. Clin. Endocrinol. Metab.* 46:169–172.
7. BRADSHAW, R. A. 1978. Nerve growth factor. *Annu. Rev. Biochem.* 47:191–216.
8. CARPENTER, G., and S. COHEN. 1976. Human epidermal growth factor and the proliferation of human fibroblasts. *J. Cell. Physiol.* 88:227–238.
9. CHEN, L. B., R. C. GUDOR, T.-T. SUN, A. B. CHEN, and M. W. MOSESSON. 1977. Control of a cell surface major glycoprotein by epidermal growth factor. *Science (Wash. D. C.).* 197:776–778.
10. COHEN, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J. Biol. Chem.* 237:1555–1562.
11. COHEN, S., and C. R. SAVAGE, JR. 1974. II. Recent studies on the chemistry and biology of epidermal growth factor. *Rec. Prog. Horm. Res.* 30:551–574.
12. DAS, M., T. MUYAKAWA, C. F. FOX, R. M. PRUSS, A. AHARONOV, and H. R. HERSCHMAN. 1977. Specific radiolabelling of a cell surface receptor for epidermal growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 74:2790–2794.
13. GOODMAN, R., and H. R. HERSCHMAN. 1978. Nerve growth factor-mediated induction of tyrosine hydroxylase in a clonal pheochromo-

cytoma cell line. *Proc. Natl. Acad. Sci. U. S. A.* 75:4587–4590.
14. HERSCHMAN, H. R., D. S. PASSOVOY, R. M. PRUSS, and A. AHARONOV. 1978. Mitogens for murine embryo cell lines. *J. Supramol. Struct.* 8:263–268.
15. HOLLENBERG, M. D., and P. CUATRECASAS. 1973. Epidermal growth factor: Receptors in human fibroblasts and modulation of action by cholera toxin. *Proc. Natl. Acad. Sci. U. S. A.* 70:2964–2968.
16. LEMBACH, K. J. 1976. Enhanced synthesis and extracellular accumulation of hyaluronic acid during stimulation of quiescent human fibroblasts by mouse epidermal growth factor. *J. Cell. Physiol.* 89:277–288.
17. LEVI-MONTALCINI, R., and P. U. ANGELETTI. 1968. Nerve growth factor. *Physiol. Rev.* 48:534–569.
18. LEVINE, L., and A. HASSID. 1977. Epidermal growth factor stimulates prostaglandin biosynthesis by canine kidney (MDCK) cells. *Biochem. Biophys. Res. Commun.* 76:1181–1187.
19. 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.
20. 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.
21. MAINS, R. E., and P. H. PATTERSON. 1973. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell. Biol.* 59:329–345.
22. MOBLEY, W. C., A. SCHENKER, and E. M. SHOOTER. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 15:5543–5551.
23. MUELLER, R. A., H. THOENEN, and J. AXELROD. 1969. Increase in tyrosine hydroxylase activity after reserpine treatment. *J. Pharmacol. Exp. Ther.* 169:74–79.
24. OTTEN, U., and H. THOENEN. 1976. Selective induction of tyrosine hydroxylase and dopamine-β-hydroxylase in sympathetic ganglia in organ culture: Role of glucocorticoids as modulators. *Mol. Pharmacol.* 12:353–361.
25. OTTEN, U., and H. THOENEN. 1977. Effect of glucocorticoids on nerve growth factor-mediated enzyme induction in organ cultures of rat sympathetic ganglia: Enhanced response and reduced time requirement to initiate enzyme induction. *J. Neurochem.* 29:69–75.
26. RHEINWALD, J. G., and H. GREEN. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.).* 265:421–424.
27. ROSE, S. P., R. M. PRUSS, and H. R. HERSCHMAN. 1975. Initiation of fibroblast cell division by epidermal growth factor. *J. Cell. Physiol.* 86:593–598.
28. SAVAGE, C. R., JR., and S. COHEN. 1972. Epidermal growth factor and a new derivative: Rapid isolation procedure and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.
29. SAVAGE, C. R., JR., T. INAGAMI, and S. COHEN. 1972. The primary structure of epidermal growth factor. *J. Biol. Chem.* 247:7612–7621.
30. STASTNY, M., and S. COHEN. 1970. Epidermal growth factor. IV. The induction of ornithine decarboxylase. *Biochim. Biophys. Acta.* 204:578–589.
31. THOENEN, H., P. U. ANGELETTI, R. LEVI-MONTALCINI, and R. KETTLER. 1971. Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine-β-hydroxylase in the rat superior cervical ganglia. *Proc. Natl. Acad. Sci. U. S. A.* 68:1598–1602.
32. WARREN, S., and R. N. CHUTE. 1972. Pheochromocytoma. *Cancer (Phila.).* 29:327–331.