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Induction of ornithine decarboxylase activity by insulin and growth factors is mediated by amino acids

(L-ornithine carboxy-lyase/polyamines/A and N transport systems/nerve, epidermal, and platelet-derived growth factors)

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ABSTRACT **The polypeptide growth factors, nerve growth factor, epidermal growth factor, and platelet-derived growth factor, as well as insulin do not induce ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) unless a minimal concentration of an ornithine decarboxylase-inducing amino acid, such as asparagine, is present in the medium. The effects of the growth factors were studied in appropriately responsive cell lines: pheochromocytoma (PC12) cells for nerve and epidermal growth factors, fibroblasts (NIH 3T3) for platelet-derived growth factor, and fibroblasts and hepatoma (KRC-7) cells for insulin. The nonmetabolizable amino acid analog α-aminoisobutyric acid can replace asparagine, indicating that the covalent modification of the inducing amino acid is not necessary for the induction of ornithine decarboxylase by these growth factors. For the same intracellular concentration of the inducing amino acid, the presence of the growth factors induces higher levels of ornithine decarboxylase. The evidence indicates that these growth factors do not induce ornithine decarboxylase by raising the intracellular concentration of amino acids but rather act synergistically with the inducing amino acid. Evidence is provided that the induction of polyamine-dependent growth by these growth factors is mediated by amino acids. The relationship of these results to the A and N amino acid transport systems and to the Na^+ influxes in relation to growth is discussed.**

Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) is present in all cells studied to date, and the polyamines, the products of its reaction, are essential metabolites (1). Inhibition of ornithine decarboxylase without the concurrent administration of polyamines leads to cessation of DNA synthesis and of cell proliferation (2, 3). Ornithine decarboxylase activity can be induced by all known classes of hormones acting on their target tissues (1, 4), and its induction is generally associated with the initiation of cell growth. Nerve growth factor (5) (NGF), epidermal growth factor (6) (EGF), and insulin have been shown to induce ornithine decarboxylase activity in various responsive cell lines, and it has been generally assumed that these hormones induce ornithine decarboxylase activity directly (7–12). We present evidence that the induction of ornithine decarboxylase by these hormones and, therefore, the subsequent ornithine decarboxylase dependent cell growth is mediated by specific amino acids.

To control the variables and to define those factors that are essential for enzyme induction, we have introduced the use of a simple salts/glucose solution as an incubation medium in place of complex growth media (13). In salts/glucose solution, L-asparagine was found to be the most effective natural amino acid to induce ornithine decarboxylase activity (13, 14); among the unnatural amino acids, analogs such as α-aminoisobutyric acid and *N*-methylasparagine, when added alone, also induce ornithine decarboxylase activity (15). The induction of ornithine decarboxylase activity by amino acid analogs has been verified (16).

Asparagine was found to be required for the induction of ornithine decarboxylase by insulin and by NGF in N15 neuroblastoma cells maintained in salts/glucose solution (17). We have extended these studies, and we show that EGF, NGF, platelet-derived growth factor (PDGF) (18, 19), and insulin will not induce ornithine decarboxylase activity in pheochromocytoma cells (PC12) or in fibroblasts (NIH 3T3) or in hepatoma cells (KRC-7) unless a minimal concentration of asparagine or of the nonmetabolizable aminoisobutyric acid is present. Amino acids are not required for the induction of all enzymes; for instance, in one well-studied case, it was shown that the induction of tyrosine amino-transferase by dexamethasone does not require amino acids (20). An interesting study on the regulation of ornithine decarboxylase activity by insulin and by asparagine in KRC-7 hepatoma cells also has been reported recently (12).

MATERIALS AND METHODS

Cell Cultures. PC12 cells were cultured in Dulbecco's modified Eagle's minimal medium supplemented with 10% fetal bovine serum and 5% horse serum. The 3T3 fibroblasts (NIH 3T3) and the hepatoma cells (KRC-7) were cultured in DMEM supplemented with 10% fetal bovine serum. All cell lines were grown in 30 × 10 mm Costar tissue culture dishes.

Measurement of Intracellular Amino Acid Pools and Ornithine Decarboxylase Activity. Confluent cell cultures were washed free of growth medium and incubated with L-[1-^{14}C]asparagine (0.2 μCi/ml, 21 μCi/mmol; 1 Ci = 37 GBq) in salts/glucose solutions at 37°C to elicit ornithine decarboxylase activity. After incubation, the culture dishes containing the cell monolayers were placed on ice, the radioactive solutions were aspirated, and the cells were washed twice with ice-cold phosphate-buffered saline. Ornithine decarboxylase assay buffer (0.4 ml of 0.1 mM Tris·HCl, pH 7.2/0.1 mM EDTA/50 μM pyridoxal phosphate/5 mM dithiothreitol) was added to the cells. The cells were lysed by freeze-thawing and centrifuged at 12,000 × *g* for 10 min. In a portion of the supernatant fraction, the trichloroacetic acid-soluble radioactivity was quantitated by liquid scintillation spectroscopy. In another portion, the ornithine decarboxylase activity was determined at saturating substrate concentrations (0.55 mM) as described (13); one unit represents 1 nmol of CO_2 released per hour. Other materials and methods used in this study have been published (21).

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Abbreviations: OrnDCase, ornithine decarboxylase; NGF, nerve growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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RESULTS

Fig. 1 shows that, in the absence of α-aminoisobutyric acid, the addition of EGF or of NGF to PC12 cells did not induce ornithine decarboxylase activity. Low concentrations of α-aminoisobutyric acid (2.5 mM) induced a low level of ornithine decarboxylase activity in PC12 cells that was appreciably enhanced by the addition of EGF. NGF was much more effective in enhancing ornithine decarboxylase activity under these conditions, raising it to the maximal level inducible by saturating concentrations (20–30 mM) of α-aminoisobutyric acid alone, as indicated by the symbol "x" (see arrow). We found that under the latter conditions, EGF and NGF produced an additional 10% and 25% increase in ornithine decarboxylase activity, respectively.

In another experiment using PC12 cells, ornithine decarboxylase was induced with asparagine in place of α-aminoisobutyric acid (Fig. 2). The presence of asparagine was essential for EGF or NGF to induce ornithine decarboxylase activity in these cells. Furthermore, the maximal ornithine decarboxylase activity induced by EGF or NGF in the presence of asparagine in salts/glucose solution was comparable to the maximal ornithine decarboxylase activity inducible by these growth factors in nutritionally complete medium. The levels of ornithine decarboxylase that we found to be inducible in PC12 cells in complete medium were similar to those obtained by other laboratories (7, 8).

Minimal concentrations of amino acids were also required for the induction of ornithine decarboxylase activity by growth factors in fibroblasts. PDGF and insulin did not induce ornithine decarboxylase activity in NIH 3T3 fibroblasts maintained in salts/glucose solution unless an inducing amino acid was included in the medium (Fig. 3). When added in combination with suboptimal levels of asparagine, PDGF and insulin increased ornithine decarboxylase activity beyond the levels induced by the asparagine alone (Fig. 3). As with the PC12 cells, we obtained similar results when α-aminoisobutyric acid was used in place of asparagine (data not presented). Consequently, the requirement for amino acids in inducing ornithine decarboxylase activity extends to both cAMP-mediated growth factors (NGF, EGF, and PDGF) and to insulin.

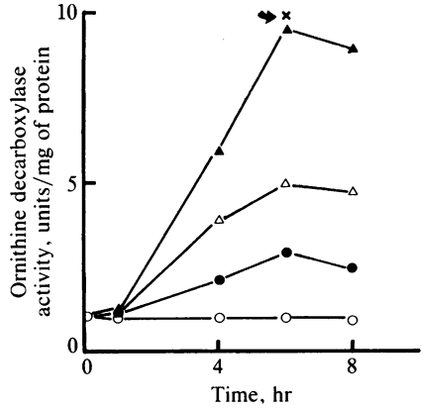

FIG. 1. The induction of ornithine decarboxylase activity by α-aminoisobutyric acid, NGF, or EGF in PC12 cells. The additions were made in salts/glucose solution: α-aminoisobutyric acid at 2.5 mM, NGF at 50 ng/ml, and EGF at 10 ng/ml. At the indicated times, the cells were harvested and assayed for ornithine decarboxylase activity; "x" (arrow) indicates the ornithine decarboxylase activity induced by 20 mM α-aminoisobutyric acid at 6 hr. The values represent the average of two experiments, each done with duplicate dishes, and vary by <10%. ○, EGF or NGF alone; ●, α-aminoisobutyric acid alone; △, EGF with α-aminoisobutyric acid; ▲, NGF with α-aminoisobutyric acid.

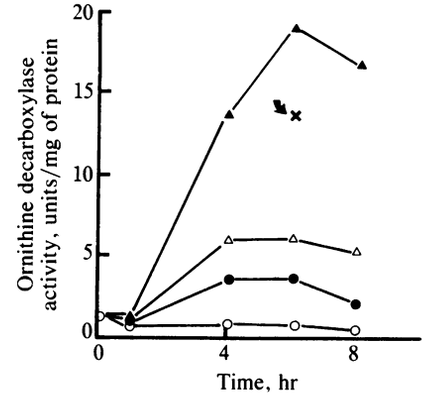

FIG. 2. The induction of ornithine decarboxylase activity by asparagine, NGF, or EGF in PC12 cells. The conditions were the same as in Fig. 1 except that asparagine at 2.5 mM was used; "x" (arrow) indicates the ornithine decarboxylase activity induced by 20 mM asparagine at 6 hr. The values represent averages of triplicate dishes from one of three experiments. Within experiments the variation was <10%. ○, EGF or NGF alone; ●, asparagine alone; △, EGF with asparagine; ▲, NGF with asparagine.

A series of experiments was performed to determine whether the growth factors induce ornithine decarboxylase indirectly, by increasing the intracellular concentration of the inducing amino acid, or whether they act synergistically with the inducing amino acid. In Figs. 4 and 5 we present the results obtained with insulin. Similar experiments were performed with other growth factors, and similar results were obtained with the other growth factors using α-aminoisobutyric acid. In all of these experiments, the values obtained represent steady-state amino acid levels and do not represent transport rates.

In agreement with the findings of other laboratories, at a given extracellular asparagine concentration, insulin increased the intracellular concentration of asparagine in KRC-7 cells (Fig. 4).

The relationship of the intracellular concentration of asparagine to ornithine decarboxylase activity in the presence and absence of insulin is shown by Fig. 5. Insulin did not induce ornithine decarboxylase activity in the absence of asparagine nor did it affect the maximal level of ornithine decarboxylase activity that was induced by high intracellular concentrations of asparagine. At intermediate levels of

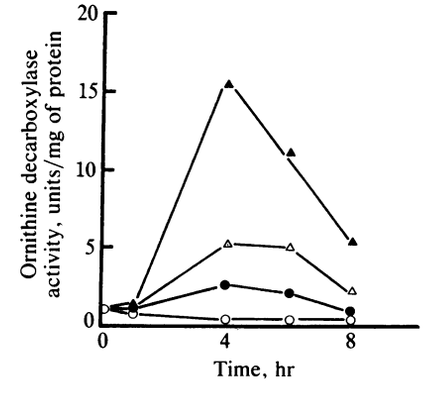

FIG. 3. The induction of ornithine decarboxylase activity by asparagine, insulin, and PDGF in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were serum-starved overnight and handled as described for Fig. 1 except that 0.1 μM insulin and partially purified PDGF were added to the cells. ○, insulin or PDGF alone; ●, asparagine; △, insulin with asparagine; ▲, PDGF with asparagine. The values are averages from triplicate dishes from one of two experiments. The variation was <10% within experiments.

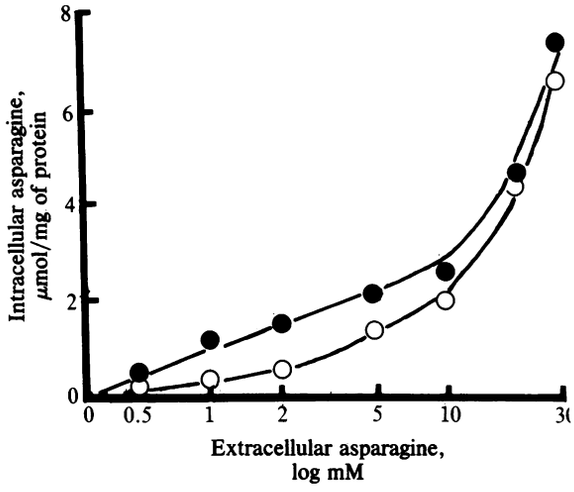

FIG. 4. Relationship of intracellular asparagine concentration to the extracellular asparagine concentration in KRC-7 cells in the presence (●) and absence (○) of 0.1 μM insulin. The intracellular asparagine concentration was determined at the end of 6 hr of incubation of KRC-7 cells in salts/glucose solution as the extracellular [^{14}C]asparagine concentration was varied from 0.5 to 30 mM. The values represent the average of triplicate dishes. Intracellular asparagine was quantitated as trichloroacetic acid-soluble radioactivity. Similar results were obtained with the nonmetabolizable radioactive amino acid analog, α-aminoisobutyric acid. ○, Asparagine alone; ●, asparagine with insulin.

asparagine, the addition of insulin allowed increased ornithine decarboxylase activity at a given intracellular concentration of asparagine, indicating a synergism between insulin and asparagine at these intracellular asparagine concentrations. Specifically, at intracellular asparagine concentrations such as 2, 2.5, 3, 4, 5, and 6 μmol of intracellular asparagine per mg of protein, the presence of insulin induced higher ornithine decarboxylase activities than did the same intracellular concentrations of asparagine in the absence of insulin. Therefore, insulin acts synergistically with asparagine.

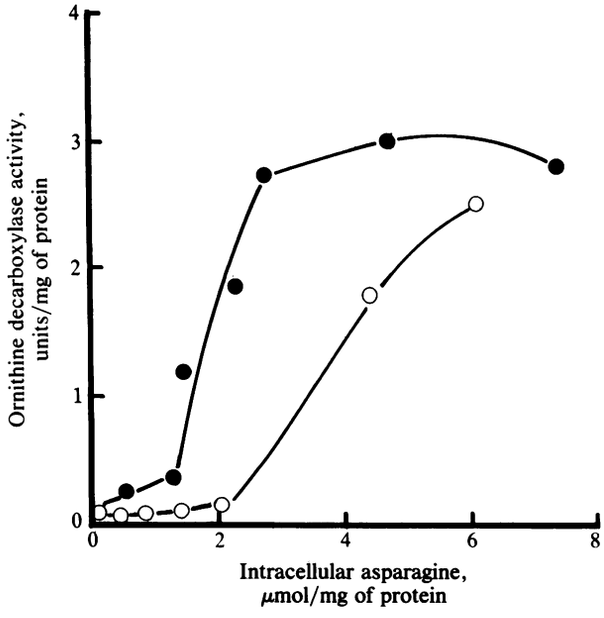

FIG. 5. Ornithine decarboxylase activity versus intracellular asparagine concentration in KRC-7 hepatoma cells in the presence (●) and absence (○) of 0.1 μM insulin. The values obtained for the intracellular asparagine described in Fig. 4 are plotted against the activity of ornithine decarboxylase at each asparagine concentration. ●, Asparagine alone; ○, asparagine with insulin.

It is evident that insulin and the growth factors could not induce ornithine decarboxylase activity in the absence of the inducing amino acid; they raised the intracellular concentration of the amino acid but, for the same intracellular concentration of the inducing amino acid, induced higher activities of ornithine decarboxylase.

The mechanism by which the amino acids facilitate the induction of ornithine decarboxylase induction activity does not include their covalent modification. Since α-aminoisobutyric acid is a nonmetabolizable amino acid, the induction of ornithine decarboxylase activity is not due to the participation of α-aminoisobutyric acid in protein synthesis or to other reactions involving covalent modification of α-aminoisobutyric acid.

Ornithine decarboxylase is induced by a limited number of amino acids, which are related by their primary reactivity with the A and N amino acid transport systems (21). The involvement of the A system seems certain, since the model amino acid substrate for this system, α-aminomethylisobutyric acid, induces ornithine decarboxylase (21). The existence of the N system (22) has been demonstrated in the liver, but its existence is as yet undetermined in other cell systems used. Na^+ influxes have been associated with growth factor action and cell proliferation (23–26), and this association becomes more interesting in the light of the fact that the ornithine decarboxylase-inducing amino acids are transported via Na^+-dependent transport systems (21).

The amino acid requirement for the induction of ornithine decarboxylase activity by the hormones demonstrated in this report, the increased induction of enzyme with increased intracellular concentrations of amino acids demonstrated in this report and in ref. 21, and the well-known stimulation of A system activity by growth factors suggest an important role for amino acids as mediators between growth factors and polyamine-requiring growth processes.

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1. Bachrach, U. (1973) *Function of the Naturally Occurring Polyamines* (Academic, New York).
2. Heby, O. (1981) *Differentiation* **19**, 1–20.
3. Pegg, A. E. & McCann, P. P. (1982) *Am. J. Physiol.* **243**, c212–c221.
4. Russell, D. H. & Durie, B. G. M. (1978) *Polyamines as Markers of Normal and Malignant Growth* (Raven, New York).
5. Yanker, B. A. & Shooter, E. M. (1982) *Annu. Rev. Biochem.* **51**, 845–868.
6. Carpenter, G. & Cohen, S. (1979) *Annu. Rev. Biochem.* **48**, 193–216.
7. Guroff, G., Dickens, G. & End, D. (1981) *J. Neurochem.* **37**, 342–349.
8. Hatanaka, H., Otten, V. & Thoenen, H. (1978) *FEBS Lett.* **94**, 313–316.
9. Stastny, M. & Cohen, S. (1970) *Biochim. Biophys. Acta* **204**, 578–589.
10. DiPasquale, A., White, P. & McGuire, J. (1978) *Exp. Cell Res.* **116**, 317–323.
11. Haselbacher, G. K. & Humbel, R. E. (1976) *J. Cell Physiol.* **88**, 239–246.
12. Potter, V. R., Evanson, T. R., Gayda, D. P. & Gurr, J. A. (1984) *In Vitro* **20**, 723–731.

13. Chen, K. Y. & Canellakis, E. S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3791–3795.
14. Costa, M. (1979) *Life Sci.* **25,** 2113–2124.
15. Viceps-Madore, D., Chen, K. Y., Tsou, H. R. & Canellakis, E. S. (1982) *Biochim. Biophys. Acta* **717,** 305–315.
16. Chideckel, E. W. & Edwards, D. (1983) *Biochem. J.* **210,** 617–619.
17. Rinehart, C. A., Jr. (1983) Dissertation (Rutgers Univ., New Brunswick, NJ).
18. Pledger, W. J., Stiles, C. D., Antoniades, H. N. & Scher, C. D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4481–4485.
19. Owen, A. S., III, Geyer, R. P. & Antoniades, H. N. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3203–3207.
20. Chen, K. Y. & Liu, A. Y.-C. (1982) *Biochim. Biophys. Acta* **755,** 244–252.
21. Rinehart, C. A., Viceps-Madore, D., Fong, W. F., Ortiz, J. G. & Canellakis, E. S. (1985) *J. Cell Physiol.* **123,** 435–441.
22. Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1980) *J. Biol. Chem.* **255,** 4011–4019.
23. Skaper, S. D. & Varon, S. (1980) *Brain Res.* **197,** 379–389.
24. Varon, S. & Skaper, S. D. (1980) *J. Supramol. Struct.* **13,** 329–337.
25. Villereal, M. C. (1981) *J. Cell Physiol.* **107,** 359–369.
26. Moolenaar, W. H., Yarden, Y., Delaat, S. W. & Schlessinger, J. (1982) *J. Biol. Chem.* **257,** 8502–8506.