DIFFERENCES IN THE MODE OF REGULATION OF ORNITHINE DECARBOXYLASE ACTIVITY IN UNDIFFERENTIATED VERSUS DIFFERENTIATED MOUSE N-18 NEUROBLASTOMA CELLS

Kuang Yu CHEN

Department of Chemistry, Rutgers University, Wright-Rieman Laboratories, Piscataway, New Jersey 08854, USA

Received 30 July 1980

1. Introduction

Ornithine decarboxylase (ODC, L-ornithine carboxy-lase; EC 4.1.1.17) is the rate-limiting enzyme in the synthesis of polyamines [1]. It is generally thought that polyamines play important regulatory roles in cellular growth [2–4]. Studies have shown that rapidly growing or cancerous tissues have a higher polyamine content [5,6]. Previous works have demonstrated that maximal ODC activity (15~30 units per mg protein) of confluent neuroblastoma cells maintained in fresh growth medium can be induced by fetal calf serum (FCS), cyclic AMP analogs and agents which increase intracellular cyclic AMP content [7–9], and asparagine [9]. However, when neuroblastoma cells are maintained in a salts/glucose medium, only asparagine can elicit a maximal increase in ODC activity; serum factors, hormones and agents which increase intracellular cyclic AMP content are completely ineffective [9]. These results suggest a possible primary function of asparagine in the induction of ODC activity in cultured cells. In view of the fact that ODC activity in cultured cells can be induced by a bewildering number of agents in growth medium [4,6], the use of salts/glucose medium may offer a suitable and simple environment for studying the regulation of ODC activity since it contains no extraneous nutrients such as serum factors, amino acids or vitamines [9].

Mouse neuroblastoma cells grown in tissue culture can be induced to differentiate by many methods including serum deprivation [10], and the addition of Bt_2cAMP (or agents which increase intracellular cyclic AMP content) to the growth medium [11,12]. Prasad and co-workers have shown that the differentiation of neuroblastoma cells is accompanied by a loss of tumorigenicity [13]. Since ODC and polyamines may be involved in cancerous growth, it is of interest to compare the mode of ODC induction in malignant and in differentiated neuroblastoma cells.

The induction of ODC activity of undifferentiated (malignant) and differentiated neuroblastoma cells maintained in salts/glucose medium was studied and compared. Results indicated that the mode of regulation of ODC activity in the differentiated neuroblastoma (ND) cells was different from that of the malignant undifferentiated (NB) cells. In contrast to the essential and sufficient role of asparagine on the induction of ODC activity in NB cells, asparagine was essential but not sufficient to produce maximal increase of ODC activity in ND cells; additional agents such as FCS or Bt_2cAMP was needed to 'potentiate' the effect of asparagine on ODC induction. Possible causes for the difference in mode of regulation of ODC activity of NB and ND cells are discussed.

Abbreviations: FCS, fetal calf serum; cyclic AMP, adenosine 3′,5′-monophosphate; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl cyclic AMP; NB, undifferentiated neuroblastoma; ND, differentiated neuroblastoma; Asn, asparagine; DM, Dulbecco's modified Eagle medium

2. Materials and methods

2.1. *Chemicals*

The following compounds were purchased: L-asparagine, Dulbecco's modified Eagle medium, fetal calf serum and Earle's balanced salt solution from Gibco, Grand Island, NY. N^6,$O^{2'}$-Dibutyryl cyclic AMP (Bt_2cAMP), insulin, L-ornithine from Sigma, St Louis, MO. L-[1-^{14}C]ornithine monohydrochloride, (59 mCi/

mmol) and L-[U-^{14}C]asparagine (105 mCi/mmol) from Amersham, Arlington, I1. L-[4,5-^{3}H(N)]Leucine (56.5 Ci/mmol), [2-^{14}C]uridine (52.4 mCi/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) from New England Nuclear, Boston, MA. All other chemicals were of reagent grade.

2.2. *Cell lines*

Mouse N-18 neuroblastoma cells were obtained from Dr E. S. Canellakis (Yale Univ.). The cells were grown as monolayer cultures in 60 mm Corning plastic dishes in 5 ml of Dulbecco's modified Eagle medium supplemented with 10% FCS and maintained at 37°C in a Forma water jacketed CO_2 incubator (95% air, 5% CO_2). To induce cell differentiation, confluent neuroblastoma cells in 30 ml culture flasks were flushed off the substratum by a stream of medium (10 ml) and the cell suspension was then transferred to 10 volumes of fresh Dulbecco's medium containing 1%, instead of the usual 10%, FCS. Cells were then plated on 60 mm dishes and maintained at 37°C. The average doubling times of cells maintained in 10% and 1% fetal calf serum were 22 h and 32 h, respectively. Full differentiation of neuroblastoma cells was observed 5 days after plating in 1% fetal calf serum. Undifferentiated (malignant) and differentiated N-18 neuroblastoma cells were designated as NB and ND cells respectively.

2.3. *Induction* of ODC activity*

For studying the induction of ODC, NB and ND cells at a stationary phase of growth were used. Cells were rinsed twice and maintained in a salts/glucose medium (Earle's balanced salt solution) [9]. The corresponding sterile salts/glucose medium without Ca^{2+} and Mg^{2+} was used as phosphate buffered saline.

Various testing agents were added, and at designated time intervals cells were harvested. Harvesting was accomplished by rinsing the cells with phosphate buffered saline twice, followed by the addition of 0.7 ml of a solution containing 50 mM Tris/HCl (pH 7.4), 0.1 mM EDTA, 50 μM pyridoxal phosphate and 5 mM dithiothreitol. The cells were scraped off the dish with a rubber policeman, freeze-thawed twice, and centrifuged at 12 000 × g for 5 min. The ODC activity was determined in the supernatant fluid by a method previously described [14]. One unit of ODC activity is defined as 1 nmol CO_2 evolved per h. The protein concentration was measured by a modified Lowry's method with bovine serum albumin as a standard [15].

* The term induction refers to an increase in enzyme activity without regard to the specific mechanism

2.4. *Metabolic and transport studies*

The incorporation of L-[4,5-^{3}H]leucine and [2-^{14}C]-uridine into acid insoluble material were used as indexes of the relative rates of protein synthesis and RNA synthesis, respectively [16].

The transport of asparagine was measured at 37°C of monolayer culture maintained in the salts/glucose medium. The concentration of [^{14}C]asparagine was 0.1 mM with a specific activity of 1 mCi/mmol. Uptake was terminated by washing the cell culture with cold phosphate buffered saline twice. Cells were scraped off the Petri dish, pelleted at 4000 × g for 5 min. Cells were resuspended in phosphate buffered saline, aliquots were used for radioactive counting and protein determination.

3. Results

Undifferentiated (NB) as well as differentiated (ND) stationary-phase neuroblastoma cells remained viable and metabolically active in the salts/glucose medium for more than 24 h. Under this condition, the basal levels of ODC activity of NB and ND cells were less than 0.3 unit/mg protein. In fig.1, the abil-

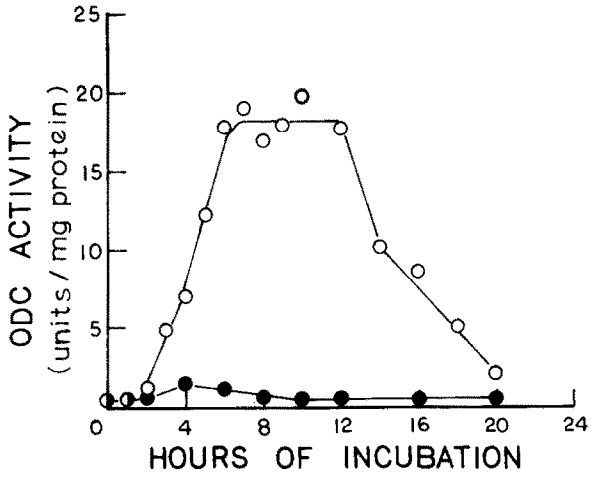

Fig.1. Time-course of the induction of ODC activity by asparagine in the salts/glucose medium. Stationary-phase cultures of NB (○) and ND (●) were rinsed with and then incubated in the salts/glucose medium. ODC activity was induced by 10 mM asparagine.

ity of 10 mM asparagine to induce ODC activity in NB and ND cells, maintained in the salts/glucose medium, was compared. In agreement with previous results [9], 10 mM asparagine produced a marked increase in the ODC activity in NB cells. Maximal activity was achieved at about 7 h after the addition of asparagine. The induced ODC activity remained at this maximal level for another 6 h. In contrast, ODC activity of ND cells was only marginally induced under identical experimental conditions. For both NB and ND cells, the optimal concentration of asparagine needed was 10 mM. Greater concentrations of asparagine did not produce any further increases in ODC activity.

The differences in the ability of asparagine to induce ODC activity in NB and ND cells could be due to: (i) diminished transport of asparagine into ND cells, (ii) alteration in macromolecular synthesis in ND cells, (iii) a defective ODC synthetic machinery of ND cells or (iv) a specific decrease in the sensitivity of the ODC synthetic machinery towards asparagine in the ND cells.

Experiments on transport of asparagine into NB and ND cells actually demonstrate that the uptake of asparagine by ND cells was greater than NB cells (fig.2). Studies on the incorporation of [^{3}H]leucine or [2-^{14}C]-uridine into acid insoluble material failed to reveal significant differences between the NB and the ND cells (figs.3A and 3B). These results suggest that the difference in the asparagine mediated ODC induction

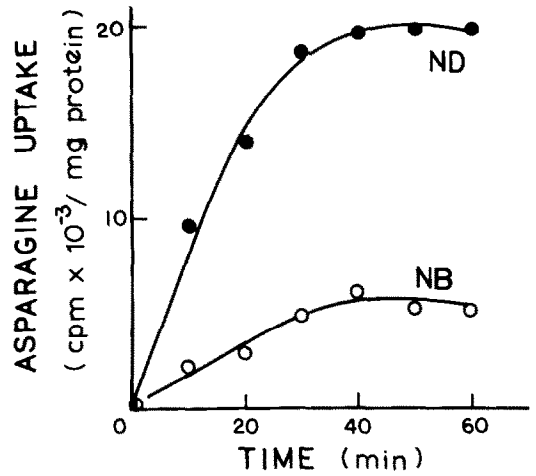

Fig.2. Time-course of uptake of [^{14}C]asparagine by NB (○) and ND (●) cells in the salts/glucose medium. The concentration of [^{14}C]asparagine was 0.1 mM with a specific activity of 1 mCi/mmol of asparagine.

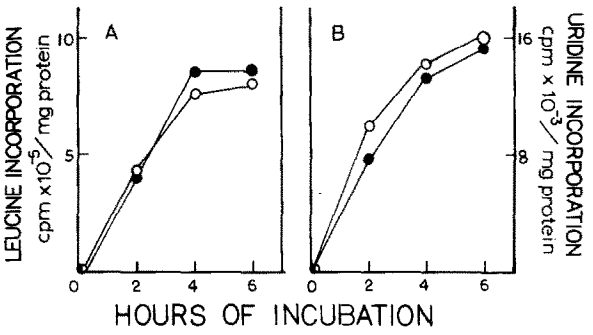

Fig.3. The incorporations of [^{3}H]leucine into protein and [^{14}C]uridine into RNA of NB (○) and ND (●) cells, maintained in the salts/glucose medium, in the presence of 10 mM asparagine. (A) [^{3}H]leucine incorporation; 10 μCi [^{3}H]leucine with a specific activity 56.5 Ci/mmol was added to 4 ml salts/glucose medium containing 10 mM asparagine. (B) [^{3}H]uridine incorporation; 1 μCi [^{14}C]uridine with a specific activity 52.4 mCi/mmol was added to 4 ml salts/glucose medium containing 10 mM asparagine.

in NB and ND cells can be attributed neither to a global difference in asparagine transport nor to gross changes in macromolecular synthesis.

It appears unlikely that the diminished response toward asparagine in ND cells, when compared to NB cells, is attributable to a defect in the ODC-synthetic machinery, as indicated in the following experiments (fig.4). Figure 4 is the time-course of asparagine-induced increase in ODC activity of NB (fig.4A) and ND (fig.4B) cells. For NB cells maintained in the salts/

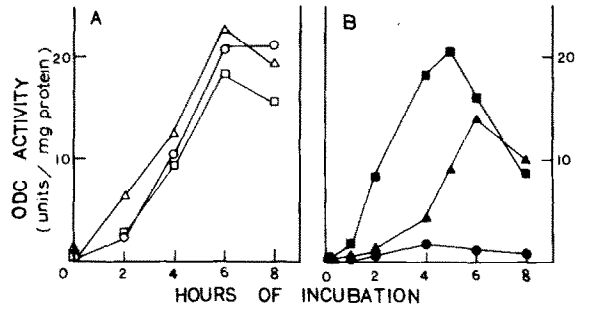

Fig.4. Effects of fetal calf serum and Bt_2cAMP on the asparagine-dependent ODC induction in NB and ND cells maintained in the salts/glucose medium. (A) Time-course of ODC induction in NB cells by 10 mM asparagine (○), 10 mM asparagine plus 10% FCS (□) or 10 mM asparagine plus 1 mM Bt_2cAMP (△). (B) Time-course of ODC induction in ND cells by 10 mM asparagine (●), 10 mM asparagine plus 10% FCS (■) or 10 mM asparagine plus 1 mM Bt_2cAMP (▲).

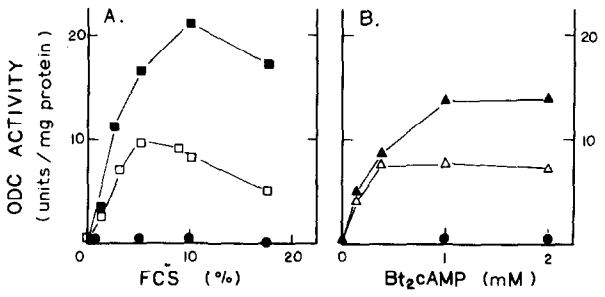

Fig.5. (A) Dose-response curves of the effect of FCS on ODC induction in ND cells. Cells were maintained for 4 h in the salts/glucose medium in the absence of (•), in the presence of 5 mM (□) and in the presence of 10 mM asparagine (■). (B) Dose-response curves of the effect of Bt_2cAMP on ODC induction in ND cells. Cells were maintained in the salts/glucose medium for 5 h in the absence of (•), in the presence of 5 mM (△) and in the presence of 10 mM asparagine (▲).

glucose medium, 10 mM asparagine was sufficient for maximal ODC induction; the addition of FCS or Bt_2cAMP did not result in further increase in ODC activity. This is to be contrasted with results obtained in ND cells, thus asparagine alone produced a small increase in ODC activity; either 10% FCS or 1 mM Bt_2cAMP was necessary to 'potentiate' the effect of asparagine in achieving maximal induction in ODC activity (fig.4B).

Figure 5 shows the induction of ODC activity in ND cells by 0, 5 and 10 mM asparagine as a function of increasing concentrations of FCS and Bt_2cAMP. The results demonstrated (i) that FCS or Bt_2cAMP alone could not induce ODC activity in ND cells maintained in the salts/glucose medium, and (ii) that the optimal concentration, of FCS and Bt_2cAMP in potentiating the effect of asparagine was about 10% (v/v) and 1 mM, respectively.

We have also studied the effects of Bt_2cAMP on asparagine transport, general protein and RNA synthesis. Bt_2cAMP at 1 mM did not increase the transport of asparagine or affect the protein synthesis and RNA synthesis in ND cells maintained in the salts/glucose medium (data not shown). These results further substantiate my contention that the decreased response of ND cells toward asparagine can neither be due to decreased transport of asparagine under the experimental conditions studied nor to an alteration in macromolecular synthesis in ND cells.

4. Discussion

The present study established an additional biochemical parameter, namely the asparagine-dependent ODC induction in the salts/glucose medium, to distinguish the differentiated state from the undifferentiated state of N-18 mouse neuroblastoma cells. In the salts/glucose medium 10 mM asparagine was essential and sufficient to elicit a maximal increase of ODC activity in NB cells, however, asparagine was essential but not sufficient to elicit a maximal increase of ODC activity in ND cells. Agents such as serum factors and Bt_2cAMP were needed to potentiate the effect of asparagine on ODC induction in ND cells.

We have previously shown that the induction of ODC activity by horse serum in L1210 mouse leukemic cells can be inhibited by microtubule-disrupting agents such as colchicine and vinblastine [14]. Similar observations have been made with other cultured cells including N-18 neuroblastoma cells [17,18]. The induction of ODC is also sensitive to a number of plasma membrane perturbing agents such as local anesthetics and ionophores [4,19]. These results prompted us to propose that the regulation of ODC activity in mammalian cells is coupled to the dynamic state of plasma membrane and cytoskeleton structure [4,20].

Changes in the plasma membrane structure and cytoskeleton structure of neuroblastoma cells after differentiation are well documented [21–23]. Neurite outgrowth in ND cells is associated with increase in tubulin and a more organized microtubule structure [23,24].

Results of the present study suggest that the altered ODC response of ND cells toward asparagine could neither be attributed to diminished transport of asparagine, alteration in general macromolecular synthesis, nor to a defective ODC synthetic machinery. The most likely cause would be specific change(s) in the sensitivity of the ODC-synthetic machinery towards asparagine in the ND cells. It is proposed that the sensitivity towards asparagine is related to the dynamic state of the plasma membrane/cytoskeleton structure. In view of the differences in plasma membrane/cytoskeleton structure of NB and ND cells, and the possible involvement of plasma membrane/cytoskeleton structure in ODC regulation, it is likely that the differences in plasma membrane/cytoskeleton structure between NB and ND cells can lead to differences in the mode of regulation of ODC activity in NB and ND cells, as shown in the present study.

Acknowledgements

I wish to thank Ms M. Ruina for her excellent technical assistance. This investigation was supported by Biomedical Research Support Grant PHS RR 7058-13 and by Grant CA 24479, awarded by the National Cancer Institute, DHEW.

References

[1] Pegg, A. F. and William-Ashman, H. G. (1968) Biochem. J. 108, 533–539.
[2] Cohen, S. S. (1971) Introduction to the Polyamines, Prentice Hall, N. J.
[3] Bachrach, U. (1973) Function of Naturally Occurring Polyamines, Academic Press, N.Y.
[4] Canellakis, E. S., Viceps-Madore, D., Kyriakidis, D. A. and Heller, J. S. (1979) Curr. Top. Cell. Regul. 15, 156–202.
[5] Russell, D. H. (ed.) (1973) Polyamines in Normal and Neoplastic Growth, Raven Press, N.Y.
[6] Jänne, J., Pösö, H. and Raina, A. (1977) Biochim. Biophys. Acta 463, 241–293.
[7] Bachrach, U. (1975) Proc. Natl. Acad. Sci. USA 72, 3087–3091.
[8] Bachrach, U. (1976) FEBS Lett. 68, 63–67.
[9] Chen, K. Y. and Canellakis, E. S. (1977) Proc. Natl. Acad. Sci. USA 74, 3791–3795.
[10] Seeds, N. W., Gilman, A. G. and Amano, T. (1973) Proc. Natl. Acad. Sci. USA 70, 160–169.
[11] Prasad, K. N. and Hsie, A. W. (1971) Nature New Biol. 233, 141–142.
[12] Richelson, E. (1973) Nature New Biol. 242, 175–177.
[13] Prasad, K. N. (1972) Cytobios 6, 163–166.
[14] Chen, K. Y., Heller, J. S. and Canellakis, E. S. (1976) Biochem. Biophys. Res. Commun. 68, 401–408.
[15] Ross, E. and Schatz, G. (1973) Anal. Biochem. 54, 304–306.
[16] Chen, K. Y., Tsai, C. M. and Canellakis, E. S. (1974) Cancer Res. 35, 2403–2412.
[17] Gibbs, J. B., Hsu, C. Y., Terasaki, W. L. and Brooker, G. (1980) Proc. Natl. Acad. Sci. USA 77, 995–999.
[18] Chen, K. Y., manuscript in preparation.
[19] Chen, K. Y. and Kyriakidis, D. (1976) Fed. Proc. 36, 2745.
[20] Canellakis, E. S., Heller, J. S., Kyriakidis, D. and Chen, K. Y. (1978) Advan. Polyamine Res. 1, 17–30.
[21] Garvican, G. H. and Brown, G. L. (1977) Eur. J. Biochem. 76, 251–261.
[22] Zisapel, N. and Littauer, U. Z. (1979) Eur. J. Biochem. 95, 51–59.
[23] Seeds, N. W. and Maccioni, R. B. (1978) J. Cell Biol. 76, 547–555.
[24] Chan, K. Y. and Baxter, C. F. (1979) Brain Res. 174, 135–152.