Involvement of Cytoskeletal Structures in Nerve-Growth-Factor-Mediated Induction of Ornithine Decarboxylase

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Induction of ornithine decarboxylase elicited in response to nerve-growth factor in target organs is greatly decreased by preincubation of these tissues with cytoskeletal poisons such as vinblastine, diamide, cytochalasin B and colchicine. These results are interpreted as evidence for the involvement of receptor-associated cytoskeletal structures in mediating the nerve-growth-factor-specific induction of ornithine decarboxylase.

Nerve-growth factor is a unique neuron-directed protein that is known to play a key role throughout the life cycle of noradrenergic neurons of the sympathetic nervous system and during a limited period of embryonic development of dorsal root sensory neurons (Levi-Montalcini, 1966; Levi-Montalcini & Angeletti, 1968). Study of the function of this protein can serve as a worthwhile model system for other unidentified neurotrophic factors for whose presence evidence is accumulating (Giller *et al.*, 1973; Monard *et al.*, 1975; Helfand *et al.*, 1978). Although much information is available on the general as well as the specific biochemical and morphological changes elicited in the target organs in response to nerve-growth factor (Levi-Montalcini & Angeletti, 1971; Varon, 1975), very little information has been obtained about the molecular mechanisms by which nerve-growth factor generates these changes. The nerve-growth-factor-mediated induction of ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17) appears to be one of the earliest and most marked common biochemical changes that is readily observed in the nerve-growth-factor-responsive organs (MacDonnell *et al.*, 1977; Lewis *et al.*, 1978). Ornithine decarboxylase catalyses the conversion of ornithine to putrescine and probably is the first rate-limiting enzyme in polyamine biosynthesis. Induction of ornithine decarboxylase seems to be an essential event in the hormone- and growth-factors-mediated cellular proliferation and macromolecular synthesis after their binding with specific receptors at the cell surfaces of the responsive organs (Stastny & Cohen, 1970; Jänne *et al.*, 1978). The present paper suggests that the cytoskeletal structures (microtubule-microfilament system) associated with the plasma membrane may mediate the nerve-growth-factor-induced increase in ornithine decarboxylase activity in target cells and thus the induction of ornithine decarboxylase is most likely under transmembrane cytoskeletal control.

Experimental

Materials

Nerve-growth factor (2.5S form) was prepared from adult male mouse submaxillary glands by the method of Bocchini & Angeletti (1969). Media and antibiotics were purchased from Grand Island Biological Co., New York, NY, U.S.A. Pyridoxal phosphate and dithiothreitol were obtained from Calbiochem, Spring Valley, NY, U.S.A. Cytochalasin B was a product of Aldrich Chemical Co., Milwaukee, WI, U.S.A., and colchicine was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Vinblastine sulphate and diamide were gifts from Dr. Susil Devare. Lumicolchicine was prepared by the method of Wilson & Friedkin (1967). DL-[1-¹⁴C]Ornithine monohydrochloride (sp. radioactivity 45 mCi/mmol) and Hyamine hydroxide were purchased from New England Nuclear Corp., Boston, MA, U.S.A. and Research Products International Corp., Elk Grove Village, IL, U.S.A. respectively. All other chemicals were of reagent grade.

Methods

Organ culture of superior cervical ganglia. Superior cervical ganglia were removed from 6-day-old rats and decapsulated under a binocular dissection microscope. They were placed (three pairs/well) in 250 μ l of BGJb medium (Biggers *et al.*, 1961) with the Fitton-Jackson modification (Grand Island Biological Co.) lacking Phenol Red. The BGJb medium, the nomenclature used by Biggers *et al.* (1961), is a relatively simple chemically defined medium containing vitamins, amino acids and salts. The medium was supplemented with 0.1% bovine serum albumin, and an antibiotic mixture that included penicillin (100 units/ml) and streptomycin (50 μ g/ml). Nerve-growth factor and drugs were always added in 5 μ l of the medium. The ganglia were maintained at 37°C in tissue-culture clusters in a

humidified atmosphere of O₂/CO₂ (19:1, v/v) for the desired length of time. At the end of incubation the ganglia were removed, rinsed in 2 ml of ice-cold 0.25 M-sucrose and pairs of ganglia were frozen in a ground-glass homogenizer at -20°C overnight. The next day the ganglia were homogenized in 400 µl of 50 mM-Tris/HCl, pH 7.6, containing 5 mM-dithiothreitol and 40 µM-pyridoxal phosphate. The homogenate was centrifuged at 30000g_{av} for 20 min and the supernatant fluid was used for the assay of ornithine decarboxylase.

Organ culture of dorsal root ganglia. Dorsal root ganglia dissected from 8-day-old chick embryo were cultured (70 ganglia/well) in 250 µl of BGJb medium by a procedure similar to the one described above for rat superior cervical ganglia. Nerve-growth factor and drugs were added in 5 µl volumes of the medium. At the end of incubation, the ganglia and the medium were aspirated through a (siliconized) Pasteur pipette and transferred to (siliconized) centrifuge tubes containing 1 ml of ice-cold 0.25 M-sucrose. The tubes were immediately centrifuged at 10000g_{av} for 5 min and the supernatant was carefully decanted. The ganglia were frozen at -20°C in the same tubes until the next day. Homogenizing medium (350 µl; 50 mM-Tris/HCl, pH 7.6, containing 5 mM-dithiothreitol and 40 µM-pyridoxal phosphate) was added to each tube and the contents of the tube were transferred quantitatively to a ground-glass homogenizer by using a (siliconized) Pasteur pipette. The ganglia were homogenized and the homogenate was centrifuged at 30000g_{av} for 20 min. The supernatant was used for enzyme assay.

Assay of ornithine decarboxylase. The activity of ornithine decarboxylase was determined by measuring the formation of ¹⁴CO₂ from DL-[1-¹⁴C]ornithine monohydrochloride as described previously (MacDonnell *et al.*, 1977). The reaction mixture

contained 75 mM-Tris/HCl, pH 7.5, containing 60 µM-pyridoxal phosphate and 111 µM-DL-[1-¹⁴C]ornithine monohydrochloride. After enzyme addition (250 µl, which is equal to 1.25 superior cervical ganglia or 50 dorsal root ganglia), mixtures were incubated at 37°C for 60 min in a total volume of 0.5 ml in rubber-stoppered glass tubes (15 mm × 100 mm) fitted with a hanging centre well that contained 200 µl of Hyamine hydroxide. The reaction was initiated by the addition of radioactive compound. Boiled enzyme solution was used as a control. After the reaction was terminated with 400 µl of 5 M-H₂SO₄ the samples were allowed to stand at room temperature overnight. Next day the centre wells of the reaction tubes were removed and the radioactivity of the trapped ¹⁴CO₂ was measured in 15 ml of scintillation fluid [5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1000 ml of toluene].

The ornithine decarboxylase activity was found to be linear with respect to incubation time under these conditions.

Results

Since previous study (MacDonnell *et al.*, 1977) showed a maximum increase in ornithine decarboxylase activity approx. 6–7 h after the addition of nerve-growth factor to organ cultures of rat superior cervical ganglia, in the present study the tissues were incubated for a period of 6.5 h with or without nerve-growth factor and the various drugs to be studied. As shown in Table 1, addition of nerve-growth factor (5 nM) resulted in an increase of ornithine decarboxylase activity to about 429 and 504% of control in immature rat superior cervical ganglia and chick embryonic dorsal root ganglia respectively. However, the preincubation of tissues for 30 min before the addition of nerve-growth factor

Table 1. *Effect of vinblastine, diamide and cytochalasin B on nerve-growth-factor-mediated ornithine decarboxylase induction in target tissues*

Superior cervical ganglia of 6-day-old rats or dorsal root ganglia obtained from 8-day-old chick embryos were pre-incubated with or without vinblastine sulphate (1 µM), diamide (1 mM) or cytochalasin B (1 µg/ml) for 30 min as described in the text. After the addition of nerve-growth factor (5 nM) the incubation was continued for another 6.5 h. The controls were incubated for a total period of 7 h. The other details are given in the Experimental section. Ornithine decarboxylase activity was expressed as pmol of CO₂ released/h per superior cervical ganglion or pmol of CO₂ released/h per 50 dorsal root ganglia. Each value represents the mean ± S.E.M. for at least 21 superior cervical ganglia or 200 dorsal root ganglia. *Value differs significantly from control value at *P* < 0.001; **value differs significantly from nerve-growth factor-treated value at *P* < 0.01.

Addition to culture	Ornithine decarboxylase activity			
	Superior cervical ganglia		Dorsal root ganglia	
	pmol of CO ₂ /h per ganglia	% of control	pmol of CO ₂ /h per 50 ganglia	% of control
Control	1.54 ± 0.18	100	1.17 ± 0.10	100
Nerve-growth factor	6.61 ± 0.20	429*	5.9 ± 0.22	504*
Nerve-growth factor + vinblastine	3.90 ± 0.10	253**	3.0 ± 0.04	256**
Nerve-growth factor + diamide	3.69 ± 0.11	239**	3.4 ± 0.10	290**
Nerve-growth factor + cytochalasin B	4.0 ± 0.12	259**	2.8 ± 0.16	239**

with vinblastine ($1\mu\text{M}$), a Vinca alkaloid that is known to induce depolymerization of microtubules (Marantz & Shelanski, 1970), decreased the induction of ornithine decarboxylase in response to nerve-growth factor. The involvement of microtubules in nerve-growth-factor-mediated ornithine decarboxylase induction was further confirmed by using diamide (1 mM), a drug shown to inhibit the polymerization as well as to cause disassembly of preformed microtubules (Mellon & Rebhun, 1976; Oliver *et al.*, 1976). The effect of nerve-growth factor on ornithine decarboxylase induction was also found to be decreased when the tissues were preincubated with diamide instead of vinblastine. Since microfilaments have been suggested to act as a link between microtubules and receptors (Edelman, 1976; Cohen, 1977), the role of microfilaments in nerve-growth-factor-mediated ornithine decarboxylase induction was tested by using cytochalasin B as a probe. This fungal metabolite is reported to disrupt the microfilament system at the cell surface (Wessels *et al.*, 1971; Spudich, 1972). Pretreatment of superior cervical ganglia and dorsal root ganglia with cytochalasin B ($1\mu\text{g/ml}$) also resulted in a decreased induction of ornithine decarboxylase in response to externally added nerve-growth factor.

The results presented in Table 2 indicate the effect of preincubation of tissues with colchicine on nerve-growth-factor-mediated ornithine decarboxylase induction. Colchicine is a potent drug that binds to the dimeric subunit of microtubule, tubulin, leading to the inhibition of microtubule assembly (Margolis & Wilson, 1977). The rate of binding of colchicine with tubulin is slow and at low concentration as long as 6–8 h is required to reach equilibrium (Byran, 1972). Accordingly the tissues were preincubated with or without colchicine ($1\mu\text{M}$) for 6 h and then nerve-growth factor was added. It was observed that the preincubation of tissues in BGJb medium alone

increased the basal enzyme activity. The reason for this increase is not clear. However the response to nerve-growth factor was similar to that obtained without preincubation. Colchicine treatment also decreased the nerve-growth-factor-mediated ornithine decarboxylase induction. However lumicolchicine ($1\mu\text{M}$), an isomer of colchicine that has no effect on microtubular assembly (Wilson & Friedkin, 1967), did not decrease the nerve-growth-factor-mediated ornithine decarboxylase induction.

Discussion

A number of peptide hormones and growth factors (Stastny & Cohen, 1970; Jänne *et al.*, 1978) are known to influence a variety of cellular functions after their binding with surface receptors on the responsive cells as the first phase of their action. Neither the nature of these nerve-growth-factor receptors nor the biochemical events after the interaction of nerve-growth factor with its receptor in the plasma membrane of different target tissues is known. However tubulin molecules on the cell surface have been suggested, but not proven, as the receptors for nerve-growth-factor molecules (Levi-Montalcini *et al.*, 1974). Only in noradrenergic sympathetic neurons does evidence exist for the presence of tubulin on the exterior of the cell surface of the membrane (Estridge, 1977). It is not yet understood whether the nerve-growth-factor receptors undergo some redistribution as suggested by studies of other cell-surface receptors (Nicolson, 1975; dePetris, 1975; Edelman, 1976). Changes in lectin agglutinability observed in clonal cell line PC12 (a nerve-growth-factor-responsive cell line) after treatment with nerve-growth factor (Schubert *et al.*, 1978) indirectly lends support for the possibility of redistribution of cell-surface receptors after treatment with nerve-growth factor. However, considerable evidence exists to show that the mobility of surface-membrane receptors and exo- and endo-

Table 2. *Effect of colchicine and lumicolchicine on nerve-growth-factor-mediated ornithine decarboxylase induction in target tissues*

Superior cervical ganglia of 6-day-old rats or dorsal root ganglia of 8-day-old chick embryos were preincubated with or without colchicine ($1\mu\text{M}$) or lumicolchicine ($1\mu\text{M}$) for 6 h as described in the text. Nerve-growth factor (5 nM) was then added and the incubation was continued for another 6.5 h. Ornithine decarboxylase activity was expressed as pmol of CO_2 released/h per superior cervical ganglion or pmol of CO_2 released/h per 50 dorsal root ganglia. Each value represents the mean $\pm$ S.E.M. for at least 18 superior cervical ganglia or 200 dorsal root ganglia. *Value differs significantly from control value at $P < 0.001$; **value differs significantly from nerve-growth-factor value at $P < 0.01$.

Ornithine decarboxylase activity

Addition to culture	Superior cervical ganglia		Dorsal root ganglia	
	pmol of CO_2 /h per ganglion	% of control	pmol of CO_2 /h per 50 ganglia	% of control
Control	3.18 ± 0.20	100	2.9 ± 0.12	100
Nerve-growth factor	12.68 ± 0.12	398*	14.91 ± 0.16	514*
Nerve-growth factor+colchicine	6.97 ± 0.08	219**	8.49 ± 0.06	292**
Nerve-growth factor+lumicolchicine	12.90 ± 0.22	405	15.18 ± 0.18	523

cytosis of receptor-ligand complex may be controlled by microtubule and microfilament systems in the cell cortex (Nicolson, 1975; Edelman *et al.*, 1976). In the present study drugs that are specifically known to interfere with the morphology and function of cytoskeletal structures were used as probes in elucidating the role of these elements in nerve-growth-factor-mediated ornithine decarboxylase induction. Results indicate that the integrity of these cytoskeletal structures is essential in relaying the signal from the cell surface to the interior of the cell. The decrease in ornithine decarboxylase activity in response to nerve-growth factor after incubation of target tissues with cytoskeletal poisons could possibly be due to: (a) lack of correct orientation of nerve-growth-factor-receptor molecules at the cell surface, so that the effective number of available receptors in 'correct' orientation is decreased; this could lead to a decreased number of nerve-growth-factor-receptor complexes entering by endocytosis, which may be a prerequisite for transmitting cell-surface stimuli to the nucleus; (b) shedding (exocytosis) of receptors or nerve-growth-factor-receptor complexes as a result of damage to the cytoskeletal structures of the membrane; and (c) a slow replacement of the lost receptors on the cell surface. On the basis of the present findings, in conjunction with the published reports on ornithine decarboxylase induction in mouse leukaemia cells (Chen *et al.*, 1976) and mouse epidermal tissue (O'Brien *et al.*, 1976) it can be suggested that one of the mechanisms by which ornithine decarboxylase induction is elicited in response to receptor-specific stimuli involves the receptor-associated cytoskeletal structures on the cell surface. However, the precise mechanism needs to be examined further.

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