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NERVE GROWTH FACTOR INDUCED TURNOVER OF PHOSPHATIDYLINOSITOL IN RAT SUPERIOR CERVICAL GANGLIA

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SUMMARY. The addition of nerve growth factor to organ cultures of superior cervical ganglia from immature rats specifically stimulated the incorporation of ^{32}P-orthophosphate into phosphatidylinositol fraction. Equimolar concentrations of other hormones such as insulin, glucagon, thyroxine and growth hormone did not cause any stimulation of the incorporation of ^{14}C-myoinositol into phosphatidylinositol. The stimulation of phosphatidylinositol turnover was observed over a concentration of nerve growth factor ranging from 10^{-10}M to 10^{-7}M. Nerve growth factor specific "inositide effect" was found to be sensitive to nerve growth factor antibody, 2,4-dinitrophenol, a high concentration of bovine growth hormones but not to Actinomycin D. The physiological significance of this finding in relation to nerve growth factor action in this target tissue is discussed.

INTRODUCTION

The growth, development and maintenance of sympathetic neurones of all ages are known to depend on nerve growth factor (NGF). Dorsal root sensory neurones require this protein only for a limited period of embryonic development (1,2). The *in vivo* effects of NGF on its target tissue include enhancement of general cell function (biosynthesis of RNA, protein and lipid, energy metabolism) and specific effects such as induction of tyrosine hydroxylase and dopamine β-hydroxylase in noradrenergic neurones (3). The intracellular requirement of NGF is possibly met by a selective retrograde axonal transport from nerve terminals to perikaryon (4)

Abbreviations: NGF, Nerve Growth Factor; SCG, Superior Cervical Ganglia; PI, Phosphatidylinositol.

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and by way of glial cells which are known to reduce the NGF requirement for the neurones in vitro systems (5). Evidence has accumulated to support the view that these target organs possess distinct surface membrane receptors for NGF as revealed by the existence of high and low affinity binding sites (6,7). However it is not clear whether some or all of pleiotypic responses of NGF are indeed mediated through these cell surface receptors. To date, no information is available on the nature of NGF receptors. Based on the specific interaction between NGF and tubulin, Rita Levi-Montalcini has recently suggested that the tubulin molecules on the membrane surface are likely to be the possible NGF binding sites (receptors) in mediating the chain of events inside the target cell (8).

The present communication deals with the NGF induced stimulation of turnover of the phosphorylinositol group of phosphatidylinositol ('PI effect' or 'Inositide effect') in immature rat SCG. Increased phosphatidylinositol turnover has been implicated in (a) selective ion transport during synaptic transmission, (b) the translocation of macromolecules across the membrane, and (c) as an intrinsic function in the cell surface receptor systems in the amplification of chemical stimuli (9).

MATERIALS AND METHODS

^{32}P-Orthophosphate (carrier-free) was purchased from Bhabha Atomic Research Center, Bombay. Myo[U-^{14}C]inositol (specific activity 225 mCi/mmole) was from Radiochemical Center, Amersham, England. Nerve growth factor, 2.5S form was prepared by the method of Bocchini and Angeletti (10). Antibody to nerve growth factor was purified from rabbit antiserum by the method of Stoeckel et al. (11). Thyroxine, cytochrome C, actinomycin D and 2,4-dinitrophenol were obtained from Sigma Chemical Co. Media and antibiotics were from Grand Biological Co. Bovine growth hormone (NIH-GH-B17bGH) was a gift from NIH. All other chemicals were of reagent grade.

Incorporation of ^{32}P-orthophosphate into individual phospholipids. The extent of incorporation of ^{32}P-orthophosphate into individual phospholipids was studied as follows. Briefly, superior cervical

ganglia from 6 day old rats were removed and decapsulated with the aid of a binocular microscope. The ganglia were cultured in 250 µl of BGJb media, Fitton-Jackson modification, without phenol red. The medium was supplemented with 0.1% bovine serum albumin, Fraction V, and an antibiotic mixture which included penicillin and streptomycin 100 units/ml, 100 µg/ml respectively. Ascorbic acid (0.1 mg/ml) and glutamine (2 mM) were added just before use. Tissues were maintained at 37° in tissue culture clusters in a humidified atmosphere of 95% oxygen and 5% carbon dioxide for the desired length of time. At the end of incubation, the tissues were removed from culture and rinsed with 0.9% saline. Two pairs of ganglia were then homogenized in a ground glass homogenizer with 1.5 ml of chloroform-methanol mixture (2:1 v/v) and transferred to conical centrifuge tubes. The lipid extracts were washed four times with 0.2 volume of 0.9% saline. It was then evaporated to dryness under a stream of nitrogen. 100 µl of chloroform-methanol extract of rat brain synaptosomal lipid (0.5 µmole lipid P) was added as carrier. The tubes were shaken carefully and the contents were quantitatively transferred to thin layer plates coated with 250 µ thick layer of silica gel H. Two dimensional separation of lipids was performed according to the procedure described by Abdel Latif et al. (12). The plates were developed using chloroform/methanol/ammonia (65/25/5, v/v) in the first dimension and n-butanol/acetic acid/water (60/10/10, v/v) in the second. After development, the plates were exposed to iodine vapor to visualize major phospholipids. The radioactive spots were scraped from the plates and counted in 10 ml toluene containing 2.5% (w/v) PPO and 0.05% (w/v) dimethyl POPOP in a liquid scintillation counter.

Incorporation of [U-^{14}C]myoinositol into phosphatidylinositol. For studying the incorporation of ^{14}C-myoinositol into phosphatidylinositol, superior cervical ganglia from 6 day old rats were removed desheathed and maintained in cultures at 37° as indicated above. The various agents to be tested were then added in 5 µl volume of BGJb medium. Ganglia were cultured without preincubation in the presence of ^{14}C-inositol (added in 2 µl volume) for the desired length of time. At the end of incubation, the ganglia were removed and rinsed three times with 2 ml of ice-cold 0.9% saline containing 10^{-4}M unlabelled myoinositol. Then, usually pairs of ganglia were homogenized in a ground glass homogenizer containing 1.5 ml of chloroform-methanol mixture (2:1, v/v) and transferred to conical centrifuge tubes. The lipid extract was then washed four times with 0.2 volume of 0.9% saline containing 10^{-4}M unlabelled myoinositol. Finally the organic layer was transferred quantitatively to scintillation vials and evaporated to dryness under a stream of nitrogen. 15 ml of scintillation cocktail was added before counting.

RESULTS

When ^{32}P-orthophosphate was used as a precursor to study the extent of phosphorus labelling into individual phospholipid fractions, maximum labelling was seen in phosphatidylcholine followed by phosphatidylinositol, phosphatidic acid, phosphatidyl-

Table 1. Effect of nerve growth factor on the incorporation of ^{32}P-orthophosphate into individual phospholipid fractions of rat superior cervical ganglia

Phospholipid	CPM/fraction		% of Control
	Control	NGF	
Phosphatidylcholine	2530±210	2620±195	103
Phosphatidylethanolamine	336±24	314±30	93
Phosphatidylinositol	1300±75	2430±120	186
Phosphatidylserine	292±32	288±26	99
Phosphatidic acid	1010±88	1105±95	109

Three pairs of ganglia (per well) were preincubated in 250 µl of BGJb medium for 60 min. At the end of preincubation, 400 µCi of ^{32}P-orthophosphate (carrier-free) was added. Five minutes after, either 5 µl of buffer or 5 µl of 2.5S NGF (final concentration 10^{-8}M) were introduced into the medium. Incubation was continued for 4 hrs. At the end of incubation the ganglia were removed and processed as described in Material and Methods. Each value represents the mean ± SD of at least four determinations.

ethanolamine and phosphatidylserine during 4 hr incubation period. Addition of NGF (10^{-8}M) specifically stimulated the entry of ^{32}P into phosphatidylinositol by 186% of that of control (Table 1). No change was observed in other phospholipid fractions under the same conditions. As can be seen in Table 2, NGF at 10^{-8}M concentration stimulated the incorporation of [U-^{14}C]myoinositol into phosphatidylinositol when incubation period was prolonged to 10 hr. The increased turnover (about 85%) of phosphatidylinositol in the presence of NGF appears to be a more specific effect, as equimolar concentration of bovine growth hormone, glucagon, insulin or thyroxine failed to bring about any stimulation. Cytochrome C, a basic protein similar in size to NGF, also did not produce any change in the incorporation of ^{14}C-myoinositol into phosphatidylinositol. It is interesting that insulin, which has some structural

Table 2. The effect of various hormones on incorporation of ^{14}C-myoinositol into phosphatidylinositol of rat SCG

Addition to culture	Concentration (M)	CPM/pair of SCG	Change from control %
None	-	600 ± 32	-
NGF	1×10^{-8}	1110 ± 40	+ 85
Growth hormone	1×10^{-8}	660 ± 58	+ 10
Insulin	1×10^{-8}	680 ± 46	+ 13.3
,,	1×10^{-5}	670 ± 66	+ 11.6
Glucagon	1×10^{-8}	674 ± 30	+ 11.2
Thyroxine	1×10^{-8}	590 ± 24	- 1.5
Cytochrome C	1×10^{-8}	610 ± 36	+ 1.5

Three pairs of ganglia (per well) were cultured in 250 µl of BGJb medium containing 0.04 µCi of ^{14}C-myoinositol (specific activity 225 mCi/mmole) with appropriate hormones for 10 hr. At the end of incubation, the ganglia were removed, washed and processed as described in Material and Methods. Values represent the mean ± SD of three determinations.

similarity to NGF (13) even at a higher concentration effected only an 11% stimulation like the other hormones tested. NGF specific turnover could be prevented by the addition of purified NGF-antibody but not by actinomycin D (Table 3). 2,4-Dinitrophenol, a mitochondrial uncoupler of oxidative phosphorylation was also found to be inhibitory. High concentration of bovine growth hormone, which is a moderate inhibitor of NGF binding to membrane fractions of rabbit sympathetic ganglia (7) brought down the stimulatory effect of NGF from 81% to 46%. The effect of various concentrations of NGF on phosphatidylinositol turnover is given in Fig. 1. It is evident that only 25% stimulation is observed at low concentration (10^{-10} M) compared to 84 to 92% at higher concentration (10^{-7}M) used here.

Table 3. Effect of various inhibitors on NGF specific stimulation of phosphatidylinositol turnover in rat SCG

Addition to culture	Concentration µg/ml	CPM/pair of ganglia	Change from control %
None	-	270 ± 20	-
NGF	0.250	490 ± 28	+ 81
NGF + NGF antibody	0.250 + 200	283 ± 34	+ 5
NGF + Actinomycin D	0.250 + 200	495 ± 27	+ 83
NGF + 2,4-Dinitrophenol	0.250 + 500	195 ± 35	- 28
NGF + Bovine Growth Hormone	0.250 + 250	395 ± 21	+ 46

Three pairs of ganglia (per well) were cultured in 250 µl of BGJb medium containing 0.025 µCi of ^{14}C-inositol (specific activity 225 mCi/mmol). The inhibitors and/or NGF were added simultaneously in 5 µl volume at the beginning of the experiment. The tissues were cultured for 10 hr. At the end, the ganglia were removed, washed and processed as described in Material and Methods. Values represent the mean ± SD of three determinations.

DISCUSSION

In presence of nerve growth factor a selective stimulation of ^{32}P-orthophosphate incorporation into phosphatidylinositol was observed. The NGF mediated stimulation of phosphatidylinositol turnover (breakdown followed by its resynthesis) was further confirmed by using ^{14}C-myoinositol. The specificity of the effect was demonstrated in that the other hormones did not elicit any significant change in the PI fraction. Furthermore the observed stimulation of PI fraction could be inhibited by the simultaneous addition of purified NGF antibody. Addition of 2,4-dinitrophenol but not actinomycin D, inhibited the stimulation. High concentration of bovine growth hormone, which inhibits the binding of NGF to membrane

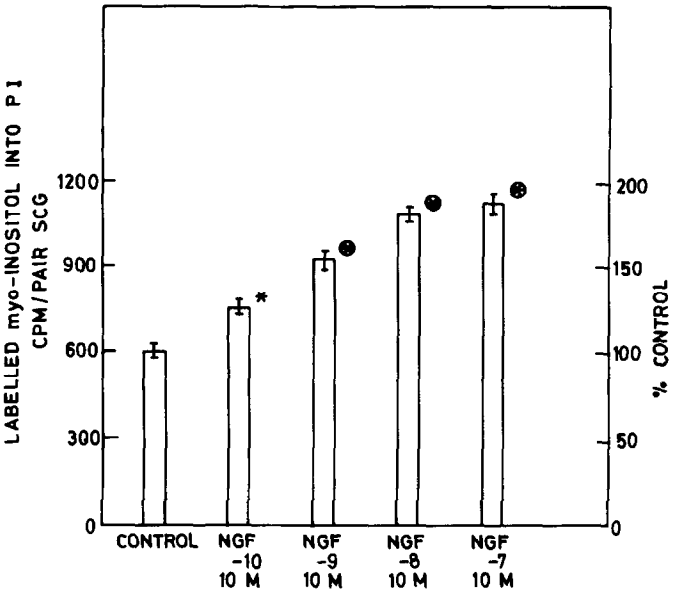

Fig. 1. Effect of various concentrations of NGF on incorporation of labelled inositol into phosphatidylinositol of rat SCG. Three pairs of superior cervical ganglia were cultured in 250 µl of BGJb medium in the presence of various concentrations of NGF (10^{-10}M to 10^{-7}M) and 0.04 µCi [U-^{14}C]myo-inositol (225 mCi/mmole) for 10 hr as described in Material and Methods. Each bar represents the mean ± SD of 12 pairs of SCG. * $p < 0.05$; ● $p < 0.01$.

preparations of rabbit sympathetic ganglia, also decreased the turnover of PI.(7).

In rat sympathetic ganglia Larrabee et al. (14) have shown an "inositide effect" by an externally applied electrical stimulation of preganglionic nerves and concluded it to be normal in situ response to physiological activity. Halstead and Larrabee observed a synaptic blockade of ganglionic transmission as an earliest event following NGF antiserum administration to young animals (15). Thus it is tempting to speculate that the observed "inositide effect" reported in the present paper could possibly be related to synaptic transmission per se. However, hormones or secretogogue are also known to produce an "inositide effect" and Hokin who

observed this phenomenon linked it with intracellular transmembrane movement of proteins (16). Evidence has accumulated to indicate that NGF or erythrocytes passively coated with NGF can be internalized by cells (17,18,8). Yet another demonstration is the association of two key enzymes involved in recycling of membrane phosphoinositides with rat brain tubulin (19,20). Thus it is likely that the increased phosphatidylinositol breakdown in response to NGF may represent its interaction with tubulin on the cell surface. Experiments are in progress to examine the physiological significance of NGF specific "inositide effect" in terms of its relevance to (a) synaptic transmission, (b) translocation of proteins across membranes, and (c) an initial event on NGF receptor sites at the cell surface leading to the amplification of chemical stimuli.

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