Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7797–7800, December 1981
Neurobiology

Nerve growth factor stimulates phospholipid methylation in growing neurites

(axonal growth/membrane biogenesis/axonal guidance/sympathetic neurons)

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Communicated by Eric R. Kandel, August 4, 1981

ABSTRACT **Cultures of neurons from rat superior cervical ganglia were deprived of nerve growth factor, loaded with [*methyl*-^{3}H]methionine, and then challenged with nerve growth factor for different periods of time. Growing neurites and perikarya were separated microsurgically and extracted with chloroform/methanol. Lipid-incorporated radioactivity in the extracts was measured and expressed on the basis of the amount of phospholipid present. The methylated species in the neurite fraction were identified by thin-layer chromatography as mono-, di-, and trimethylphosphatidylethanolamine (phosphatidylcholine). Furthermore, a small peak of lysophosphatidylcholine was detected. In the neurites, but not in the perikarya, phospholipid methylation was found to reach a peak at 10 sec after onset of stimulation. Stimulated levels were at least 4 times higher than levels of unstimulated controls. The peak was followed by rapid decline of phospholipid-incorporated radioactivity. Our result indicates that phospholipid methylation is part of a nerve-growth-factor-activated secondary messenger system in growing sympathetic neurites. The potential significance of this conclusion for directed neuritic growth and membrane expansion is discussed.**

The interaction of nerve growth factor (NGF) with cell surface receptors (1–4) stimulates various synthesis functions as well as neuritic outgrowth in neurons of the peripheral nervous system (5). We have to postulate that NGF–receptor interaction is signaled to the cell interior via one or more secondary messenger systems. However, the search for such systems—e.g., for NGF-dependent protein kinase activity or changes in cyclic nucleotide metabolism (6–11)—has not produced entirely satisfactory results so far. New perspectives have been generated by the studies of Axelrod and collaborators (12–14) who have identified receptor-activated phospholipid methyltransferases as an important link in the control of numerous cellular functions. These include leukocyte chemotaxis and histamine release from mast cells, phenomena that are of particular interest in regard to neuritic growth. Analogies may exist between leukocyte migration toward an inflammatory site and advance of a nerve growth cone toward an appropriate target area and between the potentially "exocytotic" addition of plasmalemmal precursor to the growth cone membrane (15) and the release of secretory granules. Therefore, we have investigated phospholipid methyltransferase activity in the growing neurite as a function of exposure to NGF.

METHODS

Many of the methods used for the present studies are modifications of techniques described by Axelrod and collaborators (13). Small pieces of superior cervical ganglia, explanted from late gestation rat fetus (Sprague–Dawley), were grown *in vitro* for 4 days in Leibovitz's medium (L15; GIBCO), containing glucose at 9 mg/ml, 10% human placental serum, and, as mitotic inhibitors, 10 μM 5-fluorodeoxyuridine and 10 μM cytosine-1-β-D-arabinofuranoside (Sigma). The medium also contained an experimentally determined, saturating level of NGF (0.4 μg/ml). NGF was a 2.5S preparation from male mouse submaxillary glands (16). Each experimental point consisted of the pooled material from a total of 36 explants grown in three collagen-coated dishes.

On the fifth day *in vitro*, the cultures were deprived of NGF by incubation in L15 medium with 1% bovine serum albumin (fraction V; GIBCO) and glucose at 9 mg/ml for a total of 95 min (Fig. 1). During the second half of the NGF deprivation period, the cultures were exposed to 150–200 μCi of L-[*methyl*-^{3}H]methionine (80 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) per ml in the same medium. Following two washes in NGF-free medium without radioisotope but with 1 mM L-methionine (Sigma) for a total of 5 min, the cultures were challenged with a saturating concentration of NGF in the same L15/albumin medium.

In the first experiments, the medium was simply replaced; in later experiments, greater differences between NGF stimulation and controls were obtained by infusing into a stationary culture dish (containing approximately 1.0 ml of medium) 0.5 ml of a 10-fold saturating NGF concentration (4 μg/ml) in L15/albumin medium. This was accomplished by using a manifold with 12 fine tubes evenly distributed over the area of a culture dish. At 5 sec to 5 min after onset of the NGF challenge, a large excess of 10% ice-cold trichloroacetic acid containing 50 mM L-methionine was added to the cultures. Controls were carried out by "challenging" the cultures with medium devoid of NGF or by adding trichloroacetic acid without challenge immediately following the 5-min rinsing period. Furthermore, the same experiments were executed on cultures whose neuronal perikarya (explants) had been removed microsurgically on the preceding day so that the dishes contained only degenerated neurites and a few supporting cells. All these experimental procedures were carried out in a constant-temperature room at 37°C.

The trichloroacetic acid/methionine mixture added to the challenged cultures was changed twice, and the explants containing the neuronal perikarya and supporting cells were carefully excised (leaving neuritic outgrowth intact) and collected for chloroform/methanol extraction. The remaining halos of fiber outgrowth, still attached to the collagen, were then scraped

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Abbreviations: NGF, nerve growth factor; PtdEtn, phosphatidylethanolamine; PtdMeEtn, phosphatidylmonomethylethanolamine; PtdMe$_2$Etn, phosphatidyldimethylethanolamine; PtdCho, phosphatidylcholine.

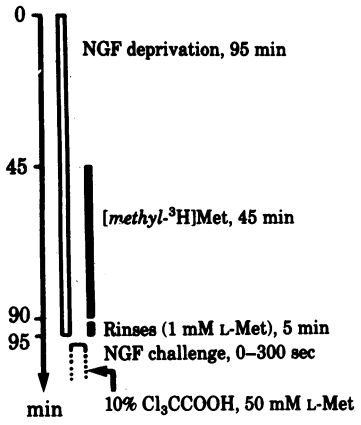

FIG. 1. Flow diagram of experimental procedures. After the reaction was stopped with 10% trichloroacetic acid, perikarya and neurites were separated microsurgically and extracted with chloroform/methanol. For further detail, see text.

from the bottom of the dishes and collected for separate extraction. The two preparations, referred to as perikaryal and neurite fraction, respectively, were again extensively washed with ice-cold trichloroacetic acid/methionine (three times) and then extracted with chloroform/methanol, 2:1 (vol/vol). Lipid phases, generated by the addition of water and chloroform, were equilibrated once more with fresh "upper phase" fluid and their surfaces were rinsed. Radioactivity in the last acid wash and in the last "upper phase" fluid was well below 5% of total radioactivity per sample.

The lower phase was divided for liquid scintillation counting and for quantitation of phospholipid by measurement of inorganic phosphate according to a modification of the method of Ames and Dubin (17). Radioactivity was expressed as cpm per μg of phospholipid and normalized so that the highest level of activity in each set of time points equalled 100. Normalization included both neuritic and perikaryal fractions. The normalized values for each point were averaged and the SEM were calculated.

Phospholipid extracts from cultures challenged with NGF were analyzed on silica gel-impregnated glass fiber strips ("Instant Thin-Layer Chromatography" strips; type SA, Gelman) or on silica gel G chromatography plates. Before they were spotted, extracts of the neuritic fraction were mixed with phosphatidylethanolamine (PtdEtn), phosphatidyldimethylethanolamine ($PtdMe_2Etn$), and phosphatidylcholine (PtdCho) (Sigma) and dried to a volume of about 10 μl under a stream of nitrogen.

For optimal separation of phospholipid from nonlipid components, the glass fiber strips were developed with chloroform/methanol/water, 60:30:4 (vol/vol), as a first solvent and, after drying, with 1% sodium borate in water for a second run past the organic solvent front (18). As an alternative, boric acid was also run in the opposite direction, past the origin. The strips were then cut into 1-cm segments and assayed for radioactivity in a liquid scintillation counter.

For optimal resolution of phospholipid species, extracts were spotted on silica gel G plates and developed with chloroform/methanol/water, 70:30:4 (vol/vol). The lanes were divided into segments, and the gel was scraped off and collected in scintillation vials for assay. Collagen-coated but tissue-free culture dishes were treated exactly as the experimental material and served as blanks; extracts of these were run in parallel in all separations. The cpm values in these blanks were near background and were subtracted from the corresponding cpm of experimental materials. The net cpm values were then plotted as a function of distance of migration.

RESULTS

Phospholipid Methylation in Neuronal Perikarya. Low levels of phospholipid-incorporated radioactivity, approximately one-third of the amount found in the unstimulated neurite fraction (see below), were detected in neuronal perikarya and supporting cells forming the explants (Fig. 2A). This radioactivity averaged approximately 990 dpm/μg of phospholipid, in a mean total of 6.8 μg of phospholipid per assayed fraction. Specific radioactivity remained unchanged during NGF challenge of 5 sec to 5 min.

Methylation of Neuritic Phospholipids. In order to ascertain that methylating activity found in the neuritic outgrowth was not due to contamination of that fraction with supporting cells (a few of these cells are always found between the fibers), some cultures were subjected to methyltransferase assays after microsurgical removal of neuronal perikarya on the day prior to the experiment. Twenty-four hours later, only remainders of degenerated neurites were visible. In these experiments, specific radioactivities of phospholipid were found to be low, approximately at the level of that of the perikaryal fraction (Fig. 2B). Furthermore, NGF challenge did not influence these values.

In another set of control experiments, intact cultures were

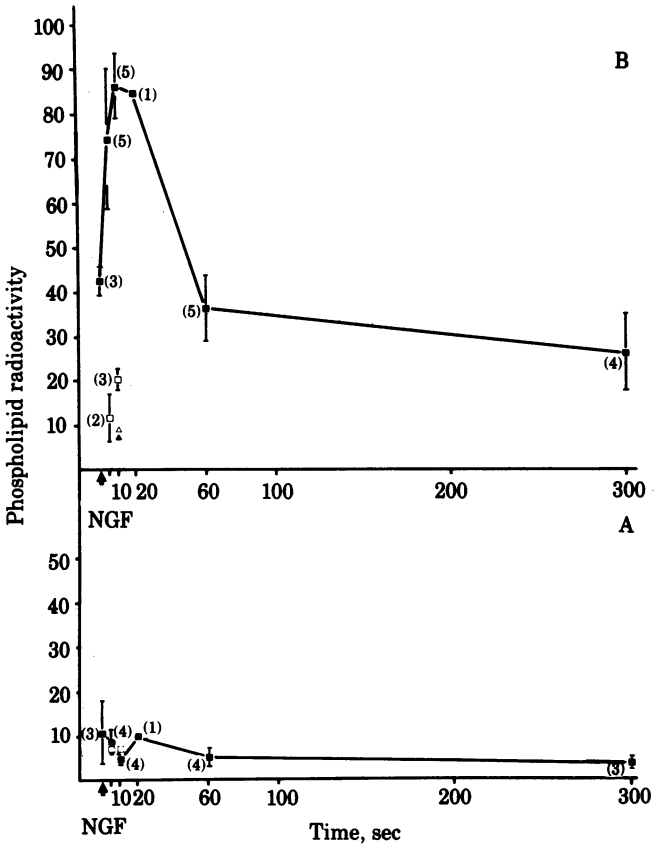

FIG. 2. Phospholipid-incorporated radioactivity (normalized arbitrary units) after NGF challenge. (A) Perikaryal fraction. Regardless of the duration of the challenge or of the presence or absence of NGF, specific radioactivity of phospholipid remained uniformly low. (B) Neurite fraction. Challenge without NGF did not increase lipid-incorporated radioactivity but NGF stimulation led to a rapid and dramatic increase in specific radioactivity. The 10-sec peak was followed by rapid decline in radioactivity. The peak in the neurite fraction corresponds to approximately 28,000 dpm/μg phospholipid. Each point is the mean ± SEM of the number of experiments indicated in parentheses. ■, Challenge with NGF; □, challenge without NGF; ▲ and △, assay of degenerating fibers with and without NGF, respectively.

challenged with medium lacking NGF. Initially, considerable difficulties were encountered because the mere medium change was found to increase phospholipid-incorporated radioactivity considerably in the 5-sec and 10-sec experiments. However, when NGF-free medium was gently and evenly infused into stationary culture dishes, specific radioactivity of phospholipid remained at base-line levels for challenge durations from 5 sec to 5 min (Fig. 2B). In contrast, challenge with saturating concentration of NGF produced a sharp increase in specific radioactivity, reaching a peak at approximately 10 sec after onset of the challenge. This peak, averaging approximately 28,000 dpm/μg of phospholipid, represents an increase in methylating activity of at least 3- to 4-fold compared with base-line levels in the neurite fraction, and of 12- to 28-fold compared with the amounts found in the NGF-stimulated perikaryal fraction. Total phospholipid in the neurite fractions averaged 4.3 μg. The methylation peak was followed by rapid decline in phospholipid-incorporated radioactivity. At 1 min after onset of NGF stimulation, the radioactivity had returned nearly to base-line levels. It continued to decrease slightly during the subsequent 4 min. At 0 sec (no challenge), lipid-incorporated radioactivity was somewhat higher than at 60 or 300 sec. This probably was due to disturbance of the cultures during the preceding rinses.

Nature of Radioactive Compounds. Chloroform/methanol extracts of neurites challenged with NGF for 7 or 15 sec were chromatographed on silica gel-impregnated glass fiber strips. A control experiment, challenge for 15 sec without NGF, was also included in the analysis. Whether boric acid was run in the same or in the opposite direction of organic solvent development, the following picture emerged. A broad double peak near the organic solvent front comprising the phospholipids (mono-, di-, and trimethyl derivatives of PtdEtn and lyso-PtdCho) contained 92–98% of all counts (Table 1). By contrast, only little radioactivity was found at the origin or the boric acid front, where nonlipid components would be located. In the 7-sec experiments, the first peak (containing methylated forms of PtdEtn) was larger and the lyso-PtdCho peak was smaller than the respective values for the neurites that were exposed to NGF longer or that served as control.

In order to resolve the radioactive phospholipid species in greater detail, extracts of neurites challenged with NGF for 7 or 15 sec were analyzed on silica gel G plates. PtdEtn, directly followed by phosphatidylmonomethylethanolamine (PtdMeEtn), ran very close to the organic solvent front and was clearly separated from PtdMe$_2$Etn (Fig. 3). A third and a small fourth peak were formed by PtdCho and lyso-PtdCho, respectively. PtdMeEtn, PtdMe$_2$Etn, and PtdCho were labeled in a time-dependent manner: after 7 sec of NGF exposure, the relative amounts of phospholipid-incorporated radioactivity were 17.0% for the PtdMeEtn peak, 52.8% for PtdMe$_2$Etn, and 18.6% for PtdCho; at 15 sec, the radioactivity was shifted to the more highly methylated compounds—13.9% in PtdMeEtn, 51.1% in PtdMe$_2$Etn, and 25.1% in PtdCho. As in the glass fiber strip analysis described above, labeling of lyso-PtdCho was also found—11.6% at 7 sec and 9.9% at 15 sec. Although better time resolution could not be achieved in this experimental system, stepwise methylation of PtdEtn to PtdCho (19) and subsequent degradation to lyso-PtdCho by phospholipase A$_2$ are evident.

Table 1. Separation of lipid and nonlipid components on silica-gel-impregnated glass fiber

	NGF exposure		Control*
	7 sec	15 sec	15 sec
Total cpm placed on strip	1796	2343	1698
Total phospholipid, %	98	95	92
PtdEtn derivatives†	78	71	59
Lyso-PtdCho	14	20	23
Between peaks	6	4	10
Water-soluble material‡	0.1	0.5	1.3
Origin	0.3	0.6	2.8
Balance§	1.6	3.9	3.9

The support was developed with chloroform/methanol/water, 60:30:4 (vol/vol), and subsequently with 1% sodium borate in water in the opposite direction, past the origin. Results are shown as net cpm in fraction as percentage of total net cpm in chromatogram.
* Challenge with medium not containing NGF.
† Mono-, di-, and trimethyl derivatives.
‡ At boric acid front.
§ Unidentified cpm between peaks.

DISCUSSION

These experiments show that NGF leads to a transient but massive increase in phospholipid methylation in the distal portion of growing neurites from rat sympathetic neurons. The various control experiments—challenges without NGF, assay of degenerated neuritic outgrowth, and chromatography of the chloroform/methanol extract—as well as separate analysis of perikarya and neurites unambiguously support this conclusion. Our initial difficulties with nonspecifically increased levels of lipid-incorporated radioactivity immediately after medium change may be explained by partial detachment of the very sensitive growth cones from the culture substratum caused by fluid currents. Improved handling of the cultures has resolved this problem.

Our data therefore lead to the identification, in a sympathetic neuron, of a secondary messenger system activated by NGF and

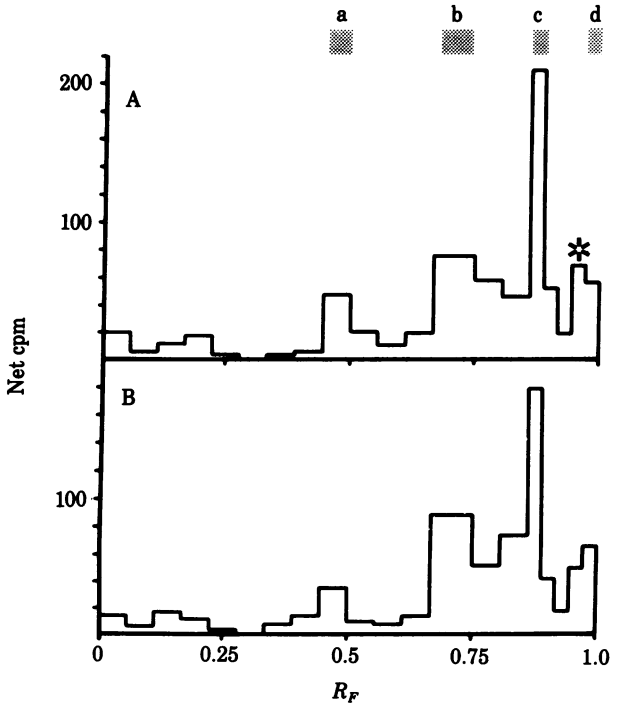

FIG. 3. Identification of methylated species 7 sec (A) and 15 sec (B) after NGF challenge. Silica gel G plates were developed with chloroform/methanol/water, 70:30:4 (vol/vol). The solvent front migrated about 180 mm. Total net radioactivity spotted onto the plates amounted to 740 cpm and 709 cpm in A and B, respectively. PtdMeEtn migrated immediately behind PtdEtn (asterisk). Lipid standards: a, lyso-PtdCho; b, PtdCho; c, PtdMe$_2$Etn; d, PtdEtn.

localize it to the distal portion of the growing neurite, probably the growth cone. This is also known to be the location of receptors that mediate the NGF stimulation of sprouting (20). The rapid time course of the NGF effect and the absence of increased methylation in the perikarya exclude the participation of perikaryal elements in the generation of the initial cellular response to NGF. These observations are of great functional significance because phospholipid methylation, as proposed by Axelrod and collaborators, appears to stimulate calcium entry and intracellular release of arachidonic acid (14). Thus, increase of intracellular calcium and arachidonic acid may be further steps in the signaling of the NGF–receptor interaction on the cell surface to cytoplasmic elements of the growth cone, neurite, and perikaryon. Indeed, the rapid appearance of tritiated lyso-PtdCho in our experiments suggests the presence of phospholipase A_2 near the methylation site.

The corollary that NGF-stimulated phospholipid methylation may increase intracellular calcium levels is consistent with earlier reports on NGF-mediated calcium entry into sprouting PC 12 cells (ref. 9, but see ref. 10) and with the turning response of growth cones toward a NGF source (21–23) or a calcium source [the latter in the presence of a calcium ionophore (23)]. Furthermore, concentration of calcium channels in distal regions of the growing neurite has been demonstrated (24–26).

The conclusions derived from the present study are of particular interest with regard to plasmalemmal expansion during neuritic growth. It has been shown that the insertion of new membrane components takes place predominantly at the nerve growth cone (ref. 15; cf. refs. 27 and 28) and that this insertion may occur by fusion of plasmalemmal precursor vesicles with the cell surface (29). The studies suggesting chemotaxis-like response of growing neurites of the peripheral nervous system to NGF (21, 22), especially Gundersen and Barrett's work (23) on the turning response of nerve growth cones, are also highly relevant.

In the light of these studies and data presented here, we propose the following hypothesis of the molecular mechanism controlling vectorial growth of axons: NGF may directly stimulate localized membrane addition to the cell surface by activating phospholipid methylation, thereby increasing calcium entry into the growth cone through the calcium channels (24–26). Locally increased calcium levels would subsequently stimulate phospholipase A_2 as proposed by Hirata and Axelrod (14). Lysolecithin (lyso-PtdCho) generated by this enzyme may then trigger fusion of plasmalemmal precursor vesicles with the cell surface (cf. ref. 30) and, therefore, exocytosis-like incorporation of new membrane components into the plasmalemma. If it were highly sensitive to NGF levels, such a system could lead to vectorial expansion of the plasma membrane. Together with appropriate changes and rearrangements of contractile elements, this could explain the turning response of nerve growth cones toward an NGF source. Similar mechanisms of directed neuritic growth could be postulated for neurons of the central nervous system (cf. ref. 24) involving stimulation of phospholipid methyltransferases by unknown NGF-like hormones.

Note Added in Proof. Heumann *et al.* (31) recently demonstrated that NGF can elicit a neuronal response only upon interaction with its receptor on the outer surface of the plasmalemma. This supports the concept of the existence of an NGF-activated secondary messenger system.

The skillful assistance of Linda B. Friedman and Kathy Silberman in the preparation of this manuscript is gratefully acknowledged. This research was supported by Grant BNS 14071 from the National Science Foundation.

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