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INCREASED PHOSPHORYLATION OF A SPECIFIC NUCLEAR PROTEIN IN RAT SUPERIOR CERVICAL GANGLIA IN RESPONSE TO NERVE GROWTH FACTOR

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SUMMARY. The addition of nerve growth factor to the media of cultures of sympathetic ganglia produces an increase in the phosphorylation of a specific nuclear protein. Similar data are obtained when nerve growth factor is administered *in vivo*. A comparable effect is produced by analogs of cyclic AMP.

One of the best characterized effects of nerve growth factor in sympathetic ganglia is its ability to increase the activity and, indeed, the differential rate of synthesis of tyrosine hydroxylase (1-4). This induction appears to involve an increase in the synthesis of the messenger RNA for tyrosine hydroxylase (4). Presumably such a specific change in the rate of RNA synthesis is preceeded by some change in the nuclear proteins regulating the rate of the transcription.

Recently we have shown that nerve growth factor produces a rapid increase in cAMP concentration in the ganglia (5) and others have reported the presence of nuclear receptors for nerve growth factor (6). Which of these routes for the action of nerve growth factor is directly related to the induction of tyrosine hydroxylase in this system is not known. The findings do suggest that nuclear changes are likely to occur in response to nerve growth factor, and the cAMP data indicate that among them might be a phosphorylation.

Accordingly, we have investigated the pattern of nuclear phosphorylation in superior cervical ganglia in the presence and absence of nerve growth factor. In this paper we present evidence that nerve growth factor increases the phosphorylation of a specific nuclear protein both *in vivo* and *in vitro* and that this effect is mimicked by derivatives of cAMP, but not by other hormones.

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MATERIALS AND METHODS Two different protocols were used in this study. The in vitro experiments were done with ganglia from 5-8 day old rats incubated in 0.3 ml of BGJ_b medium as previously described (2). The ganglia were cultured with or without nerve growth factor, cAMP derivatives, or other agents for 4 hours. During the last hour of the incubation (^{32}P) KH_2PO_4, (670 μCi per ml incubation medium, 1000 mCi per mmole, New England Nuclear) was added. For in vivo studies, nerve growth factor, 10 μg per gram body weight, or buffer was administered subcutaneously. After 6 hours, the ganglia were removed and placed in culture for 1 hour with (^{32}P)inorganic phosphate.

Whichever protocol was used the ganglia were removed in groups of 10 and rinsed twice with 5 ml of 0.32 M sucrose containing 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM sodium phosphate, pH 6.5 (Buffer A). They were then homogenized in 1 ml of buffer A. The homogenates were centrifuged at 800 x g for 10 min, the pellet resuspended in 1 ml of Buffer A containing 0.2% Triton X-100, and the centrifugation repeated. The pellet was suspended once again in 1 ml of Buffer A and the suspension was layered on a 3.9 ml discontinuous sucrose gradient composed of 1.3 ml portions of 0.8 M, 1.2 M, and 2.4 M sucrose, all brought to the ionic composition of Buffer A, and centrifuged at 58,400 x g for 1 hour. The nuclear pellet was then prepared for gel electrophoresis.

Each nuclear pellet was suspended in 150 μl of SDS sample buffer (0.0625 M Tris·HCl, pH 6.8, containing 10% glycerol, 5% β-mercaptoethanol, and 2.3% SDS) and the suspensions heated for 10 min at 90°. The samples were centrifuged, counted, and portions of the supernatant fractions were placed in sample wells of a 10% SDS-polyacrylamide slab gel. The gel was run in SDS running buffer (0.025 M Tris base, 0.19 M glycine, and 0.1% SDS) at 100 volts for 3 hours. Appropriate standards were placed in adjacent wells. After the electrophoresis, the gels were removed, stained for 15 hours with 0.5% Coomassie blue in 45% methanol and 9% acetic acid and destained in 40% ethanol-7% acetic acid followed by 7% acetic acid. The gels were dried and then exposed to X-ray film for 15 hours or more. The films were developed and fixed by standard techniques and scanned with a densitometer.

Nerve growth factor in the 2.5 S form was prepared according to the method of Bocchini and Angeletti (7). Rats were obtained from Zivic-Miller (Allison Park, Pennsylvania). Insulin, dibutyryl cAMP, and 8-bromo-dibutyryl cAMP were purchased from Sigma Chemical Co. Glucagon was supplied by Eli Lilly Laboratories.

RESULTS AND DISCUSSION The addition of nerve growth factor to the media of cultures of ganglia from young rats markedly increased the amount of radioactive phosphate found in one of the bands on the autoradiogram (Fig. 1). Some increase in the incorporation into this peak was seen with as little as 30 ng of nerve growth factor per ml, and a maximal effect was apparent at 300 ng per ml. The material represented by this band has a molecular weight of about 30,000 and migrates in this system to about the same position as does f_1 histone. The band appears to represent a protein since it was not altered by prior treatment of the sample with RNase or DNase, but disappeared upon treatment with Pronase.

A similar increase in the phosphorylation of this protein could be obtained by injecting nerve growth factor into the animals and then culturing the ganglia

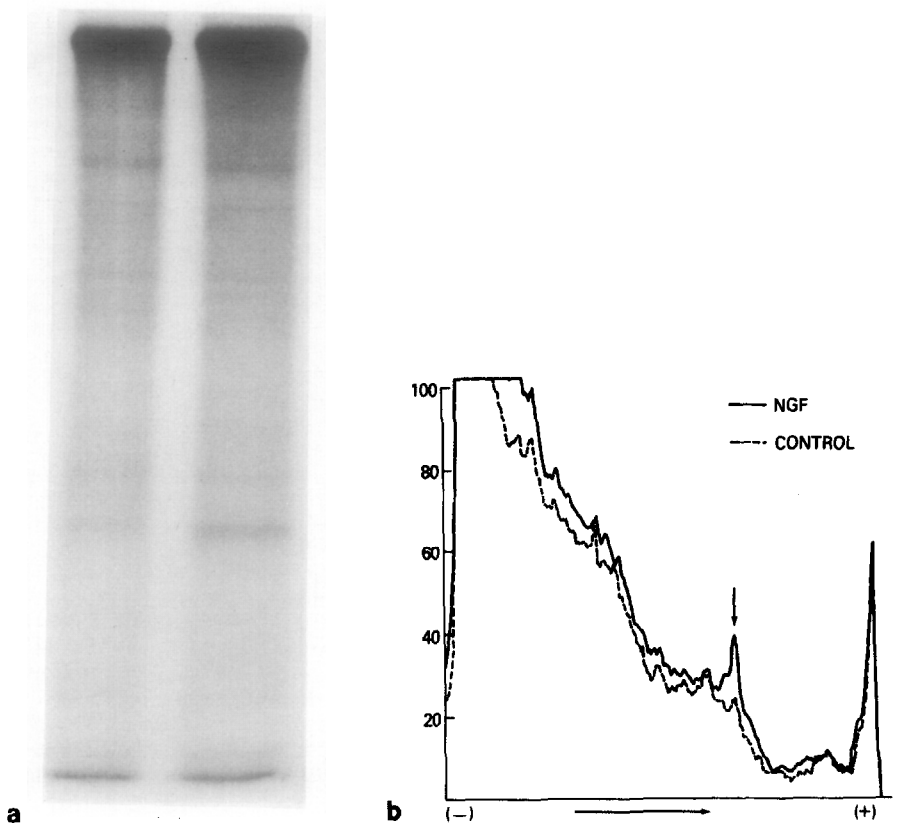

Fig. 1a. The phosphorylation of nuclear proteins from rat superior cervical ganglia cultured in the absence (left) or in the presence (right) of 2.5 S nerve growth factor (300 ng/ml) for 4 hours. Radioactive phosphate (670 µCi per ml culture medium) was added after 3 hours. Thirty-six thousand cpm (40 µl) of each nuclear extract was applied.
Fig. 1b. Densitometer tracing.

in the presence of radioactive phosphate (Fig. 2).

The effect of nerve growth factor in vitro is quite specific in that neither insulin nor glucagon produced an increase in the radioactivity in the band in the f_1 region (Fig. 3). It is clear, however, that the effect of nerve growth factor on the 30,000 dalton species can be mimicked to some extent by 1 mM 8-bromo-cAMP (Fig. 3). The addition of dibutyryl cAMP also produced an increase in the relevant band (data not shown).

In addition to the increased synthesis of tyrosine hydroxylase (4) produced in ganglia by nerve growth factor, we have also observed an induction of ornithine

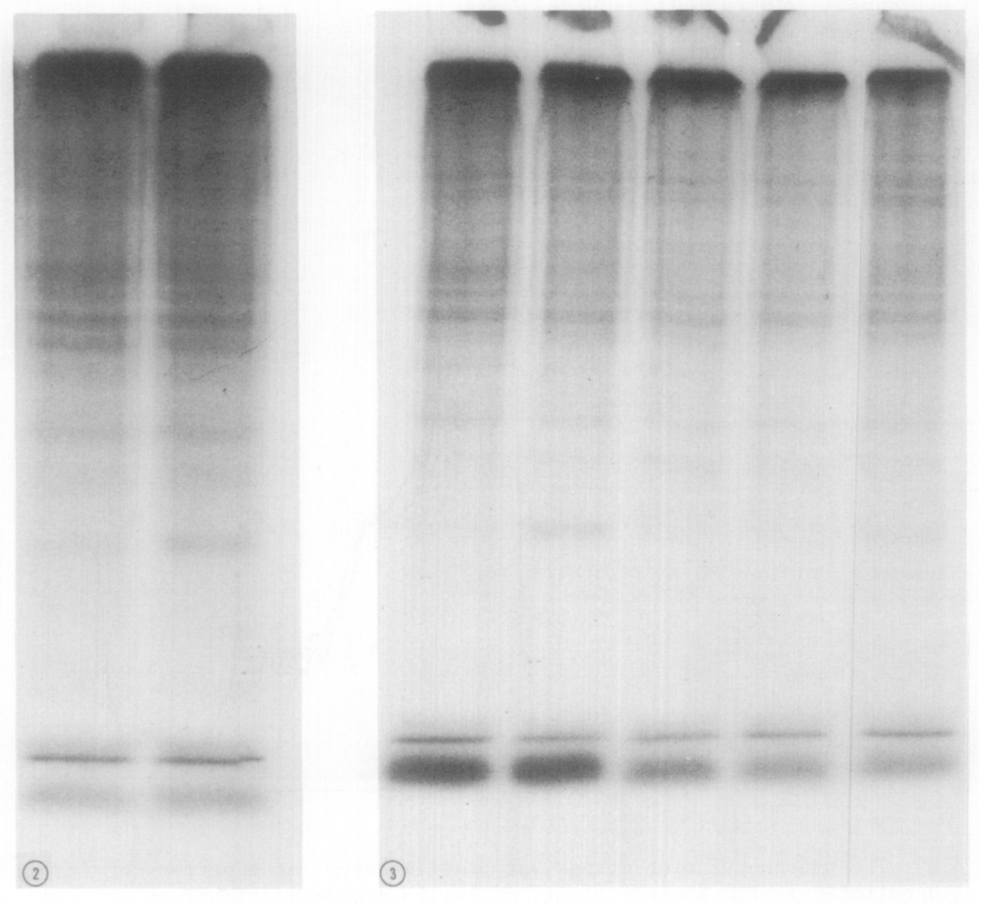

Fig. 2. The phosphorylation of nuclear proteins from rat superior cervical ganglia 4 hours after the administration of buffer (left) or of 2.5 S nerve growth factor (10 µg per gram body weight) (right). The ganglia were removed and cultured for 1 hour with radioactive phosphate. Twenty-four thousand cpm (30 µl of control, 34 µl of NGF-treated) of each nuclear extract was applied.

Fig. 3. The phosphorylation of nuclear proteins from rat superior cervical ganglia cultured in the presence of nerve growth factor or of various other agents. The gels represent (left to right) ganglia cultured with: Buffer, nerve growth factor (3 µg per ml; 1.1×10^{-7}M), insulin, (1.1×10^{-7}M), glucagon (1.1×10^{-7}M), 8-bromo-cAMP (1 mM) + 3-isobutyl-1-methyl-xanthine (0.5 mM). Thirty-one thousand cpm (between 54 and 79 µl) of each nuclear extract was applied.

decarboxylase (8), and an increase in the activity of the RNA polymerases (9). It is resonable to expect that these effects are reflected in changes in the characteristics of some of the nuclear proteins. The present data represent the first observation of just such a specific change.

The identity of the protein involved is currently being sought. Its

migration in SDS-acrylamide is similar to that of f_1 histone, but other chromatographic systems will be needed for positive identification. Also, current experiments are designed to determine whether it is simply the phosphorylation of this protein which is increased; alternatively, its synthesis or its translocation to the nucleus could be stimulated by nerve growth factor. Finally, it will be necessary to know whether the time course of this increased phosphorylation is consistent with the other nerve growth factor-induced changes that take place in the ganglia.

It is of interest that even insulin, although structually related to nerve growth factor (10), does not promote the increased phosphorylation. On the other hand, the ability of cAMP derivatives to mimic this action of nerve growth factor would seem to strenthen the case for cAMP as a second messenger for nerve growth factor.

There are now several examples of specific nuclear phosphorylations which occur as a result of the effects of various hormones on their target tissues (11-14). One of the most interesting in this context is the recent report of the action of β-adrenergic agonists on phosphorylation in the nuclei of cultured pineal glands (15). In that case, as in this, the increased phosphorylation was found in a protein migrating as does f_1 histone, cAMP derivatives were found to mimic the action of the agent, and the phosphorylation preceeded the induction of a specific enzyme involved in the synthesis of a cell-specific product.

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