POTENTIATION OF THE NGF-MEDIATED NERVE FIBER OUTGROWTH BY GINSENOSIDE Rb₁ IN ORGAN CULTURES OF CHICKEN DORSAL ROOT GANGLIA

Hiroshi SAITO*, Kitaru SUDA, Martin SCHWAB and Hans THOENEN

Department of Pharmacology, Biocenter of the University, Basel, Switzerland

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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Abstract The fiber outgrowth induced by ACh, dibutyryl cyclic AMP and dibutyryl cyclic GMP in explanted chick embryonic dorsal root ganglia differed distinctly from that by nerve growth factor (NGF) and submandibular gland extract of adult male mice. Ginsenoside Rb₁ potentiated the effects of NGF and submandibular extract at concentrations of 3 and 30 μM, but did not potentiate the effects of ACh, dibutyryl cyclic AMP and dibutyryl cyclic GMP. NGF-antibody inhibited the effects of NGF, but not the effects of ACh, dibutyryl cyclic AMP and dibutyryl cyclic GMP. Concanavalin A and KCl did not promote fiber outgrowth.

Stimulation of fiber outgrowth from chick sympathetic and dorsal root ganglia led to the detection of NGF (1, 2). This physiological effect of NGF is still one of the most frequently used criteria for estimating the biological activity of exogenously administered or endogenously produced NGF in organ cultures of dissociated cells of sympathetic or dorsal root ganglia. In spite of a long history and extensive experimental use, the mechanism of fiber outgrowth and modulation is poorly understood. The specificity of the nerve fiber outgrowth effected by NGF has been questioned in light of the fact that other substances such as concanavalin A, high potassium concentrations, ACh and cyclic AMP also produce similar fiber outgrowth (3, 4, 5, 6, 7).

We attempted herein to determine the differences between the fiber outgrowth produced by NGF and that produced by other substances and to delineate the possible modifications of the fiber outgrowth. We confirmed that a series of other substances also produce a slight fiber outgrowth, however, the shape of the fibers and the maximal extent of fiber outgrowth differed from that produced by NGF. Moreover, we found that the effect of NGF is markedly potentiated by a saponin (ginsenoside Rb₁) whereas the fiber outgrowth resulting from other substances remained unaffected.

MATERIALS AND METHODS

NGF was isolated as the 2.5 S subunit according to the procedure of Bocchini and Angeletti (8). The purity was determined by SDS gel electrophoresis. NGF-antibody was isolated from sheep antiserum according to the procedure of Stockel et al. (9). ACh was purchased from Dr. E. Baeschlin AG (Switzerland), dibutyryl cyclic AMP and GMP from Boehringer Ltd. (Mannheim, Germany) and concanavalin A from Miles-Yeda Ltd. The
GRb1, one of the saponins contained in the root of Panax Ginseng C.A. Meyer, was kindly provided by Prof. Shibata, University of Tokyo and Prof. Shoji, Showa University (Japan). Protein concentrations of the supernatant fractions from adult male mouse submandibular glands (2 months old) were determined according to the method of Lowry et al. (10).

Spinal dorsal root ganglia from 10 day old chick embryos (Optigal, Fribourg, Switzerland) were incubated in Maximov depression slides in a culture medium consisting of 25 μl of Dulbecco's modified eagle medium (Gibco Bio-cult. Co. Ltd.) with 10° W/V bovine thrombin (SAF Hoffmann-La Roche & Co., Ltd.), 25 μl of physiological saline containing NGF or the test substances in various concentrations, and 25 μl of freshly heparinized chicken serum (11). Three ganglia were placed on each slide and incubated at 37 C in 95° air and 5° CO₂ for 24 hr. The fiber outgrowth from the ganglia was observed under dark-field illumination and numerical scores (index 0-8) of intensity were assigned to each ganglion. Optimal fiber outgrowth (dense halo of fibers, maximal fiber length) was designated as index 4 and corresponded to one biological unit (BU) of NGF (11). Supraoptimal concentrations resulted in increased density of the fiber halo concomitant with a reduced fiber length (index 5-8).

RESULTS

Effect of purified NGF on fiber outgrowth

2.5 S NGF at various concentrations from 0.1 nM (2.6 ng ml) to 6.4 nM (165 ng ml)

![Image of fiber outgrowth with numerical scores](image-url)
resulted in a gradually increased fiber outgrowth in embryonic chick dorsal root ganglia after 24 hr in culture. The optimal response (index 4) i.e. a dense halo of long, straight nerve fibers, was obtained with 0.8 to 1.6 nM 2.5 S NGF. Supraoptimal doses (index 5-8) resulted in a dense outgrowth, at which time the length of the individual fibers was progressively reduced. The outgrowth with suboptimal doses was sparse (0.1-0.8 nM) (Fig. 1, 2).

**Effect of crude extracts from male mouse submandibular gland**

Crude extract from adult male mouse submandibular glands stimulated neurite outgrowth in our system in concentrations of 0.08 (index 1.5) to 20 µg protein per ml tissue.
culture medium (index 6). The optimal response (index 4) was obtained with 2.4 µg/ml (Fig. 3).

**Effects of ACh, cyclic nucleotides, concanavalin A and depolarization by potassium**

In a second set of experiments the effect of ACh, dibutyryl cyclic AMP and GMP, concanavalin A and KCl on fiber production by embryonic chick dorsal root ganglia in culture was tested over a wide range of concentrations. Optimal effects were obtained with ACh (3.2 µM), dibutyryl cyclic GMP (0.5 mM) and dibutyryl cyclic AMP (4.0 mM) (Fig. 4, 5b). The outgrowth observed under these conditions was clearly different from that obtained with NGF: only a few curved nerve fibers were present (index 2). KCl (0.01–10 mM) and concanavalin A (0.1–100 µg/ml) had no stimulatory effect on neurite outgrowth by embryonic chick dorsal root ganglia. ACh (1.6 and 3.2 µM), dibutyryl cyclic AMP (0.25 and 0.5 mM), dibutyryl cyclic GMP (2 and 4 mM), concanavalin A (6 and 12 µg/ml) or KCl (0.1 and 1 mM) had no influence on either the NGF-mediated fiber outgrowth nor that evoked by submandibular gland extract.

![Figure 4](image-url)  
**Fig. 4.** Effects of NGF, ACh, dibutyryl cyclic AMP and dibutyryl cyclic GMP on fiber outgrowth of dorsal root ganglia from chick embryos. Each point represents the mean of 6 experiments (Symbols as in Fig. 2).

**Potentiation of the effect of NGF by GRβ1**

Effects on nerve fiber production were nil when GRβ1 in concentrations of 0.3 to 30 µM was given alone. On the other hand, a marked potentiation of the effect of NGF occurred in the presence of 3 and 30 µM GRβ1, resulting in a pronounced shift of the dose-response curve for NGF (Fig. 2, 5a). Thus, optimal fiber outgrowth in the presence of 30 µM GRβ1 was achieved with 0.16 nM NGF, or with 0.39 nM NGF at a concentration of 3 µM GRβ1. The smallest concentration at which an unequivocal response could be obtained was 0.006 nM NGF in the presence of 30 µM GRβ1 (Fig. 2). These results indicate a marked potentiation of the original effect of NGF by GRβ1 (about 30-fold at low concentrations of NGF (index 2) and about 7-fold at optimal concentrations (index 4). A lesser but still significant potentiation (about 4-fold) of the effect of crude submandibular gland extract by GRβ1 is shown in Fig. 3. The optimal concentration of submandibular gland extract (index 4) was shifted...
from 2.4 \( \mu g \) protein/ml to 0.64 \( \mu g \) ml by 30 \( \mu M \) GRb1. The smallest concentration for which an unequivocal response could be obtained was 0.04 \( \mu g \) protein/ml submandibular gland extract in the presence of 30 \( \mu M \) GRb1 (Fig. 3). These results indicate a 4-fold potentiation of the original effect of NGF by GRb1. There was no potentiation of the effect of ACh, dibutylryl cyclic AMP, dibutylryl cyclic GMP, concanavalin A and KCl on nerve fiber production by 3 or 30 \( \mu M \) GRb1.

**Effect of NGF-antibody on fiber outgrowth produced by ACh and cyclic nucleotides**

To determine whether or not the slight fiber outgrowth seen with ACh and cyclic nucleotides

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**Fig. 5.** (a): Effects of GRb1 on NGF elicited neurite outgrowth from chick embryonic dorsal root ganglia 24 hr in organ cultures. Optimal outgrowth (score 4) was obtained with 0.1 nM NGF in presence of 30 \( \mu M \) GRb1. (b): Fiber outgrowth produced by ACh 3.2 \( \mu M \).

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**Fig. 6.** Effects of various concentrations of NGF-antibody on NGF (0.8 nM) stimulated fiber outgrowth of dorsal root ganglia from chick embryos. Each point represents the mean of 6 experiments (Symbols as in Fig. 2).

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**Fig. 7.** Effects of NGF-antibody (800 ng/ml) on fiber outgrowth of embryonic chick dorsal root ganglia elicited by various concentrations of NGF. Each point represents the mean of 6 experiments. b: Symbols indicate the significant difference (P 0.01) between the response to NGF in presence of NGF-antibodies and c: (P 0.05).
nucleotides did in fact result from stimulation of the production of NGF and its release, we investigated the effect of NGF-antibody on neurite outgrowth effected by NGF, ACh, dibutyryl cyclic GMP and dibutyryl cyclic AMP. A marked inhibition of the effect of NGF at various concentrations did occur in the presence of NGF-antibody (Figs. 5, 6, 7). In contrast, there was no inhibitory effect of NGF-antibody for the fiber outgrowth produced by ACh (3.2 and 6.4 μM), dibutyryl cyclic GMP (0.5 mM) and dibutyryl cyclic AMP (4.0 mM).

DISCUSSION

The present experiments confirm the well established fact that both purified β-NGF and crude extracts of the male mouse submandibular gland evoke a concentration-dependent outgrowth of nerve fibers from chick dorsal root ganglia (1, 11). Quantitative differences in fiber outgrowth occurring on either side of the optimum in the dose-response curve, were observed to be the same as those reported in the literature (12). The fiber outgrowth produced by ACh and cyclic nucleotides differed distinctly from that produced by NGF. The maximal fiber outgrowth achieved by these substances was much smaller than that produced by NGF. Moreover, ACh and cyclic nucleotides produced a massive radial outgrowth of non-neuronal fibroblast-like cells which during superficial inspection could be confused with outgrowing nerve fibers. Optimal effects obtained with ACh, dibutyryl cyclic AMP and dibutyryl cyclic GMP were not inhibited by NGF-antibody. In contrast to preliminary reports from other laboratories (6, 7) there were no effects on fiber outgrowth induced by concanavalin A and K+ over a wide range of concentrations.

GRβ1 alone produced no fiber outgrowth whatever over a large range of concentrations. However, this saponin potentiated markedly the response of the dorsal root ganglia to NGF. This potentiating effect was specific for NGF and did not occur when fiber outgrowth was elicited by ACh or cyclic nucleotides. The mechanism of the potentiating effect remains to be established; it is not even amenable to pertinent speculations, since the mechanism of the NGF-mediated fiber outgrowth is unknown as are the mechanisms of a large variety of biological effects of the Ginseng saponins. The latter include stimulation of RNA and protein synthesis in rat liver cells, an enhanced cholesterol synthesis in serum and liver (13, 14, 15, 16) and behavioral effects (17). The chemical properties of this saponin suggest a membrane effect influencing either the uptake of NGF into the ganglionic neurons or the consequences of the NGF-receptor interaction in the neuronal membrane.

Although the molecular mechanism of the potentiating effect of GRβ1 remains to be established, the potentiating effect provides a tool for increasing the sensitivity of the chick dorsal root ganglion preparation when used for the biological determination of NGF activity.

REFERENCES

1) REVI-MONTALCINI, R. AND ANGELETTI, P.U.: Physiol. Rev. 48, 534 (1968)
2) Nerve Growth Factor and its Antiserum, Edited by ZAIMIS, E. AND KNIGHT, J., p. 46, Athlone Press, Univ. of London (1972)
45 (1972)


15) Yamamoto, M.: Metabolism and Disease, Japan 10, 531 (1973)
