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Does Antinerve Growth Factor Affect Isolated Ileal Contractility in Rat?

E. KOÇ, S. YAVUZER, B. OCAKCIOĞLU

Department of Physiology, School of Medicine, Ankara University, Ankara, Turkey

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Summary

This study investigates the effects of antinerve growth factor (anti-NGF) application on isolated ileal contractility in the rat. For this purpose, rats were divided into four groups. The control animals (n=8) received only intraperitoneal injection of an isotonic NaCl solution (i.p). Anti-NGF was daily administered intraperitoneally at the dose of 1 ng/g level in the first experimental group (n=8), and at doses of 10 ng/g (n=7) and 40 ng/g (n=7) in the second and third experimental groups, respectively. Seven days after the injections rats were sacrificed and ileum segments were isolated. Responses to acetylcholine (ACh) were evaluated by using standard Tyrode, double-calcium Tyrode and calcium-free Tyrode solutions. The average peak amplitude of ACh-induced contractions recorded in standard Tyrode solution was significantly decreased in all three experimental groups as compared to the control group ($p<0.05$). When double-calcium Tyrode solution was used as the perfusion medium, the responses to ACh were also lower in all anti-NGF applied groups as compared to its control group ($p<0.05$). Our results showed that the application of anti-NGF reduced the contractile responses of the rat isolated ileum apparently by decreasing the calcium influx from the extracellular medium.

Key words

Nerve growth factor • Ileal contractility • Acetylcholine • Rat isolated ileum

Introduction

Nerve growth factor (NGF) is a protein essential for the development and healthy functioning of some sensory neurons in the peripheral and sympathetic nerve system (Otten 1984, Thoenen *et al.* 1987). In the peripheral nervous system, NGF is synthesized predominantly in the target organs, taken up into the axons of these neurons and retrogradely transported to the neuronal cell bodies (Yanker and Shooter 1982). Previous studies showed the presence of NGF in the intestine of adult rats (Weskamp and Otten 1987) and its production *in vitro* by intestinal epithelial cells (Varilek *et al*. 1995). However, a recent study of rat with experimental colitis showed that NGF has a protective role (Reinshagen *et al*. 2000).

There is substantial evidence indicating that the Trk family of tyrosine protein kinase receptors, Trk A, Trk B, and Trk C play a significant role in the development and maintenance of neural tissue by mediating the trophic effect of the NGF family of neurotrophines (Kaplan and Stephens 1994, Manness *et al.* 1994). Trk is a receptor tyrosine kinase, the activity of which is stimulated by the binding of NGF (Klein *et al.*

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

1991). Once activated, Trk stimulates the activity of the small GTP-binding protein Ras *via* a pathway that has not been completely elucidated (Qiu and Green 1991). Shibayama and Koizumi (1996) showed that in the small intestine and colon, there was strong immunoreactivity for all Trk proteins in small cells with neuroendocrine cell-like morphology.

Although members of the neurotrophin family are known to have profound effects – including the promotion of survival and differentiation – on neurons of both peripheral and central nervous system, the exact mechanisms of the NGF effects on neurons remain partially understood. Numerous studies have analyzed the NGF action on the pheochromocytoma cell line and central nervous system.

However, little is known about the effects of NGF on the enteric nervous system in relation to intestinal motility. This provided the motivation for our experiments which aimed to examine the effects of NGF on intestinal motility through application of anti-NGF. The present study was designed 1) to test the effects of anti-NGF on ACh-induced contractions of the isolated ileum, and 2) to investigate possible mechanisms of NGF effects on smooth muscle contraction and the role of extracellular calcium on these events.

Methods

Our study was performed with the permission of the local ethics committee. Healthy adult Wistar rats (n=30) weighing 150-200 g and including both sexes were used in these experiments. The animals were obtained from The Animal Care and Research Center of Ankara University, School of Medicine. They were housed under standard conditions. Four experimental groups were designed. The control group of animals (n=8), received an injection of isotonic NaCl solution (SP). Anti-NGF was daily administered intraperitoneally (i.p) in a dose 1 ng/g (n=8), 10 ng/g (n=7) and 40 ng/g (n=7) levels in the first, second and third experimental groups, respectively. Seven days after the injections, rats were anesthetized lightly with pentobarbital (35 mg/ kg i.p) and killed by decapitation and exanguination. Terminal ileum segments (1 cm long pieces) were removed rapidly and their contents was flushed with an ice-cold Tyrode solution. Afterwards, tissue segments were suspended in an isolated tissue bath containing 40 ml standard Tyrode solution (in mM: NaCl 137, KCl 2.68, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.5) and bubbled with 95 % O_2, 5 % CO_2 mixture at 37 °C, pH 7.4. For recording tension, the samples were mounted vertically between a fixed holder and a force transducer (Ugo Basile isometric transducer No. 7003). The tissue segments were brought into equilibrium for 30 min under optimal resting tension of 0.3 g. After equilibration, the ileum pieces were contracted with ACh ($2.7x10^{-7}$ M). This dose elicits maximal contractions in the control group after the cumulative addition of ACh (data not shown). Changes in isometric tension were recorded on the Ugo Basile No. 7050 recorder. The ACh-induced contraction amplitude was measured in millimeters from recorded traces and calibrated as 1 g per 10 mm with a resting tension of 0.3 g. Tyrode solutions used in these experiments were of three types: (i) standard, (ii) with a double calcium concentration, and (iii) calcium-free. Non-standard Tyrode solutions were obtained by changing the concentrations of $CaCl_2$ and NaCl in standard Tyrode solution. In separate experiments, the bath medium was exchanged either for double-calcium or calcium-free solution and the tensions were recorded in a similar manner.

Statistical analyses were performed by Mann-Whitney-U and Wilcoxon tests. The results were expressed as means ± S.D. The level of statistical significance was set for $P<0.01$ or $P<0.05$.

Results

The effects of anti-NGF administration on isolated ileal contractility were examined. At the end of the experimental period (7 days) no significant difference was observed in the average weight gain between the three experimental groups and the control group. To investigate the effects of extracellular calcium ion concentration on the ileal contraction, we studied the response to ACh in standard, double-calcium and calcium-free bath solutions. As shown in Table 1, the average peak amplitude of ACh-induced contractions recorded in standard Tyrode solutions of were significantly lower in all three experimental groups as compared to the control group ($p<0.05$). The contractile response to ACh was also lower in all anti-NGF groups as compared to their control group when double-calcium Tyrode solution was used ($p<0.05$). When double-calcium medium was used, the contractile responses were increased in both control and all anti-NGF-pretreated groups as compared to the responses observed in standard

Tyrode perfusion medium.

In calcium-free medium, the contractile response to ACh was decreased both in control and all experimental groups in comparison with the the responses seen in standard Tyrode medium. There was no significant difference between control and anti-NGF groups in the calcium-free medium.

Table 1. Contraction amplitude (mm) of isolated ileum from control and experimental groups, in different Tyrode solutions.

	Contraction amplitude with standard Tyrode	Contraction amplitude with 2xCalcium Tyrode	Contraction amplitude with calcium -free
Control (n=8)	31.625±16.256	41.000±.545	13.250±8.795
1 ng/g Anti-NGF (n=8)	12.000±8.264 *	17.500±11.892**	9.125±7.530
10 ng/g Anti-NGF (n=7)	20.429±10.565 **	26.429±11058 **	14.000±5.416
40 ng/g Anti-NGF (n=7)	14.143±10.383 **	20.143±13.643**	12.000±9.626

Data are means ± S.D. Significantly different from control group: * $p<0.01$, ** $p<0.05$.

Discussion

Our results showed that the application of anti-NGF (1 ng/g, 10 ng/g or 40 ng/g for one week) significantly decreased ACh-induced contractions in isolated rat ileum in both standard and double-calcium Tyrode solutions. Several investigations support our findings. Levine *et al*. (1995) reported that increased conductance is due to an increase in the number of channels *per* neuron. They suggested that NGF modulates several pharmacologically distinct components of whole-cell calcium current in neurons. Hence, different trophic effects of NGF may be mediated by calcium entry *via* particular channels (Levine *et al*. 1995). Moreover, a recent study along lines similar to ours has pointed out that NGF plays an important role in the development of motor alterations (Torrents *et al*. 2002). They showed that anti-NGF treatment completely blocked the development of spontaneous hypermotility. NGF overexpression regulates nerve remodeling that modifies the sensitivity of intestinal motor reflexes. This remodeling affects afferent as well as motor innervation. Although we were unable to examine calcium entry or calcium concentration in smooth muscle cells due to technical reasons, our data has clearly shown that decreased calcium influx into the cytoplasm resulted in decreased contractile response. When anti-NGF was applied, these effects were inhibited. Furthermore, Furukawa *et al.* (1993) postulated that NGF has strong and moderate effects on the expression of voltage-dependent sodium and calcium channels, respectively. Some investigators with a different opinion about the effects of NGF on ionic-channels hypothesized that the action of NGF is exerted on channels other than the L-type calcium channels or the ATP-activated calcium channels (Nikodijevic and Guroff 1991). It was also postulated that NGF may also interact with other factors, e.g. neurotransmitters and neurotrophins, to induce calcium influx (Rudy *et al*. 1987). In fact, the effects of NGF are not fully understood. When anti-NGF is applied, calcium channels may reduce the interactions of some neurotransmitters by possible neuromodulatory effects of NGF or due some reduction in ACh receptors.

On the other hand, it has been shown that NGF has a stimulating effect on the pericaryal mechanisms synthesizing substance P in dorsal root ganglion cells and an inhibitory effect upon the VIP-synthesizing machinery in these cells (Knyihar-Csillik *et al.* 1991). It is also known that the VIP stimulate enzymatic breakdown of glycogen to glucose (Said 1984) probably by stimulating cyclic AMP production (McCulloch and Kelly 1983).

The investigations about NGF and enteric neuropeptides reveal that there is an increase in vasoactive intestinal polypeptides (VIP), galanin (GAL) and substance P-like immunoreactivity in the myenteric plexus of the ileum from rats treated with NGF antiserum compared with the controls. The researchers raise the possibility that NGF may differentially regulate the expression of enteric neuropeptides during the postnatal stage of development (Belai *et al*. 1992). Therefore, it may be hypothesized that anti-NGF application may result in an increase of VIP synthesized and that this may decrease the contractile activity of the small intestine.

The influx of extracellular calcium is a prerequisite for motility. In gastrointestinal smooth

muscles, the upstroke action potential is principally mediated by calcium influx through voltage-dependent L-type calcium channels and is responsible for the initiation of contraction. A variety of neurotransmitters and hormones modulate calcium channel activity through protein phosphorylation (Levintan 1994). On the other hand, accumulating evidence suggests that tyrosine kinases are involved in the regulation of smooth muscle contraction. For instance, activation of growth factor receptors leads to smooth muscle contraction. This is accompanied by tyrosine phosphorylation of a number of proteins (Pavalko *et al*. 1995). The fact that calcium current is decreased by tyrosine kinase inhibitors and enhanced by growth factors in smooth muscle cells (Hatakeyama *et al*. 1996) points toward a novel mechanism for tyrosine kinases in the regulation of smooth muscle function. Berse *et al*. (1999) described that there are two signaling pathways which are activated by NGF: Trk A-snc interaction and calcium signaling. It was shown that anti-NGF antibodies and also Trk A blocker downregulate the side, number and location of cholinergic synapses (Debeir *et al*. 1999). It was recently shown that the basal calcium current is modulated by c-Src and focal adhesion kinase (FAK) in different smooth muscles (Hu *et al*. 1998). Our results indicate that when calcium is omitted in the perfusion solution, ACh-induced contractions decrease significantly in 1 ng/g and 10 ng/g anti-NGF groups compared to standard Tyrode perfusion medium. Although it is well known that smooth muscle uses extracellular calcium, Furukawa *et al*. (1993) showed that ACh induced three kinds of ionic currents; (i) a rapid transient inward current, (ii) a subsequent transient current and (iii) a long-lasting slow inward current which were mimicked by muscarine, whereas rapid transient current was mimicked by nicotine. These investigators stated that it was possible to generate the muscarinic ACh-response only once in a calcium-free external solution because the content of calcium in intracellular organelles is sufficient to generate only one muscarinic response. Extracellular calcium is, however, necessary to produce more than two muscarinic responses. Their results also indicated that the activation of calcium/calmodulin complex is essential for generating the muscarinic ACh-response in PC-12 cells (Furukawa *et al*. 1993).

In addition, our results showed that ACh-induced contractions increased in control and all experimental groups as compared to the standard Tyrode groups when double-calcium perfusion media were used. Although it was shown that the Trk family receptors play an important role in mediating the effects of NGF, it has also been demonstrated that a multicomponent receptor complex mediates the actions of GDNF at the cell surface (Baloh *et al*.1997, Creedon *et al*. 1997).

It has been well documented that catecholamines exert an inhibitory influence on intestinal peristalsis. Although the inhibitory mechanisms are still not clear, some investigators suggested that an adrenergic component modulates the release of ACh from the Auerbach plexus, probably presynaptically through its alpha-adrenoceptor (Drew 1978, Yoshimura *et al*. 1986) while beta adrenoceptors do not participate in the modulation of cholinergic transmission (Yoshimura *et al*. 1986). It was also shown that the effects of NGF on sympathetic neuronal processes may also involve calcium (Rogers and Hendry 1990). In turn, we suggest that the effects of NGF on the contractile mechanism of the ileum may be mediated by muscarinic ACh receptors and/or nicotinic ACh receptors by second messengers and calcium/ calmodulin complexes.

In our experiments, we also demonstrated that a significant decrease in the amplitude of ACh-induced contractions was accompanied by a marked increase in duration of the tonic component of contractile responses and there is an increase in contraction time in the anti-NGF treated groups in standard Tyrode medium. We presume that there is a reduction in the calcium pump activity that transports calcium out of the cell or that there are calcium channels activated by anti-NGF treatment. We assume the height of contraction after 12 s as the phasic component and the actual contraction as tonic component of contractions as was postulated by Aloamaka *et al.* (1984). We showed that when calcium was removed from the medium, the initial phasic component of contraction was abolished and the tonic component was remarkably reduced in these preparations. We therefore thought that NGF can modulate both phasic and tonic components of contraction and thus can modulate contractile activity by changes in the permeability of calcium channels.

The effects produced by anti-NGF in the rat suggest that its application results in a decrease in ACh-induced ileal contractility *in vitro*. Since treatment with an antibody against NGF prevents most of the abnormal motor changes, it may be useful in the therapy of motor disorders caused by hypermotility. Moreover, the exact intracellular signaling process of NGF remains to be clarified by further investigations.

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Reprint requests

Emine Koç, Department of Physiology, Faculty of Medicine, Ankara University, Sihhiye 06100, Ankara, Turkey, e-mail: ekoc@medicine.ankara.edu.tr