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Neurotrophin induced cAMP and IP_3 responses in PC12 cells

Different pathways

Marlies Knipper[b], Anja Beck[a], Jane Rylett[b] and Heinz Breer[a]

[a]*University of Hohenheim, Institute of Zoophysiology, Garbenstraße 30, 7000 Stuttgart 70, Germany* and [b]*Department of Physiology, University of Western Ontario, London, Ont N6A 5C1, Canada*

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NGF and BDNF elevate cAMP and IP_3 levels in membranes of PC12 cells within a subsecond time period. The cAMP formation induced by NGF and BDNF pretreatment for 2 s was reduced by GDP-β-S and PTX, but not the trk^{NGFR} inhibitor K 252a. NGF, but not BDNF, induced IP_3 formation. IP_3 formation was reduced by K 252a, but not by GDP-β-S and PTX. Using $p75^{NGFR}$ expressing, but trk^{NGFR}-deficient PCNA cell membranes, NGF and BDNF induced cAMP formation, but not IP_3 formation. We suggest that NGF and BDNF induced cAMP formation is mediated via a $p75^{NGFR}$/G-protein mediated mechanism, and IP_3 formation via a K 252a sensitive pathway.

NGF; BDNF, cAMP; IP_3; $p75^{NGFR}$; trk^{NGFR}

1. INTRODUCTION

Nerve growth factor (NGF) is a protein that is required for the survival and development of sympathetic and sensory neurons in the peripheral nervous system [1,2]. Recently it was shown that NGF belongs to a family of nerve survival-promoting factors, the neurotrophins [3,4]. Two structurally unrelated receptors have now been characterized, the $p75^{NGFR}$ serves as receptor for all known neurotrophins [5–7]. The second receptor is a member of the trk family of tyrosine kinases and shows specificity for the different neurotrophins, as there is the receptor for NGF: $p140^{trkA}$ [8]; for BDNF: $p145^{trkB}$ [9]; and for NT-3: $p145^{trkC}$ [10]. At present little is known about the mode of actions which couples NGF receptors to different intracellular pathways leading to the various physiological reactions. Both NGF receptors offer different transduction mechanisms via their included C-terminal sequences; thus it has been postulated that $p75^{NGFR}$ via its mastoparan-like domain [11] might interact with G-proteins to generate a signal transduction mechanism (for review, see [12]) contributing to NGF induced modifications of serine and threonine residues in target proteins; in comparison, the $p140^{trkA,B,C}$ receptor contains a cytoplasmic domain with tyrosine kinase activity, which upon activation results in autophosphorylation [13] and contributes to neurotrophin effects via modification of tyrosine residues in target proteins with SH2/SH3 domains [14,15]. A number of recent reports point to a possible role of second messengers in early steps of NGF-signalling within seconds and minutes [16–19]. However, at present the results are highly controversial. As growth factor induced second messenger formation may be relatively rapid, compared to the rather slow processes following the internalization of the receptor/ligand complex, we have tried to monitor changes in the concentrations of cAMP and IP_3 after pretreatment of either PC12 cell membranes or membranes of $p75^{NGFR}$ expressing, TRK-deficient cell lines in subsecond-to-second time periods using a rapid kinetic methodology.

Correspondence address M. Knipper, Department of Physiology, University of Western Ontario, London, Ont., Canada N6A 5C1. Fax: (1) (519) 661 3827.

Abbreviations: cAMP, 3′5′-cyclic adenosine monophosphate; EDTA, ethylene-diaminetetraacetate; EGTA, ethyleneglycol bis(aminoethylether)tetraacetate; IP_3, inositoltrisphosphate, NGF, nerve growth factor; BDNF, brain-derived neurotrophin factor; $p75^{NGFR}$, low-affinity NGF receptor; trk^{NGFR}, high-affinity NGF receptor.

2. MATERIALS AND METHODS

2.1. *Preparation of PC12/PCNA/LTK-cell membranes*

A stable cell line of $p75^{NGFR}$ transfected mouse LTK^- fibroblasts [20], PCNA cells, or the appropriate non-transfected LTK^- cells were kindly supplied by Dr. E.W. Shooter, Stanford University. PC12 cells, PCNA, and LTK^- cells were collected in 120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 7.5 mM glucose, pH 7.4, frozen in liquid nitrogen and stored for a maximum of 4 days until use. Cells were thawed and lysed for 20 min in hypo-osmotic medium (10 mM Tris, 5 mM EGTA, 1 mM $MgCl_2$, pH 7.4) containing antiproteases in the following final concentrations. 0.1 mg/ml bacitracin, 4 μg/ml leupeptin, 5 μg/ml pepstatin A, 4 μg/ml antipain, 5 μg/ml soybean trypsin inhibitor, 2 mM iodoacetamide, 0.1 mM benzethonium chloride, 1 mM benzamidine, and centrifuged for 15 min at 27,000 × *g*. The resulting pellet was resuspended in lysis-buffer and quickly used for second messenger determination.

The pertussis toxin-sensitive G proteins in membrane fractions were

ADP-ribosylated directly with the A-subunit of pertussis toxin as described [21]. Membrane protein (1 mg) was incubated in 1 ml of 10 mM L-arginine, 1 mM ATP, 2.5 mM $MgCl_2$, 1 mM EDTA, 100 mM HEPES, pH 8.0, 10 mM NADP, 100 μM NAD^+, 1 μg/ml pertussis toxin A-subunit, 0.5 mg/ml BSA at 4°C for 15 min, then 30°C for a further 10 min. Control membranes were treated with the same ribosylation buffer in the absence of the pertussis toxin A-subunit. The reaction was terminated by centrifugation at 14,000 × g for 2 min and resuspension of the pellet in lysis buffer. Membranes were used quickly for second messenger determination. For various studies membranes were preincubated for the following time and temperature. Walsh-inhibitor (40 μM), 20 min, 4°C; Calphostin (1 μM), 3 min, 30°C; K 252a (100 nM), 3 min, 30°C. K 252a and Calphostin were diluted from a stock solution in DMSO, stored at −20°C. Final concentration of dimethyl sulfoxide was not greater than 0.001%. Controls were preincubated under the same conditions and similar concentrations of DMSO were added.

2.2. *Cell culture*

PC12 cells were maintained on collagen/poly-lysine (10 μg/ml)/poly-ornithine (10 μg/ml)-coated culture dishes in a culture medium comprised of DMEM containing 6.5% (v/v) fetal bovine serum (Gibco), 6.5% (v/v) horse serum (Gibco) and 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were maintained in a culture medium comprised of DMEM containing 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin (LTK^-), or HAT 2% (PCNA) [21]. The cells were grown at 37°C in a 5% CO_2-enriched, humidified atmosphere. Stock cultures were routinely subcultured at a ratio of 1:5 at 7-day intervals and the medium changed once between each subculturing.

2.3. *Determination of cAMP and IP_3*

Measurement of cAMP and IP_3 were performed according to Boekhoff et al. [22]. Syringe I (SI) contained PC12 cell membranes (1 mg protein/ml) in 70 μl lysis buffer. Syringe II (SII) was filled with buffer composed of 200 mM NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM $MgCl_2$, 1 mM ATP, 2 μM GTP and $CaCl_2$ to give a free Ca^{2+} concentration of 0.02 μM containing NGF, BDNF or GDP-β-S at appropriate concentrations. 7S NGF or 2.5S NGF and rhBDNF were diluted from BSA containing stock solution, to give a 1.4 nM concentration in 20 μg/ml BSA. Syringe III (SIII) contained 10% perchloric acid (0°C). Reaction was started by mixing solutions from SI and SII and was stopped after an appropriate incubation with perchloric acid from SIII. Determination of cAMP and IP_3 was performed as previously described [22]. cAMP and IP_3 radioligand assay kits were supplied from Amersham. 7S NGF came from Boehringer and 2.5S NGF came from Gibco. Protein was determined according to Bradford [23].

Table I

Effect of Walsh-inhibitor and calphostin on NGF induced cAMP formation in PC12 cell membranes

NGF (nM)	cAMP concentration (% of control)		
	NGF	NGF + PKA-I	NGF + PKC-I
0.7	132 ± 10*	133 ± 14*	137 ± 8*
3.5	100 ± 16	219 ± 40**	108 ± 5

PC12 cell membranes were prepared and treated for 2 s with either control medium, 40 μM Walsh-inhibitor (PKA-I) or 1 μM calphostin (PKC-I) as described in section 2. NGF was added for 2 s and cAMP concentrations were determined as described above. The effect is indicated as change in % of control. Control represents mean of basal level from all experiments = 64 ± 8 pmol/mg protein, ± S.E.M., n = 8. Different from control group; *P < 0.05; **P < 0.001, Student's t-test.

3. RESULTS AND DISCUSSION

The application of NGF to PC12 cell membrane preparations induced a time dependent elevation of the cAMP and IP_3 level (Fig. 1). NGF induced a concentration dependent elevation of the cAMP level within a tested range between 0.07 nM and 1.4 nM 7S NGF. The concentration of cAMP was elevated significantly after 100 ms and continuously increased over time, resulting in a formation of 27 ± 5 pmol cAMP/pmol protein above the control level of 110 ± 15 pmol cAMP/pmol protein (± S.E.M., n = 4) when 0.07 nM NGF was applied for 5 s (not shown) and a maximal response of 110 ± 11 pmol cAMP/mg protein above the control level when 1.4 nM NGF was applied for 5 s (Fig. 1A). Concentrations of NGF higher than 1.4 nM resulted in a reduced cAMP formation of 30.0 ± 10 pmol cAMP/mg protein after 5 s (Fig. 1A). NGF concentrations in a range between 0.7 nM and 3.5 nM stimulated a concentration dependent elevation of the IP_3 level, resulting in an elevation of 63 ± 9 pmol (± S.E.M., n = 8) IP_3/mg protein above the control level of 115 ± 6 IP_3/mg protein when 3.5 nM NGF was applied for 5 s (Fig. 1B). In contrast to the kinetics of NGF induced cAMP formation, stimulated IP_3 formation reached a saturation point within 500 ms (Fig. 1B). Concentrations below 10^{-7} nM NGF did not induce significant IP_3 formation. In further experiments we used a concentration of 1.4 nM NGF and an exposure time of 2 s, where significant changes in cAMP and IP_3 formation could be determined.

Hypothesizing that the reduced cAMP formation in the presence of 3.5 nM NGF (Fig. 1a) might be due to phosphorylation induced inactivation or desensitization phenomena of the receptor/effector [24], we studied whether kinase inhibitors influence the kinetics of NGF stimulated cAMP formation. As indicated in Table I,

Table II

rhBDNF and 7S NGF induced cAMP and IP_3 formation in PC12 cell membranes

Growth-factor (0.7 nM)	Second messenger concentration (pmol/mg protein)					
	cAMP	n	P	IP_3	n	P
Control	181 ± 6	8		115 ± 6	8	
BDNF	298 ± 19	3	< 0.01	127 ± 13	8	> 0.05
NGF	299 ± 28	5	< 0.01	156 ± 8	6	< 0.05

Effect of 1.4 nM rhBDNF and 1.4 nM 7S NGF on cAMP and IP_3 formation in PC12 cell membranes. Membrane preparations were prepared as described in section 2. BDNF and NGF were incubated with membranes for 2 s. Reactions were stopped and cAMP and IP_3 concentrations were determined as described below. The effect is indicated as pmol/mg protein. Control represents mean of basal levels from all experiments. Values are means ± S.E.M., n = 8. P = different from control group, Student's t-test.

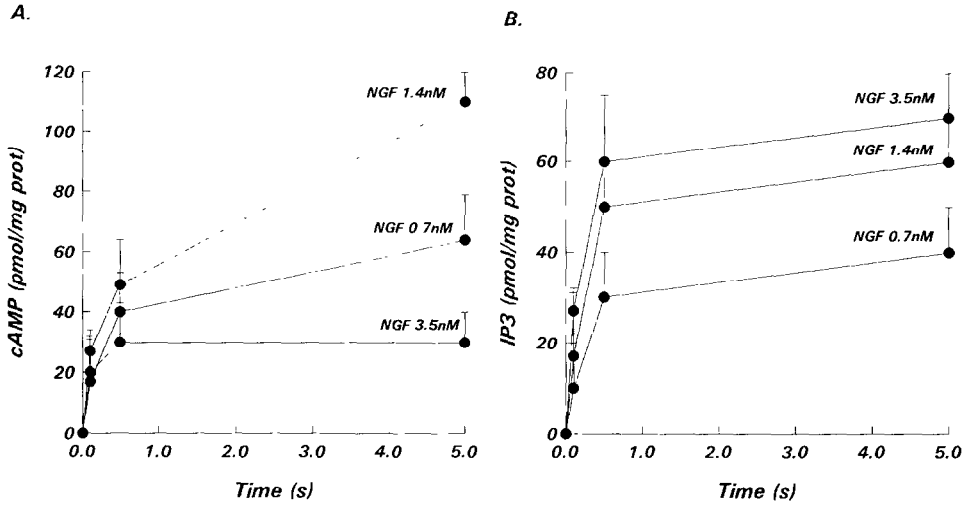

Fig. 1. Time course of NGF induced cAMP (A) and IP_3 (B) formation in membrane preparation from PC12 cells. NGF at indicated concentrations was applied to PC12 cell membrane preparations and cAMP and IP_3 formation were determined as described in section 2. The effect is indicated as change in pmol/mg protein cAMP over a basal level of 110 pmol cAMP/mg protein or IP_3 over a basal level of 115 pmol IP_3/mg protein, which is indicated as means of control value from all experiments. Data are mean ± S E.M. values from 6–8 experiments

the reduction of cAMP response could be reversed upon pretreatment with 40 μM Walsh inhibitor, a specific kinase A inhibitor [25], however not upon pretreatment with 1 μM Calphostin, a specific kinase C inhibitor [26]. Neither pretreatment with Walsh-inhibitor nor Calphostin using the same assay conditions as for cAMP, had any effect on the IP_3 response (data not shown). Thus, NGF induced cAMP formation might be controlled by a protein kinase A induced feed-back inhibition or desensitization mechanism. Further experiments will be necessary to elucidate the molecular mechanism involved. 7S NGF was used for routine experiments; all experiments were confirmed using 2.5S NGF with no difference in the result. Control of the specificity of NGF induced second messenger production was verified by the inability of inactivated NGF to induce significant cAMP and IP_3 responses (data not shown). Inactivation of NGF was performed upon boiling the NGF stock solution for 5 min in 5 mM DTT. The increase in cAMP and IP_3 caused by NGF in PC12 cell membranes was statistically significant within the first 10 s.

Further experiments were aimed at testing the receptor subtype involved in NGF and BDNF induced second messenger responses in PC12 cell membranes. As these cells do not normally respond to BDNF since they lack the high-affinity receptor for BDNF, trk^{NGFRB} [7],

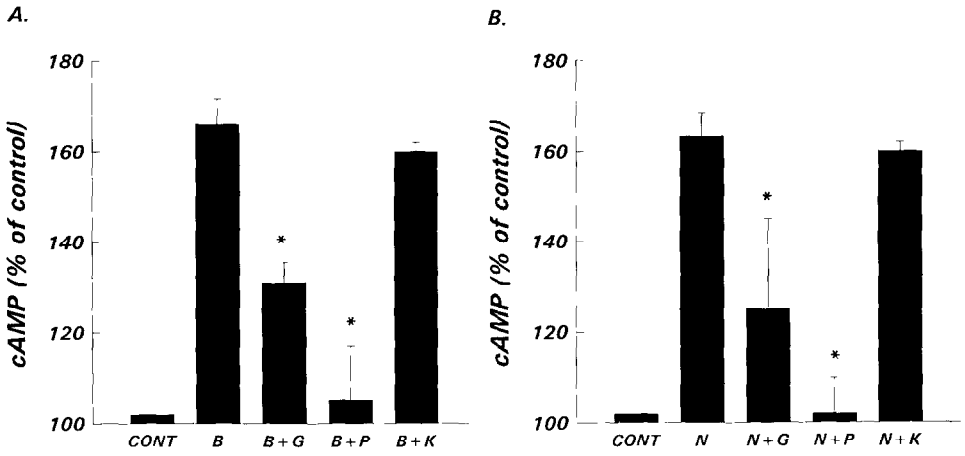

Fig. 2. BDNF (A) and NGF (B) induced cAMP formation in PC12 cell membranes in the presence of GDP-β-S (G), PTX (P), K 252a (K) after 2 s. Membranes were incubated with 50 μM GDP-β-S, 100 nM K 252a and 1 μg/ml PTX as described in section 2. PC12 cell membrane fractions were treated with 1.4 nM BDNF or 1.4 nM NGF for 2 s. The reaction was stopped and the cAMP concentration determined as described in section 2. The effect is indicated as % of control. Mean of control value from all experiments is 111 ± 6 pmol cAMP/mg protein. ± S.E.M., $n = 6$. Data are mean ± S.E.M. values from 3–6 experiments. *$P < 0.05$ when compared to B in Fig. A or to N in Fig. B.

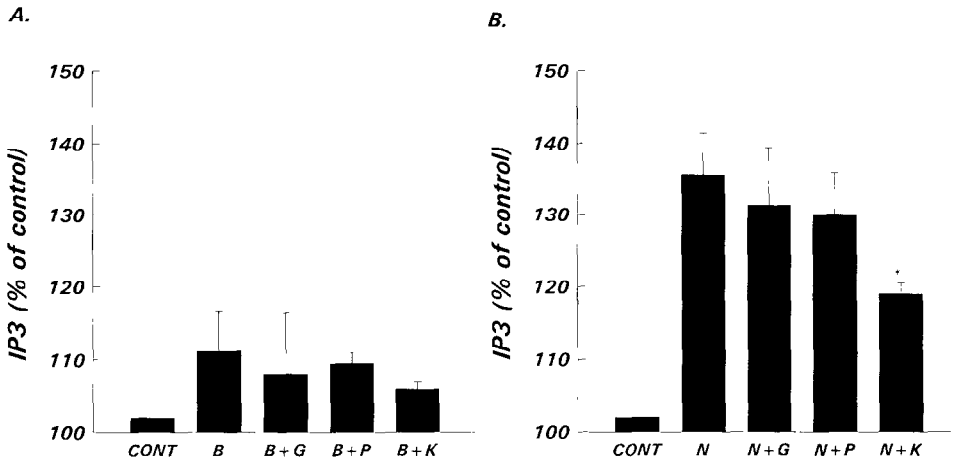

Fig 3. BDNF (A) and NGF (B) induced IP_3 formation in PC12 cell membranes in the presence of GDP-β-S (G), PTX (P), K 252a (K) after 2 s. All procedures were performed as in Fig. 2 When reactions were stopped, IP_3 concentrations were determined as described in section 2. The effect is indicated as % of control Mean of control value from all experiments is 115 ± 6 pmol IP_3/mg protein, ± S.E.M., $n = 5$. Data are mean ± S.E M. values from 3–8 experiments

they offer the possibility to distinguish between $p75^{NGFR}$ and trk^{NGFR} involved transduction pathways. rhBDNF (1.4 nM) or NGF (1.4 nM 7S NGF) induced a comparable and significant increase of cAMP about 170% of control (± S.E.M., $n = 4$–5) over the basal level of 181 ± 6 pmol cAMP/mg protein (± S.E.M., $n = 7$–8) in PC12 cell membranes (Table II). The same concentration of NGF induced an IP_3 formation of 136 ± 6% (± S.E.M., $n = 6$, $P < 0.05$) over the basal level of 115 ± 6 pmol/mg protein (± S.E.M., $n = 8$); 1.4 nM BDNF did not induce any significant IP_3 formation (Table II). This finding suggests that the IP_3, but not the cAMP responses, might be mediated by trk^{NGFR}. As it is known that the amino acid sequences of the C-terminal region of $p75^{NGFR}$ propose a transduction mechanism of G-protein coupling [11] and the trk^{NGFR} subtype propose a transduction process via its endogenous tyrosine kinase activity [14] we tested the effect of substances which are known to interrupt these transduction pathways. Thus the effect of either stable GDP-β-S analogs and ADP-ribosylation of the alpha subtype of G-proteins by pertussis toxin, or the effect of the potent inhibitor of the tyrosine protein kinase activity of the trk^{NGFR} family, K 252a [27], was studied on NGF and BDNF induced second messenger responses in PC12 cell membranes. BDNF (Fig. 2A) and NGF (Fig. 2B) induced cAMP formation could be blocked by either 50 μM GDP-β-S and 1 μg/ml PTX, but not 100 nM K 252a.

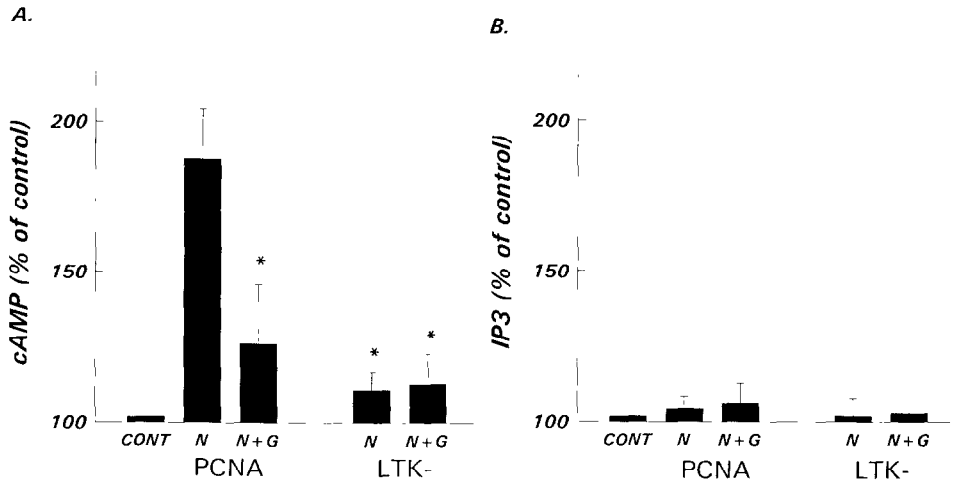

Fig. 4. NGF (N) induced cAMP (A) and IP_3 (B) formation in $p75^{NGFR}$ L-cell transfectants (PCNA) and non-transfected control cells (LTK^-) in the presence of GDP-β-S after 2 s. Membranes were prepared as described. Membranes were treated with 1 4 nM NGF in the absence or presence of 50 μM GDP-β-S for 2 s The reaction was terminated and cAMP and IP_3 determined as described in section 2. The effect is indicated as % of control. Means of control values from all experiments is 50 ± 7 pmol cAMP/mg protein, ± S.E M , $n = 5$ and 168 ± 21 pmol IP_3/mg protein, ± S E.M , $n = 4$. Data are mean ± S.E M. from 3–6 experiments. *$P < 0.01$ when compared to N value, Student's t-test

BDNF did not induce any significant IP_3 formation (Fig. 3A), supporting the concept that trk^{NGFRB}, which is lacking in PC12 cells [7], mediated BDNF stimulated IP_3 formation. NGF induced IP_3 formation, however, could not be influenced by either GDP-β-S or PTX, but was significantly reduced by K 252a (Fig. 3B). In further experiments we used $p75^{NGFR}$ expressing, trk^{NGFR}-deficient PCNA cells, which were probed for their cAMP and IP_3 responses, respectively, upon NGF application. A significant cAMP response, but no IP_3 response, was elicited in PCNA cell membranes (Fig. 4). The cAMP response upon application of NGF for 2 s was significantly reduced in the presence of stable GDP-β-S. Nontransfected LTK^- cells showed no significant cAMP formation upon NGF application (Fig. 4A). NGF could not elicit significant IP_3 formation, either in PCNA or in LTK^- (Fig. 4B). These results support the concept that the rapid formation of cAMP by nerve growth factors is transduced via the low affinity NGF receptor subtype $p75^{NGFR}$ upon a G-protein coupled transduction pathway, while the IP_3 response might be induced via activation of a tyrosine kinase, probably trk^{NGFR}, mediated pathway. Though the inhibition of the IP_3 response via K 252a, and the lack of BDNF induced IP_3 formation in trk^{NGFRB}-deficient PC12 cells, support the idea of a transduction via trk^{NGFR}, higher NGF concentrations than expected for activation of trk^{NGFR} were needed for the initiation of the IP_3 response. This may indicate the involvement of uncommon conditions in this transduction process, requiring further exploration.

The $p75^{NGFR}$ has a broad distribution not only within the CNS but also in other tissues [28]. Recently, physiological functions of this receptor type were described in transgenic mice, including involvement in morphogenesis of kidney [29] and an essential role in the development and function of sensory neurons which innervate cutaneous tissues [30]. It will be interesting to determine whether any of these functions can be connected to rapid cAMP responses. Furthermore, the known functions of NGF induced phosphorylations through the actions of protein kinase A, such as an increase in tyrosine hydroxylase activity [31], an increase in the number of sodium channels [32] and an increase in ornithine decarboxylase activity [33], should be reevaluated again as a putative response of $p75^{NGFR}$. Previous results elucidating the role of cAMP in NGF functions become now more significant; thus cAMP was unable to induce priming of PC12 cells to NGF-stimulated neurite regeneration [34], however was able to mimic some of the NGF induced phosphorylation steps upon kinase A. Bearing in mind that NGF transduces its cAMP-mediated response through the $p75^{NGFR}$ and that it has been shown recently that this does not contribute to the biological effects of neurotrophins as growth and transformation or induction of differentiation [7], could elucidate some of the contradictory results concerning the role and function of cAMP. The studies of Halegoua et al. [35], in which they did not find significantly inhibitory effects for neurite outgrowth in pertussis toxin-treated PC12 cells either before or during NGF treatment, support our findings that only the cAMP response was blocked upon PTX pretreatment.

Our finding that the rapid IP_3 formation, induced by nerve growth factors is brought about via a tyrosine kinase mediated pathway, suggests the mediation via the tyrosine kinase containing high affinity NGF receptor trk^{NGFR}. This idea is supported by the finding of Kim et al. [36], who recently found that the trk^{NGFR} is coupled to phospholipase C.

It will be interesting to see in future experiments how these rapid second messenger responses are connected to the different biological activities of either the $p75^{NGFR}$ or trk^{NGFR}.

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