Nerve growth factor stimulates MAPK via the low affinity receptor $p75^{LNTR}$

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Abstract Apart from its high affinity receptor TrkA, nerve growth factor (NGF) can also stimulate the low affinity receptor $p75^{LNTR}$ and induce a Trk-independent signaling cascade. We examined the possible involvement of mitogen-activated protein kinase (MAPK) in this signaling pathway in neuronal cultures of the cerebellum of P2-aged rats and PCNA cells; both cell types express $p75^{LNTR}$ but not TrkA. We found a fast and transient phosphorylation of p42- and p44-MAPK after stimulation with NGF or C_2-ceramide which proved to be sensitive to inhibition of MAPK kinase and protein kinase A (PKA). As stimulation with NGF also activated p21Ras it can be concluded that at least part of the observed MAPK activation was effected via p21Ras and via PKA.

Key words: Nerve growth factor; Mitogen-activated protein kinase; $p75^{LNTR}$; C_2-ceramide

1. Introduction

Nerve growth factor (NGF) and similar neurotrophins have been extensively characterized as survival and differentiation factors and as modulators of synaptic strength and neurotransmission [1,2]. All these functions could be linked to specific stimulations of neurotrophin Trk receptors, resulting in an activation of the mitogen-activated protein kinase (MAPK) cascade, of phospholipase Cγ, and of phosphatidylinositol 3-kinase [3,4].

MAPK is a serine/threonine kinase whose activity is regulated by tyrosine/threonine phosphorylation. The best characterized subtypes, p42-MAPK and p44-MAPK, are specifically phosphorylated by MAPK kinases which in turn are serine-phosphorylated by MAPK kinase kinases like Raf-1 [5,6]. p42-MAPK and p44-MAPK are also stimulated by tumor necrosis factor (TNF) inducing mitogenesis in fibroblasts [7–10].

All neurotrophins can also bind to the low affinity neurotrophin receptor $p75^{LNTR}$, and at least NGF is supposed to induce apoptosis by stimulation of this receptor [11,12]. $p75^{LNTR}$ lacks kinase activity, and only two proteins have been reported as intracellular binding partners: NRIF, a zinc finger protein with unknown function [13], and TRAF6, belonging to the TRAF proteins which bind to TNF receptors [14]. Functional and structural similarity of $p75^{LNTR}$ and TNF receptors suggests similar signaling, and Dobrowsky et al. [15] could demonstrate activation of the sphingomyelinase pathway after $p75^{LNTR}$ stimulation. Recently Boone and co-workers [7] observed activation of MAPK by the receptor p55 of TNF with participation of the receptor's 'death domain'; an analogous participation of the death domain of $p75^{LNTR}$ in the signaling of this receptor may be conjectured.

In the present study we wanted to investigate if $p75^{LNTR}$ stimulation involves MAPK activation. We chose neuronal cultures of the cerebellum of P2-aged rats (cerebellar granule cells, CGC). At this stage of development these neurons produce relatively high levels of $p75^{LNTR}$ while no TrkA is detectable. We compared the obtained results with those on PCNA cells (L-fibroblasts transfected stably with $p75^{LNTR}$ cDNA [16]) where $p75^{LNTR}$ signaling can be analyzed without interference from TrkA signaling. We found that stimulation of $p75^{LNTR}$ with NGF or application of C_2-ceramide leads to an activation of MAPK via activated MAPK kinase which in turn probably was activated by p21Ras since NGF stimulation also increased activated p21Ras.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium, glutamine, trypsin–EDTA, and penicillin/streptomycin were obtained from Gibco (Eggenstein, Germany), fetal calf serum from Greiner (Frickenhausen, Germany) and 2.5S β-NGF from Alomone (London, UK). Antibodies, used in the indicated dilution, were obtained from the following sources: anti-phospho-MAPK (1:1000): New England Biolabs (Schwalbach, Germany); anti-phosphotyrosine (1:2000): Upstate Biotechnology/Biomol (Hamburg, Germany); anti-$p75^{LNTR}$ (MC192, 800 ng/ml): Roche Diagnostics (Mannheim, Germany); anti-mouse horseradish peroxidase (HRP; 1:2000): Sigma (Munich, Germany). [^{32}P]Phosphatidic acid was purchased from Du-Pont NEN (Zaventem, Belgium). C_2-ceramide was from Biomol, PKI-tide from Bachem (Heidelberg, Germany), protein A-Sepharose from Sigma/Fluka (Munich, Germany), and IBMX and all other agents were obtained from Sigma (Munich, Germany) if not otherwise indicated.

2.2. Cell culture

Primary cultures of CGC were prepared from Wistar rats at the developmental stage P2 as described for hippocampal neurons [17]. PCNA cells (a kind gift from E.M. Shooter, Stanford, CA, USA) were grown as a monolayer in Dulbecco's modified Eagle's medium/10% fetal calf serum/1 mM Glutamax. PC12 cells (H. Thoenen, Martinsried, Germany) were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum/5% horse serum/1 mM Glutamax. For experiments, all types of cells were seeded on 35 mm dishes (Sarstedt, Nümbrecht, Germany) at a density of 5×10^5 and on 24 well plates at 2.5×10^5 cells per well and used for experiments on the third day in culture.

2.3. RT-PCR (reverse transcriptase PCR)

For the detection of TrkA and $p75^{LNTR}$ expression, RNA was isolated, transcribed with Superscript (Gibco BCR, Eggenstein, Germany) and oligo-dT primer (24-mer) into cDNA, and PCR was per-

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formed using the primers 5′-AGAAGAAGGACGAAACACCT-3′ and 5′-TCACTGAAGTACTGTGGGTT-3′ for TrkA detection and 5′-CGCCATGAGGAGGGGAGGTG-3′ and 5′-AGACCTTGGGA-TCCATCGAC-3′ for $p75^{LNTR}$ detection. The PCR program for TrkA (hot start) was: 5 min 95°C; 30 cycles of 95°C (1 min), 60°C (1 min, +0.1°/cycle), 72°C (1 min); 10 min 72°C. An analogous program was executed for $p75^{LNTR}$ but 40 cycles with an annealing temperature of 62°C (1 min, +0.1°/cycle) were used.

2.4. Stimulation, lysis, immunoprecipitation, analysis by Western blot and analysis of p21Ras activation

Cells were stimulated by adding neurotrophins or agents (dissolved in medium) to the cells (without changing the medium). Inhibitors were added 30 min prior to stimulation. For the sake of brevity, the expression 'after stimulation' will be used instead of 'after the start of stimulation'.

After stimulation cells were placed on ice and lysed with 60 μl per 35 mm dish and 30 μl per well of 24 well plates with a buffer containing 50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, 1% (v/v) Nonidet P40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride for 10 min (4°C) on a shaker. Lysates were centrifuged for 10 min at 14 000 rpm (4°C), and the supernatant was frozen for further use at −20°C.

For immunoprecipitation, equal amounts of protein were incubated with the antibody at concentrations suggested by the suppliers for 4 h at 4°C on a shaker. Protein A coupled to Sepharose was added at a concentration of 100 μg/ml and the incubation was continued overnight at 4°C; the antibody–protein–protein A complex was separated by centrifugation (14 000 rpm, 4°C, 5 min), washed once with 500 mM LiCl in 100 mM Tris–HCl, pH 7.4, and twice in 10 mM Tris–HCl, pH 7.4, then denatured in Laemmli sample buffer (10 min at 95°C) and analyzed with SDS-PAGE.

Equal amounts of protein (10–15 μg/slot) were analyzed with SDS-PAGE. Western blots were performed as suggested by the suppliers of the antibodies, and detected with enhanced chemiluminescence (Pharmacia/Amersham Corp., Freiburg, Germany). For semiquantification, Western blots were scanned and densitometrically analyzed with the program TINA 2.09 (Raytest Isotopenmeßgeräte; Straubenhardt, Germany).

Analysis of p21Ras activation was performed in PCNA cells or CGC, seeded at a density of 10^6 per 35 mm dish on day 2 prior to experiments, according to Valius and Kazlauska [18]. The amount of radioactivity present in GTP and GDP was quantified with a phospho-imager (BioImager Bas 100C) and evaluated with the program TINA 2.09.

3. Results and discussion

MAPK activation plays a significant role in the signaling of neurotrophins. Since MAPK is transferred into the nucleus soon after activation, cytosolic as well as nuclear targets can be influenced by the enzyme. Typically tyrosine kinase-induced MAPK activation will be sustained over hours, even days. In the present study we describe activation of MAPK after stimulation of $p75^{LNTR}$ with NGF which is relatively fast and short-lived in contrast with Trk-mediated activation.

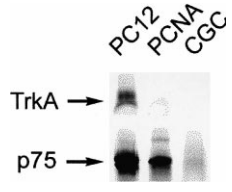

Fig. 1. RT-PCR of TrkA and $p75^{LNTR}$ was performed with mRNA of PC12 cells (serving as a positive control expressing TrkA and $p75^{LNTR}$), PCNA cells and CGC. The PCR products are marked by arrows.

3.1. MAPK is activated after stimulation of $p75^{LNTR}$ with NGF

We used PCNA cells and CGC (developmental stage P2) for our experiments. With RT-PCR we ensured that both cell types did not express TrkA (Fig. 1). In PCNA cells and K252a-pretreated CGC cultures, p44-MAPK and p42-MAPK (in the following collectively referred to as MAPK) were activated by NGF-mediated stimulation of $p75^{LNTR}$ within 3 min (PCNA, Fig. 2A) and 5 min (CGC, Fig. 2B). This effect subsided within 30–60 min to control levels. In CGC pretreatment with K252a was necessary to inhibit activation of MAPK via Trk receptors which are stimulated by other neurotrophins (BDNF or NT-3) spontaneously released within the culture.

MAPK activation can be affected by the activation of sphingomyelinase [9,10]. $p75^{LNTR}$ is known to activate sphingomyelinase and elevate ceramide levels [15]; the receptor-mediated onset of ceramide production takes place 10–15 min [15,19] after stimulation. Since we observed a fast MAPK activation after NGF stimulation (3–5 min) ceramide cannot be the only cause of MAPK activation during $p75^{LNTR}$ signaling. To examine how ceramide influences this MAPK activation we stimulated PCNA cells and CGC with C_2-ceramide, a membrane-passing, short form of the natural long acyl chain ceramide. In PCNA cells C_2-ceramide caused stimulation of MAPK similar to NGF (Fig. 2A) but with a slight temporal shift. With CGC, however, the observed activation pattern was more complex. Without inhibition of Trk receptors by K252a we first observed a slight reduction of activated MAPK (5–15 min), then levels comparable to controls, and after about 1 h a distinct reduction. We performed a semiquantification using densitometric analysis of the Western blots. Compared to the control (100% ± 32), p44-MAPK was reduced to 57% (± 12) at 15 min, slightly increased to 158% (± 19) at 60 min, and decreased again to 60% (± 23) and 45% (± 15) at 2 and 4 h, respectively. Treatment with K252a resulted in a peak at 5 min (137% ± 29) and in a pronounced elevation of activated MAPK after 30 min (283% ± 34), and subsided after 1 and 4 h to 160% (± 41) and 127% (± 38), respectively. These data (obtained in four independent experiments) suggest an interference between TrkA and sphingomyelin pathway, which requires separate investigation. Several authors [20,21] described inhibitory effects of Trk signaling on $p75^{LNTR}$, and conversely inhibition of TrkA activation by serine phosphorylation via C_2-ceramide has been described [22]. While the same authors [22] recently reported a ceramide-induced activation of TrkA-tyrosine autophosphorylation in PC12 cells after longer treatment (>1 h) with C_2-ceramide [23], our data for CGC (cells that express TrkB and TrkC but not TrkA) do not support this result as we observed inhibition of MAPK activation after 1 h of treatment with C_2-ceramide and Trk stimulation should induce MAPK activation.

3.2. Some elements of the mechanism of MAPK phosphorylation

To analyze the mechanism of MAPK activation via $p75^{LNTR}$, we examined the involvement of MAPK kinase which could be blocked with PD-098059. This pretreatment nearly completely inhibited NGF- and C_2-ceramide-mediated MAPK activation (Fig. 2A). Therefore certainly MAPK kinase steps between $p75^{LNTR}$ and MAPK. As this observation indicated a Ras-Raf-dependent pathway we studied a possible

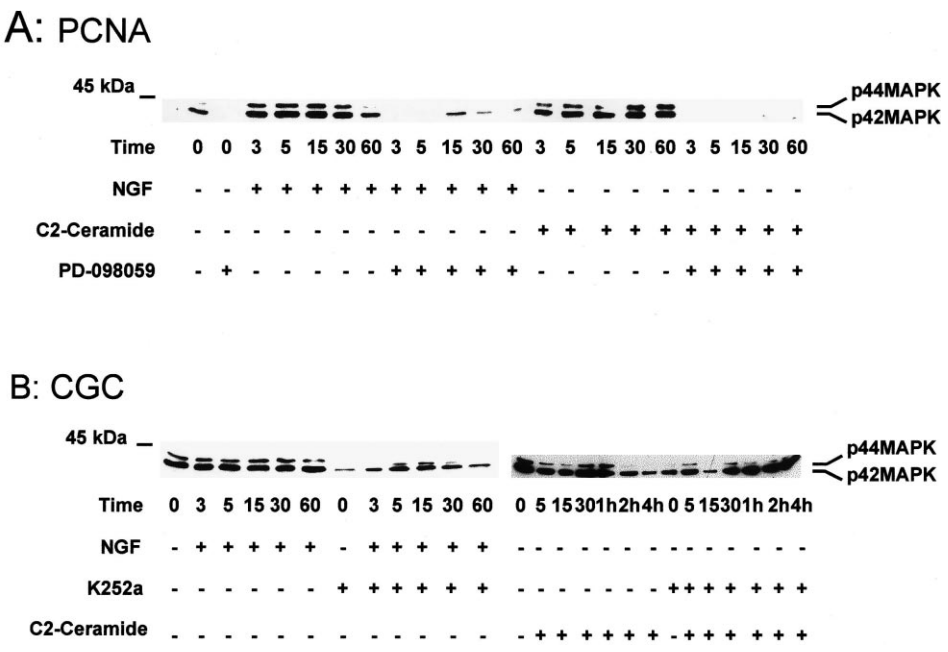

Fig. 2. In PCNA cells (A) and CGC (B), NGF and C_2-ceramide induce MAPK phosphorylation. Cells were stimulated over the indicated time (min) either with NGF (100 ng/ml) or with C_2-ceramide (100 μM); MAPK inhibitor PD-098059 (10 μM) or tyrosine kinase inhibitor K252a (100 nM) were added 30 min prior to stimulation. +/− signs indicate application/absence of the respective agent. Cells were lysed and equal amounts of protein were analyzed by Western blotting with monoclonal phospho-specific MAPK antibodies. The results shown are representative for four independent experiments.

interaction of $p75^{LNTR}$ with p21Ras. Using a very sensitive radioactive assay we observed a p21Ras activation which was similar to that in positive controls where PCNA cells were stimulated with platelet-derived growth factor (PDGF), known for signaling via p21Ras [18], and CGC with neurotrophin-4 (Table 1).

In PC12 cells (with TrkA and $p75^{LNTR}$) Volonté and co-authors [24] observed an association of activated MAPK with $p75^{LNTR}$ mediated by TrkA. For our experiments we can rule out such an interaction between TrkA, $p75^{LNTR}$ and MAPK as there was no TrkA. We could co-precipitate phosphorylated MAPK and tyrosine-phosphorylated $p75^{LNTR}$ (Fig. 3) which suggests a common compartment or even a complex of the two proteins although $p75^{LNTR}$ is tyrosine-phosphorylated and certainly cannot be phosphorylated by MAPK, a serine/threonine kinase.

Other protein kinases might possibly be involved in MAPK activation. Impey et al. [25] reported that MAPK phosphorylation can depend on protein kinase A (PKA) and that translocation of MAPK into the nucleus is reduced by PKA inhibitors. Indeed, we found a reduced and delayed MAPK phosphorylation after PKA inhibition (peak at 15 min after stimulation with NGF) and elevated activation after application of IBMX, which activates PKA (by inhibiting phosphodiesterase), suggesting PKA as upstream activator of MAPK (Fig. 4); PKA-mediated MAPK activation might be connected with translocation of MAPK into the nucleus.

We conclude that stimulation of $p75^{LNTR}$ with NGF or application of C_2-ceramide leads to an activation of MAPK requiring activated MAPK kinase. As p21Ras activity also increases after stimulation of $p75^{LNTR}$, the receptor signaling probably proceeds via p21Ras to activation of MAPK kinase and MAPK. Part of the observed MAPK activation should be effected via PKA.

Table 1
NGF activates p21Ras via $p75^{LNTR}$

Treatment	PCNA (% GTP)	CGC (% GTP)
0 min NGF	4.77 ± 0.78 (n = 3)	12.16 ± 2.76 (n = 2)
2 min NGF	8.54 ± 1.30* (n = 3)	n.d.
5 min NGF	6.36 ± 2.67 (n = 3)	17.39 ± 6.22 (n = 2)
5 min PDGF	12.49 ± 2.00 (n = 2)	n.d.
5 min NT-4	n.d.	35.44 ± 10.44 (n = 2)
K252a/0 min NGF	4.47 ± 0.98 (n = 3)	15.46 ± 1.17 (n = 2)
K252a/2 min NGF	10.67 ± 1.21 (n = 2)	18.90 ± 2.26 (n = 2)
K252a/5 min NGF	7.36 ± 1.76 (n = 3)	26.88 ± 4.92 (n = 2)

PCNA cells and CGC were stimulated with NGF (100 ng/ml), PDGF (10 ng/ml) and NT-4 (10 ng/ml) for the indicated time and p21Ras activation was measured as described. Results are presented as percentage of GTP to total guanine nucleotides. Some cultures were pretreated with K252a (100 nM). n.d. = not determined; *$P < 0.05$ to control (t-test).

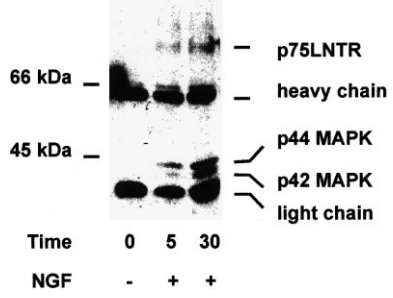

Fig. 3. The receptor $p75^{LNTR}$ is phosphorylated by NGF stimulation and can be co-precipitated with phosphorylated MAPK. PCNA cells were stimulated with NGF (100 ng/ml) for 5 min. $p75^{LNTR}$ was precipitated with the monoclonal antibody MC192 from the cell lysate and analyzed by Western blotting with a monoclonal phosphotyrosine-specific antibody.

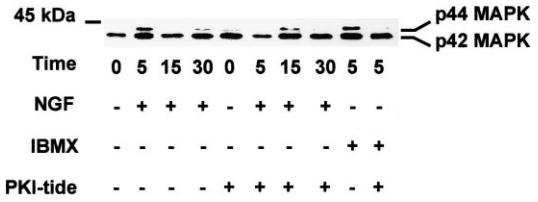

Fig. 4. Influence of PKA on NGF-induced phosphorylation of MAPK. PCNA cells were stimulated with NGF (100 ng/ml) over the indicated time (min). For inhibition of PKA, PKI-tide (100 μM) was added 30 min prior to stimulation. To activate PKA the cells were stimulated with the inhibitor of phosphodiesterase IBMX (200 μM). The cells were lysed and equal amounts of protein were analyzed by Western blotting with monoclonal phospho-specific MAPK antibodies. The results shown are representative for two independent experiments.

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