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MS-430, a Synthetic Pyrimidine Derivative, Influences the Intracellular Signal Transduction Pathway Leading to Neuronal Differentiation of PC12h Cells[1]

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Although NGF (nerve growth factor) induces neuronal differentiation of PC12 cells, EGF (epidermal growth factor) acts as a mitogen for these cells. We have studied the effects of a synthetic pyrimidine derivative, MS-430, on the NGF and EGF actions on PC12h cells. We found that MS-430 accelerated NGF-induced neurite extension of PC12h cells and that, in the presence of MS-430, PC12h cells extended neurites in response to EGF. Next, we investigated the tyrosine phosphorylation of NGF receptor TrkA and the EGF receptor (EGFR) as well as mitogen-activated protein kinase (MAPK), which is a key protein in the intracellular signal transduction pathway. It was found that MS-430 prolonged the EGF-induced phosphorylation of EGFR and MAPK compared to that without MS-430. MS-430 also prolonged the NGF-induced phosphorylation of MAPK, but the phosphorylation of TrkA induced by NGF was not affected by MS-430. These results suggest that MS-430 influences the intracellular signal transduction pathway which causes the neuronal differentiation of PC12h cells.

Key words: EGF, MAP kinase, MS-430, neuronal differentiation, NGF.

Nerve growth factor (NGF) is a well-characterized target-derived neurotrophic factor for sympathetic and sensory neurons in the peripheral nervous system, and for certain kinds of neurons in the central nervous system (*1, 2*). NGF is the prototype of a family of NGF-related proteins known as neurotrophins, which include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and so on. The neurotrophins bind to and activate specific receptor tyrosine kinases of the Trk family (*3, 4*): NGF binds to and activates the TrkA receptor.

Epidermal growth factor (EGF) is one of the well-known conventional mitogenic factors and stimulates the proliferation of various types of cells (*5, 6*). In the central nervous system, EGF is known to act as a neurotrophic factor for several types of neurons. For example, EGF enhances the survival and process outgrowth of cultured subneocortical telenpalic, cerebral cortical and cerebellar neurons (*7-9*).

The rat pheochromocytoma cell line, PC12, has proved to be a useful model for investigating the mechanisms of action of NGF and EGF (*10*). PC12 cells differentiate into sympathetic neuron-like cells in response to NGF. PC12 cells treated with NGF show marked cellular changes including extension of neurites, cessation of mitosis, increased neurotransmitter biosynthesis, and development of Na^+ and Ca^{2+} channels. Unlike NGF, EGF does not induce neuronal differentiation but promotes the proliferation of PC12 cells (*11*).

NGF and EGF bind to and activate TrkA and the EGF receptor (EGFR), respectively, which each possess a tyrosine kinase domain (*12*). The activated receptor tyrosine kinases are autophosphorylated at tyrosine residues, and then initiate intracellular signal transduction pathways which culminate in specific programs of gene expression and cellular responses (*4*). The tyrosine-phosphorylated receptor serves as scaffolding for the recruitment of several enzymes and effectors, including phospholipase C-γ (PLC-γ) (*13-15*), phosphatidylinositol 3-kinase (PI3-K) (*16, 17*), and the adapter protein, Shc (*15, 17*). Each of these molecules activates a distinct pathway which has a different function. The Ras-MAP kinase pathway initiated by Shc is considered to be involved in the neuronal differentiation and proliferation of PC12 cells caused by NGF and EGF, respectively. On the other hand,

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Abbreviations: AChE, acetylcholinesterase; DMEM, Dulbecco's modified Eagle's medium; EGFR, EGF receptor; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MEK, MAPK-ERK kinase; NGF, nerve growth factor; PI3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecyl sulfate; Shc, Src homologous and collagen protein.

the PI3-K pathway may be involved in neuronal survival promoted by NGF (*18*). The Ras-MAP kinase pathway is composed of Shc, Grb2, Sos, Ras, Raf, MAP kinase kinase (MAPKK, also known as MEK), and MAP kinase (MAPK, also known as Erk) (*4*). Shc is phosphorylated at tyrosine residues by the activated Trk receptor and MAPK is phosphorylated by the activated MAPKK.

As mentioned above, both NGF and EGF activate the Ras-MAPK pathway, but they can induce different cellular responses of PC12 cells (*3, 12*). It has been proposed that the duration of signaling activated by these factors decides the specificity of cellular responses (*19*). NGF induces sustained activation of Ras and MAPK, but EGF does so only transiently in PC12 cells (*20, 21*). In addition, the overexpression of constitutively active Ras, Raf, or MAPKK evokes neurite outgrowth in PC12 cells (*22-24*). We recently reported that PC12h-R, a subclone of PC12 cells, differentiated into neuron-like cells in response to EGF as well as NGF (*25, 26*). PC12h-R cells treated with EGF extended neurites, and exhibited attenuated cell proliferation, and increased levels of tyrosine hydroxylase protein synthesis and acetylcholinesterase (AChE) activity as those treated with NGF. In addition, EGF induced sustained tyrosine phosphorylation of EGFR and MAPK, and prolonged activation of MAPK in PC12h-R cells, but not in the parent PC12h cells, which do not show EGF-induced differentiation. Taken together, these observations indicate that induction of the neuronal differentiation of PC12 cells requires sustained activation of the receptor tyrosine kinase-Ras-MAPK pathway. However, it remains unknown how the duration of NGF- and EGF-induced signaling is regulated.

In the present study, we have examined the effects of a synthetic pyrimidine derivative, MS-430 (*27, 28*), on the NGF- and EGF-induced responses and signaling pathways in PC12h cells. MS-430 influences the neuronal responses and prolongs the signaling in PC12h cells.

MATERIALS AND METHODS

Materials—NGF was prepared from male mouse submandibular glands according to the method of Bocchini and Angeletti (*29*) modified according to Suda *et al.* (*30*). EGF was purchased from Toyobo. Anti-pan-Trk antiserum was raised in rabbits against a synthetic peptide corresponding to the 14 carboxyl-terminal amino acids of human TrkA (*25, 26*). The anti-phosphotyrosine monoclonal antibody, 4G10, was purchased from Upstate Biotechnology. The anti-phosphorylated MAP kinase antibody was obtained from New England Biolab. MS-430 was dissolved in distilled water as described previously (*27, 28*).

Cell Culture—The PC12h cell line is a subclone of PC12 cells which differentiates into neuron-like cells in response to NGF similarly to PC12 cells (*31*). PC12h cells were maintained in 75 cm^2 flasks (Costar) using Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% (v/v) precolostrum newborn calf serum (PNCS; Mitsubishi Kagaku) and 5% (v/v) heat-inactivated (56°C, 30 min) horse serum (HS; Gibco). Cells were cultured in 100-mm collagen-coated Falcon dishes (surface area, 55 cm^2; to examine tyrosine phosphorylation of TrkA), 60-mm collagen-coated Falcon dishes (surface area, 21 cm^2; to detect tyrosine phosphorylation of MAPK or EGFR), collagen-coated 6-well Costar plates (surface area, 9 cm^2; to assay AChE activity), or collagen-coated 24-well Costar plates (surface area, 2 cm^2; to observe neurite outgrowth). Cells were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin and 20 nM progesterone (TIP/DMEM) (*32*) (to observe neurite outgrowth and to assay AChE activity), or in DMEM containing serum (to detect the tyrosine phosphorylation of TrkA, EGFR or MAPK). To detect tyrosine phosphorylation of TrkA, EGFR, or MAPK, the medium was changed to that without serum 4 h before adding NGF or EGF. To investigate the effect of MS-430 on the tyrosine phosphorylation of these molecules, it was added (or not), at a concentration of 1 mM, 2 h before the addition of NGF or EGF.

Assaying of Acetylcholinesterase (AChE) Activity—The cells were washed once with phosphate-buffered saline and then lysed by sonication in ice-cold 20 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100, 10 mM $MgCl_2$, and 150 mM NaCl. The lysates were assayed for acetylcholinesterase (AChE) activity using 0.5 mM acetylthiocholine (Sigma) and 0.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (*33*). The enzyme reaction proceeded at 37°C and was stopped by the addition of an equal volume of 5% SDS. The absorbance was measured photometrically at 412 nm. The specific activity was calculated on the basis of the protein concentrations of the enzymatically assayed samples determined with a BCA protein assay kit (Pierce).

Immunoprecipitation and Immunoblotting—PC12h cells were washed once with ice-cold Tris-buffered saline (TBS), and then lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 5 μg/ml aprotinin to detect the tyrosine phosphorylation of TrkA. Lysates were centrifuged at 10,000 × *g* at 4°C for 30 min, and then the protein concentrations of the clarified lysates were determined by means of the BCA protein assay. Excess antibodies were added to the lysates; *i.e.* anti-pan-Trk antiserum (10 μl) for the detection of TrkA tyrosine phosphorylation, was added, followed by incubation at 4°C for 1-3 h. Protein A-Sepharose (30 μl) was then added, and the tubes were rotated at 4°C for 30-60 min. The immune complexes were pelleted by centrifugation at 10,000 × *g* at 4°C for 1 min, and then washed 3-5 times with lysis buffer. The immune complexes were eluted with the sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β-mercaptoethanol], boiled for 3 min, and then recovered by centrifugation for 5 min in a microfuge. The eluates were resolved by electrophoresis on 4-20% SDS-polyacrylamide gels. The gels were then immunoblotted with anti-phosphotyrosine antibody 4G10 to detect the tyrosine phosphorylation of TrkA.

Cells were washed once with ice-cold TBS, lysed in a buffer containing 1% SDS, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM Tris-HCl pH 7.5, and 1 mM PMSF to detect the tyrosine phosphorylation of MAPK or EGFR, and then boiled for 5 min. The lysates were clarified by centrifugation at 10,000 × *g* at 4°C for 1 h. The protein concentrations were determined by means of the BCA protein assay, and then 20 μg aliquotes of protein was resolved by electrophoresis on 4-20% SDS-polyacrylamide gels.

Proteins were transferred to nitrocellulose membranes in 0.1 M Tris base, 0.192 M glycine, and 20% (v/v)

methanol using a semi-dry electrophoretic transfer system. The membranes were blocked with 5% (w/v) nonfat dried milk and 0.1% Tween 20 in TBS at room temperature for at least 1 h, washed three times in 0.1% Tween 20/TBS (T-TBS), and then incubated with anti-phosphotyrosine antibody 4G10 diluted to 1:1,000, or the anti-phosphorylated MAP kinase antibody diluted to 1:1,000 in T-TBS, at room temperature for 1-2 h. After 3 washes with T-TBS, the membranes were incubated with the horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham) diluted to 1:1,000 with T-TBS at room temperature for 1 h. The membranes were then washed four times with T-TBS, and visualized using an ECL chemiluminescence system (Amersham).

RESULTS

Effect of MS-430 on NGF-Induced Neuronal Differentiation of PC12h Cells—MS-430 (2-piperidino-5,6-dihydro-7-methyl-6-oxo(7*H*)pyrrolo[2,3-*d*]pyrimidine maleate) is a synthetic pyrimidine heterocyclic compound (*28*) and is illustrated in Fig. 1. Awaya *et al.* reported that its isomeric analogue MS-818 promoted the neurite outgrowth of neuroblastoma cell lines and potentiated NGF-induced neurite sprouting of PC12 cells (*27*). To study the effect of MS-430 on NGF-induced neurite outgrowth in PC12h cells, we cultured PC12h cells for 3 d after NGF addition in the presence or absence of 0.3 mM MS-430. As shown in Fig. 2 (C and D), the percentage of neurite-bearing cells treated with both NGF and MS-430 was larger than that treated with NGF alone. However, when cells were cultured for longer than 3 d, the percentage of neurite-bearing cells treated with NGF alone reached that in the case of cells treated with both NGF and MS-430 (data not shown). Figure 3 shows the dose-dependent stimulation by MS-430 of neurite outgrowth in NGF- treated cells. While only 35% of the PC12h cells treated for 3 d with NGF alone extended neurites, about 65% of the cells extended neurites in the presence of NGF and 0.3 mM MS-430. These results indicate that MS-430 accelerates NGF-induced neurite extension in PC12h cells. However, concentrations of MS-430 higher than 0.3 mM had a toxic effect on PC12h cells (data not shown).

MS-430 Induces Neurite Outgrowth in EGF-Treated

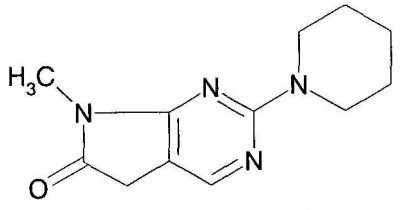

Fig. 1. **Chemical structure of MS-430.**

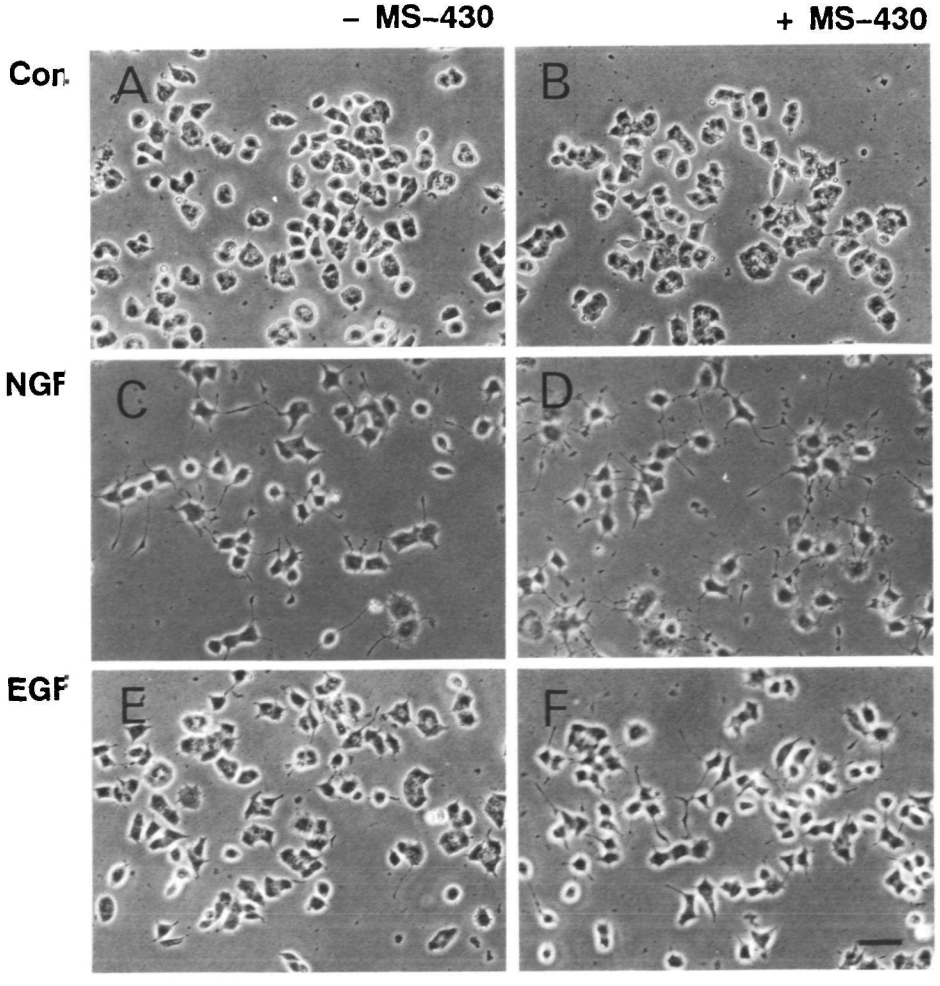

Fig. 2. **Effects of MS-430 on the morphological responses induced by NGF and EGF.** PC12h cells were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 20 nM progesterone in the absence (A, C, E) or presence (B, D, F) of 0.3 mM MS-430, without (A, B) or with 50 ng/ml NGF (C, D) or 50 ng/ml EGF (E, F) for 3 d. Photographs were taken under a phase-contrast microscope. The bar represents 10 μm.

PC12h Cells—Next, we investigated the effect of MS-430 on EGF-treated PC12h cells. EGF is mildly mitogenic for PC12h cells, and PC12h cells grown with EGF alone scarcely extended neurites (Fig. 2E). In contrast, approximately 20% of the PC12h cells extended neurites of longer than one cell body when treated with the combination of EGF and MS-430 for 3 d (Figs. 2F and 3). These data indicate that MS-430 can induce neurite outgrowth in EGF-treated PC12h cells. However, even when PC12h cells treated with EGF and MS-430 were cultured for longer than 3 d, the length of neurites did not increase thereafter (data not shown).

Effect of MS-430 on NGF- and EGF-Induced AChE Activity—When PC12h cells differentiate into neuron-like cells in response to NGF, the activity of acetylcholinesterase (AChE), as a marker enzyme, in these cells is known to increase. Therefore, AChE activity was assessed *in vitro* in PC12h cells treated for 4 d with NGF alone or NGF plus MS-430. As shown in Fig. 4A, AChE activity after treatment with NGF alone was ~2-fold that in untreated cells, whereas AChE activity after treatment with NGF plus MS-430 was increased to 3-4-fold. MS-430 alone had no effect on AChE activity. These results indicate that MS-430 does not only stimulate neurite outgrowth, but also increases the AChE activity in NGF-treated PC12h cells.

Next, we measured AChE activity in PC12h cells treated for 6 d with EGF alone or the combination of EGF and MS-430 (Fig. 4B). While AChE activity was scarcely increased by EGF alone, it was induced over 2-fold by EGF plus MS-430. On the other hand, MS-430 alone had only a little effect on AChE activity. These data demonstrate that EGF can induce neuronal differentiation of PC12h cells with the coexistence of MS-430, although EGF alone induces proliferation of the same cells.

Effect of MS-430 on NGF-Induced Tyrosine Phosphorylation of MAPK—As mentioned above, MS-430 was found to influence the neuronal responses induced by NGF and EGF. To elucidate the mechanisms underlying these synergistic responses induced by MS-430 and NGF, we examined the effect of MS-430 on the tyrosine phosphorylation of MAPK in NGF-treated PC12h cells. It has been reported that NGF induces sustained tyrosine phosphorylation and activation of MAPK, but that EGF does so only transiently in PC12 cells (*19-21*). First, to detect the tyrosine phosphorylation of MAPK, PC12h cells were cultured for 5 min, 1.5, 3, or 5 h in the presence of NGF following pretreatment for 2 h with or without MS-430, and then total lysates were analyzed by Western blotting with an anti-phosphotyrosine antibody (4G10). As shown in Fig. 5A, the tyrosine phosphorylation of two MAPKs, Erk1 and Erk2, was observed 5 min after NGF addition in PC12h cells pretreated with or without MS-430. The level of tyrosine phosphorylation of MAPK in MS-430-treated PC12h cells 1.5 h after NGF exposure was higher compared with that in control MS-430-untreated PC12h cells. In the experiment in Fig. 5C, on the other hand, we used an anti-phosphorylated MAPK antibody instead of the anti-phosphotyrosine antibody. It should be noted that the former is more sensitive than the latter, and thus it can be used to detect low levels of tyrosine phosphorylation of MAPK. Figure 5C shows that MS-430 could induce the tyrosine phosphorylation of MAPK in PC12h cells in the absence of NGF, and as a result it could increase the level of tyrosine phosphorylation of MAPK in NGF-treated PC12h cells from 5 min to 3 h after NGF addition.

Effect of MS-430 on NGF-Induced Tyrosine Phosphorylation of TrkA—To determine whether or not MS-430 has some effect on the NGF-induced autophosphorylation of

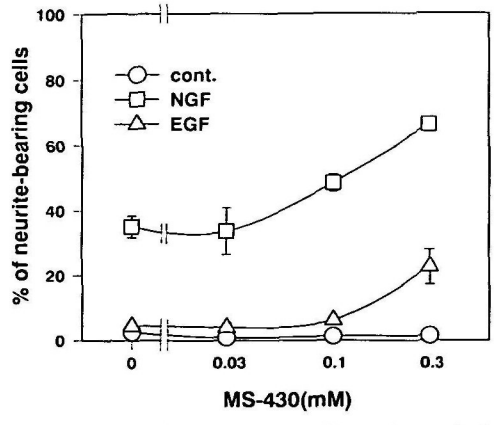

Fig. 3. **Effect of MS-430 on the neurite outgrowth in PC12h cells.** PC12h cells were cultured in DMEM supplemented with 5 μg/ml human transferrin, 5 μg/ml bovine insulin, and 20 nM progesterone in the presence of the indicated concentrations of MS-430, without (cont.) or with 50 ng/ml NGF (NGF) or 50 ng/ml EGF (EGF) for 3 d. The percentage of cells that extended neurites of longer than one cell body was determined. The values represent means ± SD for four individual cultures.

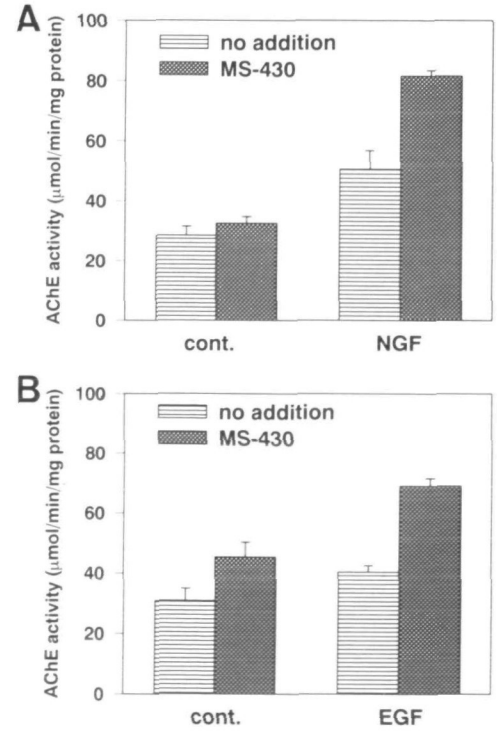

Fig. 4. **Effect of MS-430 on the activity of acetylcholinesterase (AChE).** A: The AChE activity in PC12h cells treated without (cont.) or with 50 ng/ml NGF (NGF) for 4 d, in the absence (no addition) or presence of 0.3 mM MS-430 (MS-430), was determined. B: The AChE activity in PC12h cells treated without (cont.) or with 50 ng/ml EGF (EGF) for 6 d, in the absence (no addition) or presence of 0.3 mM MS-430 (MS-430), was determined.

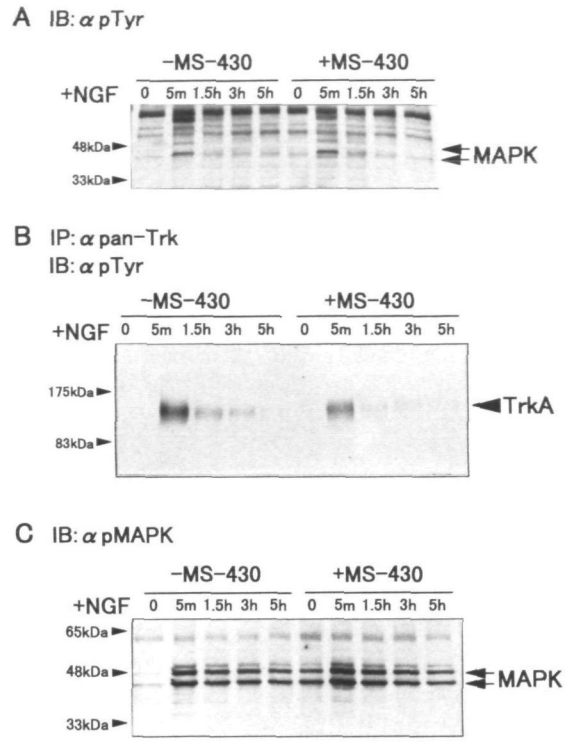

Fig. 5. **Effects of MS-430 on the NGF-induced tyrosine phosphorylation of TrkA and MAPK.** PC12h cells were cultured for 0 min (0), 5 min (5 m), 1.5 h (1.5 h), 3 h (3 h), or 5 h (5 h) in the presence of 100 ng/ml NGF following pretreatment for 2 h without (−MS-430) or with 1 mM MS-430 (+MS-430), and then the total lysates (A, C) or immunoprecipitates obtained with the anti-pan-Trk antibody (B) were analyzed by Western blotting with the anti-phosphotyrosine antibody (A, B) or the anti-phosphorylated MAPK antibody (C). The positions of MAPK (Erk1 and 2) are indicated by filled arrows. The position of TrkA is indicated by a filled arrowhead.

TrkA, lysates of PC12h cells treated with NGF alone or NGF plus MS-430 were immunoprecipitated with an anti-pan-Trk antibody and then analyzed by Western blotting with the anti-phosphotyrosine antibody (4G10). As shown in Fig. 5B, MS-430 neither increased the level of tyrosine phosphorylation of TrkA nor prolonged its duration. These results indicate that MS-430 influences the tyrosine phosphorylation of MAPK, but not that of TrkA, in the NGF-induced signal transduction pathway. The enhancing effect of MS-430 on NGF-induced differentiation of PC12h cells, including neurite extension and AChE activation, may be due to the increased level of tyrosine phosphorylation of MAPK.

Effect of MS-430 on EGF-Induced Tyrosine Phosphorylation of MAPK—To elucidate the mechanisms of synergistic neuronal responses induced by MS-430 and EGF, we examined the effect of MS-430 on the tyrosine phosphorylation of MAPK in EGF-treated PC12h cells. PC12h cells were cultured for 5 min, 30 min, 1 h, or 3 h in the presence of EGF following a pretreatment for 2 h with or without MS-430, and then total lysates were analyzed by Western blotting with the anti-phosphotyrosine antibody. As shown in Fig. 6A, the tyrosine phosphorylation of two MAPKs, Erk1 and Erk2, became maximum within 5 min after exposure to EGF in both MS-430-treated and untreated PC12h cells. The level of tyrosine phosphorylation of

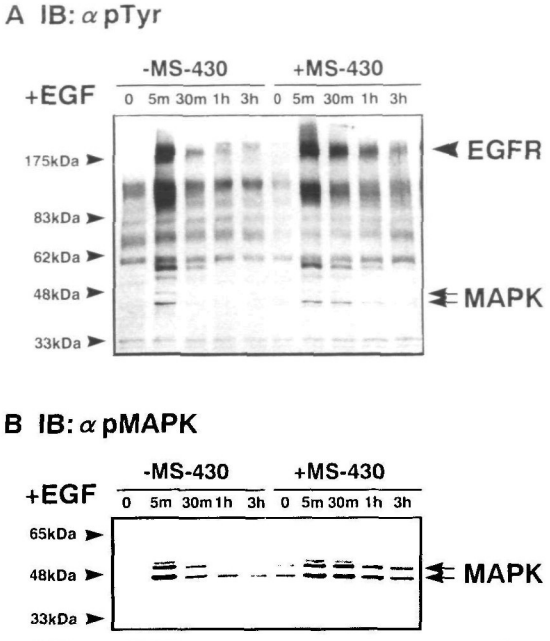

Fig. 6. **Effects of MS-430 on the EGF-induced tyrosine phosphorylation of EGFR and MAPK.** PC12h cells were cultured for 0 min (0), 5 min (5 m), 30 min (30 m), 1 h (1 h), or 3 h (3 h) in the presence of 100 ng/ml EGF following pretreatment for 2 h without (−MS-430) or with 1 mM MS-430 (+MS-430), and then the total lysates were analyzed by Western blotting with the anti-phosphotyrosine antibody (A) or the anti-phosphorylated MAPK antibody (B). The positions of MAPK (Erk1 and 2) are indicated by filled arrows. The position of EGFR is indicated by a filled arrowhead.

MAPK decreased to the basal level within 30 min in untreated PC12h cells. In MS-430-treated PC12h cells, however, EGF-induced tyrosine phosphorylation of MAPK was detected even 1 h after exposure to EGF. The experiment in Fig. 6B, in which we used the anti-phosphorylated MAPK antibody instead of the anti-phosphotyrosine antibody, shows that the level of EGF-induced tyrosine phosphorylation of MAPK was sustained in MS-430-treated PC12h cells, as compared with that in untreated PC12h cells. These results indicate that EGF induces the prolonged tyrosine phosphorylation of MAPK with the coexistence of MS-430, although EGF alone induces only transient tyrosine phosphorylation.

Effect of MS-430 on EGF-Induced Tyrosine Phosphorylation of EGFR—Next, we investigated whether or not MS-430 has some effects on the duration of EGF-induced autophosphorylation of the EGF receptor (EGFR). As shown in Fig. 6A, the level of EGF-induced tyrosine phosphorylation of EGFR decreased to the basal level within 1 h in PC12h cells not pretreated with MS-430. In MS-430-pretreated PC12h cells, on the other hand, EGF-induced tyrosine phosphorylation of EGFR was detected even 3 h after exposure to EGF. Taken together, these results suggest that MS-430 prolongs the duration of tyrosine phosphorylation of EGFR upstream of MAPK in the signal transduction pathway in EGF-treated PC12h cells, and as a result it can induce neurite extension in EGF-treated PC12h cells.

DISCUSSION

An analogue of MS-430, MS-818, was previously reported to promote neurite outgrowth in neuroblastoma cell lines and to potentiate NGF- induced neurite sprouting in PC12 cells (*27*). Here, we have found that MS-430 induced neurite outgrowth in EGF-treated PC12h cells (Figs. 2F and 3). MS-430 and EGF synergistically induced neuronal differentiation, including neurite extension and AChE activation (Fig. 4B). From these results, we speculate that MS-430 influences the common signal transduction pathway induced by NGF and EGF.

Both NGF and EGF are known to activate the common Ras-MAPK pathway, although they can induce different cellular responses in PC12 cells (*3, 12*). It was proposed that the duration of signaling activated by these factors decides the specificity of cellular responses between neuronal differentiation and proliferation (*19*). NGF induces sustained activation of the Ras-MAPK pathway, but EGF induces only transient activation in PC12 cells (*20, 21*). Therefore, we examined the effect of MS-430 on the duration of tyrosine phosphorylation of MAPK in NGF- or EGF-treated PC12h cells. We found that MS-430 prolonged the duration of tyrosine phosphorylation of MAPK in EGF- as well as NGF-treated PC12h cells (Figs. 5A and 6A). These results suggest that MS-430 may influence the mechanism which regulates the duration of activation of MAPK in PC12h cells.

In the experiments involving the anti-phosphorylated MAPK antibody, which is more sensitive than the anti-phosphotyrosine antibody, we observed that MS-430 alone induced the tyrosine phosphorylation of MAPK in the absence of growth factors (Figs. 5C and 6B). This suggests that MS-430 increases the basal level of tyrosine phosphorylation of MAPK, and as a result it stimulates the level of tyrosine phosphorylation of MAPK and apparently prolongs the duration of tyrosine phosphorylation of MAPK in NGF-treated PC12h cells (Fig. 5C). On the other hand, MS-430 really prolonged the duration of tyrosine phosphorylation of MAPK in EGF-treated PC12h cells (Fig. 6B), consistent with the observation that MS-430 prolonged the duration of tyrosine phosphorylation of EGFR (Fig. 6A).

MS-430 did not prolong the duration of tyrosine phosphorylation of TrkA in NGF-treated PC12h cells, in fact, it seemed to slightly decrease the level of tyrosine phosphorylation of TrkA (Fig. 5B). But we consider that this decrease in the level of phosphorylation of TrkA caused by MS-430 is not significant because MS-430 increased the level of phosphorylation of MAPK downstream of TrkA in the signaling pathway (Fig. 5C). Although MS-430 did not sustain the duration of phosphorylation of TrkA in NGF-treated PC12h cells (Fig. 5B), it prolonged that of EGFR in EGF-treated PC12h cells, as mentioned above (Fig. 6A). One possible mechanism by which MS-430 may act to increase tyrosine phosphorylation of EGFR is by activating MAPK, which subsequently phosphorylates EGFR at serine/threonine residues. The serine/threonine-phosphorylated EGFR may be easily tyrosine-phosphorylated by EGFR itself or by some other tyrosine kinases. It was reported that MAPK could phosphorylate EGFR at serine/threonine residues (*34*), but, at present, the meaning of this phosphorylation of EGFR by MAPK is unclear. Alternatively, MS-430 may inhibit the activity of some tyrosine phosphatases which dephosphorylate EGFR and MAPK, and thereby prolong the duration of tyrosine phosphorylation of these molecules. If this is the case, however, these tyrosine phosphatases must not act on TrkA in NGF-treated cells.

Another mechanism that could account for the action of MS-430 is that it may act to inhibit the down-regulation of EGFR. EGFR is endocytosed and degraded in lysosomes, following its activation (*35, 36*). The decreased rate of down-regulation of EGFR on internalization may lead to prolonged phosphorylation of EGFR. To evaluate this possibility, the protein level of EGFR in the presence or absence of MS-430 should be determined in further studies.

We observed that NGF induced the extension of small neurites only 2 h after its addition to PC12h cells pretreated with MS-430 for 2 h (data not shown). This rapid extension of neurites in response to NGF in MS-430–treated PC12 cells resembled that in NGF-primed PC12 cells (*37, 38*). When PC12 cells are cultured for 7–10 d in the presence of NGF, they differentiate into neuron-like cells with extended neurites. If these differentiated PC12 cells are removed and recultured in fresh medium, they lose their neurites but can rapidly extend neurites in response to NGF, like explanted sympathetic and sensory ganglion neurons (*37, 38*). These PC12 cells are known as NGF-primed PC12 cells. The rapid NGF-induced neurite outgrowth in NGF-primed PC12 cells is RNA synthesis-independent and may reflect NGF-stimulated regeneration rather than the generation of neurite outgrowth (*38*). On the other hand, PC12h-R cells, a subclone of PC12h cells, showed a rapid NGF response and thus resembled the NGF-primed PC12 cells (*39*). Surprisingly, we found that PC12h-R cells extended neurites in response to EGF alone as well as to NGF (*25*). In addition, EGF induced prolonged tyrosine phosphorylation of EGFR and MAPK, and prolonged activation of MAPK in PC12h-R cells compared with in the parent PC12h cells. These results suggest that MS-430 may have an effect similar to the priming by NGF. However, MS-430 was added 2 h before the addition of EGF or NGF in the experiments presented in Figs. 5 and 6. Therefore, the expression of genes other than immediate early genes (such as c-*fos* and c-*jun*) is considered not to occur (*40, 41*). If priming by NGF influences the expression of some immediate early genes, it is possible that MS-430 may have the same effect as priming by NGF has in PC12 cells.

Finally, MS-430 may act on some molecules in signaling pathways other than the Ras-MAPK pathway. If this is the case, the other MS-430-activated pathways may crosstalk to activate the Ras-MAPK pathway. Raf, which phosphorylates and activates MAPKK, is known to be phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) (*42-44*). PKC phosphorylates and activates Raf-1, and PKA phosphorylates and activates B-Raf, a homologue of Raf-1. On the other hand, PKA phosphorylates and inactivates Raf-1. If MS-430 could activate PKA or PKC, it might crosstalk to activate MAPK. Further studies are necessary to elucidate the molecular mechanism of the MS-430 action which causes the neuronal responses of PC12 cells.

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