Mouse Semaphorin H Induces PC12 Cell Neurite Outgrowth Activating Ras-Mitogen-activated Protein Kinase Signaling Pathway via Ca\(^{2+}\) Influx*

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We recently showed that mouse semaphorin H (MSH), a secreted semaphorin molecule, acts as a chemorepulsive factor on sensory neurites. In this study, we found for the first time that MSH induces neurite outgrowth in PC12 cells in a dose-dependent manner. Comparison of Ras-mitogen-activated protein kinase (MAPK) signaling pathways between MSH and nerve growth factor (NGF) revealed that these pathways are crucial for MSH action as well as NGF. K-252a, an inhibitor of tyrosine auto-phosphorylation of tyrosine kinase receptors (Trks), did not inhibit the action of MSH, suggesting that MSH action occurs via a different receptor than NGF. L- and N-types of voltage-dependent Ca\(^{2+}\) channel blockers, diltiazem and ω-conotoxin, inhibited MSH-induced neurite outgrowth and MAPK phosphorylation in a Ca\(^{2+}\)-dependent manner. A transient elevation in intracellular Ca\(^{2+}\) level was observed upon MSH stimulation. These findings suggest that extracellular Ca\(^{2+}\) influx, followed by activation of the Ras-MAPK signaling pathway, is required for MSH induced PC12 cell neurite outgrowth.

Nervous system function is dependent upon highly specific connections that form between neurons during development. The patterning and specificity of these connections requires neurite extension toward the proper targets guided by the growth cone in response to environmental signals. However, the process that involves signal-induced morphological changes resulting in coordinated cytoskeletal remodeling in the specialized growth cone is poorly understood (1).

The semaphorins/collapsins are a large family of structurally distinct secreted and transmembrane proteins characterized by the presence of a conserved sema domain of about 500 amino acids (2–4). Chick collapsin-1 and its mammalian homolog, mouse semaphorin D (MSD)\(^1\) (initially named sema III), act in vitro as collapsing factors on growth cones and as selective chemorepellents for subpopulations of spinal and cranial, sensory, and motor axons (5, 6). Thus, semaphorins are thought to be involved in the axon guidance mechanism during neuronal development. To investigate the mechanism of neural network formation, we identified several novel semaphorins (7–9). One of them, mouse semaphorin H (MSH) is structurally similar to MSD and acts as a chemorepellent on sensory axons (9, 10). To investigate the signaling pathway for secreted semaphorin MSH, we used PC12 cells (a clone derived from a pheochromocytoma tumor of the rat adrenal medulla), which are known to differentiate into neuronal cells in response to NGF.

Studies on PC12 cells have demonstrated that a variety of extracellular signals can lead to neurite outgrowth and morphological differentiation. In this cell line, the signaling pathway via the NGF receptor is the most well characterized (11–13). Ligand binding to its tyrosine kinase receptor (Trk) causes activation of Ras-dependent MAPK cascades resulting in cellular differentiation and neurite outgrowth (14).

In this study, we have found that MSH induces neurite outgrowth in PC12 cells through a Ras-MAPK signaling pathway similar to NGF. It has been described that the conventional extracellular Ca\(^{2+}\) concentration (1 mM) is required for chemotactic growth cone action, induced by brain-derived neurotrophic factor, acetylcholine, and bradykinin, and also for chemorepulsive action induced by soluble fraction of myelin-associated glycoprotein (15, 16). In this study, we investigated whether Ca\(^{2+}\) influx is involved in MSH-induced PC12 cell neurite outgrowth.

MSD acts as a chemorepellent on sensory and sympathetic axons via neuropilin-1 receptor (17, 18). Antibodies against the extracellular domain of neuropilin-1 block the effects of MSD in vitro. However, the chemorepulsive action of MSD can be converted to chemotraction by membrane-permeable cyclic GMP agonists via the same neuropilin-1 receptor (19). These findings show that different actions, for instance growth cone attraction and repulsion, can occur via the same receptor. We examined the involvement of neuropilins in MSH action on PC12 cells. In this study, we describe the neurite outgrowth effect of MSH on

\(^{1}\) The abbreviations used are: MSD, mouse semaphorin D; MSH, mouse semaphorin H; MSE, mouse semaphorin E; NGF, nerve growth factor; AP, alkaline phosphatase; MAPK, mitogen-activated protein kinase; Trk, tyrosine kinase receptor; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium.
PC12 cells and the signaling pathways that this action occurs through.

EXPERIMENTAL PROCEDURES

Materials—NGF (7S) was purchased from Roche Molecular Biochemicals. PD98059 was purchased from Research Biochemicals International (Natick, MA). K-252a and G6983 were purchased from CAL-BIOCHEM (La Jolla, CA). Calphostin C was purchased from Wako (Osaka, Japan). Diltiazem was purchased from Life Technologies, Inc. α-Conotoxin GVIA was purchased from Alomone Labs (Jerusalem, Israel). All reagents were used at concentrations established in literature to substantially block their respective targets. Other materials used in this study were described previously (9, 10).

Cell Culture—PC12 cells, NIH3T3 cells, and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.35% glucose, 10% fetal bovine serum, and 100 units/ml penicillin. All cells were grown at 37 °C in 5% CO2.

Preparation of MSH-AP, MSE-AP, AP, and MSD—The conditioned media containing MSH-AP, mouse semaphorin E (MSE)-AP, AP or MSD were obtained from NIH3T3 cells and/or 293T cells expressing them and concentrated with Centricon (Amicon) as described previously (9, 10). The concentration of the proteins was determined from AP activity assayed at 405 nm, and AP activity was equalized in MSH-AP and AP (19).

Determination of Neurite Outgrowth—PC12 cells were dissociated in phosphate-buffered saline solution followed by incubation at 37 °C for ~2 min with 0.05% (w/v) trypsin in the same buffer. A single-cell suspension was obtained by trituration of pelletized trypsinized cells, resuspended in DMEM. For culture experiments, 2 × 103 cells were added to each well of a 24-well plate (Costar) or a 48-well plate (Costar) that had been coated with poly-l-lysine. After plating for 1 h, cells were treated with various reagents (PD98059, K-252a, G6983, calphostin C, diltiazem, or α-conotoxin GVIA) at the indicated concentrations. To activate calphostin C, the cells were irradiated with a cool white fluorescent lamp located 20 cm away for preincubation (20). PC12 cell differentiation was determined by scoring for neurite length. Cells possessing one or more neurites of a length greater than 1.5-fold the diameter of the cell body were scored as positive (21). Each value is the mean ± S.E. for 100–150 PC12 cells sampled from three independent experiments.

To determine whether MSH-AP action is specific to MSH-AP, the conditioned media containing MSH-AP or AP were absorbed with anti-AP mouse antibody or normal mouse IgG (control) immobilized on beads at 4 °C for 1 h. After removal of beads, the resulting conditioned media were assayed for neurite outgrowth as described above.

Detection of Activated p44/42 MAPK—To detect phospho-MAPK proteins stimulated by NGF, AP, or MSH-AP, PC12 cells (2 × 103/60-mm dish) were treated with 50 ng/ml NGF, 1 μM AP, or 1 μM MSH-AP at 37 °C for the duration indicated in the text. Cells were lysed in lysis buffer Tris, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin). Aliquots of the lysates (10–15 μg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (0.45-μm pore size Immobilon-P, Millipore). The blots were probed with the phospho-p44/42 MAPK antibody (PhosphoPlus p44/42 MAPK antibody kit, New England Bio-Labs) at a dilution of 1:1000 in blocking buffer (10 mg/ml bovine serum albumin) for 60 min at room temperature. The blots were probed with secondary antibody, horseradish peroxidase-linked anti-rabbit IgG, at a dilution of 1:2000 in blocking buffer for 60 min at room temperature. The blots were stained for 1 min using the nucleic acid chemiluminescence reagent (LumiGLO chemiluminescent reagent, Kirkegaard and Perry Laboratories) and exposed to x-ray film. Blots were stripped with 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 30 min and reprobed with p44/42 MAPK antibody to verify that the protein levels were uniform.

Monitoring of Intracellular Ca2+ Levels—For monitoring intracellular Ca2+ levels, cells were loaded by exposing them to 10 μM (final concentration) Fura 2-AM (Molecular Probes) in the dark at 37 °C for 60 min. Cultures were then rinsed and perfused continuously in a modified Kreb’s solution containing 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 13.8 mM glucose, and 20 mM HEPES, pH 7.4. Fluorescence measurements calculated with a real time image processor, ARGUS-50/CA (Hamamatsu Photonics KK), were shown as 340/380 nm (excitation) ratios obtained from groups of six cells. Measurements shown are representative of at least three and, in most cases, a larger number of independent experiments.

RESULTS

MSH Induces Neurite Outgrowth in PC12 Cells—PC12 cells were incubated with 1 μM MSH-AP, 50 ng/ml NGF, 1 μM AP, MSD, or 1 μM MSE-AP for 24 h. The majority of PC12 cells cultured with AP, MSD, or MSE-AP showed no neurite growth (Fig. 1A). In contrast, many PC12 cells showed morphological differentiation in response to MSH-AP or NGF. The percent-
MSH. We then used immunoblot analysis with anti-phospho-p42/44 MAPK antibody to detect activated MAPK in PC12 cells stimulated by NGF or MSH-AP. 1 nM MSH-AP induced an increase in MAPK1/2 activation in PC12 cells within 2 min (Fig. 2B). This activation reached peak level after 5 min and lasted at least 60 min. NGF similarly stimulated sustained activation of MAPK in PC12 cells as described previously (14) (Fig. 2B), whereas AP did not cause any significant activation of MAPK. These findings suggest that sustained activation of MAPK is crucial for MSH-induced neurite outgrowth of PC12 cells as well as NGF.

Ras activation is upstream of the MAPK pathway in NGF-induced neurite outgrowth of PC12 cells. Thus, we investigated whether Ras regulation is involved in the signal transduction of MSH-induced neurite outgrowth of PC12 cells. Dominant-negative Ras overexpressing PC12 cells (PC12Ha RHK cells) and mock cells (PC12Ha XMV cells) were incubated with MSH-AP or NGF. Overexpression of dominant-negative Ras almost completely inhibited MSH-AP induced neurite outgrowth as well as NGF-induced neurite outgrowth, whereas the majority of mock PC12Ha XMV cells showed morphological differentiation in response to MSH-AP as well as NGF (Fig. 3). These findings suggest that Ras activation is necessary for MSH-induced neurite outgrowth in analogy with NGF. Activation of the Ras-MAPK cascade may therefore be essential for neurite outgrowth activity induced by MSH.

To investigate whether Trks and their signal transduction are involved in MSH-induced neurite outgrowth, K-252a was used to block tyrosine autophosphorylation of Trks and their downstream signal transduction (24, 25). PC12 cells were incubated with the indicated concentration of K-252a for 15 min prior to MSH-AP addition (Fig. 4). K-252a at 100 nM markedly inhibited neurite outgrowth activity induced by MSH.
inhibited NGF-induced neurite outgrowth but did not exert any inhibition on MSH-AP-induced neurite outgrowth, suggesting that the neurite outgrowth action of MSH is not mediated by Trks. Thus, MSH and NGF actions are mediated by distinct upstream receptors sharing a common signaling pathway via Ras-MAPK cascades.

Protein Kinase C Inhibitors Reduce Neurite Outgrowth Activity of MSH and NGF—Because activation of PKC stimulates NGF-induced neuronal differentiation of PC12 cells (26, 27), we investigated the involvement of PKC in the signal transduction of MSH-induced neurite outgrowth. PC12 cells were treated with a PKC inhibitor, calphostin C (20) or Go6983 (28), for 30 min prior to addition of MSH-AP or NGF. Both treatments blocked MSH-AP action as well as NGF action (data not shown). 20 nM calphostin C markedly inhibited neurite outgrowth induced by MSH-AP and NGF; 50 nM Go6983 showed similar inhibition. These data suggest that PC12 cell neurite outgrowth induced by MSH and NGF is dependent on PKC activation.

Ca2+ Influx Is Required for Action of MSH, but Not for NGF—By culturing PC12 cells in 25 mM KCl and 100 mM BayK8644, it has been reported that depolarization-induced neurite growth requires Ca2+ entry via voltage-dependent Ca2+ channels (29). To determine whether Ca2+ influx is crucial for MSH-induced neurite outgrowth, two types of Ca2+ channel blockers, diltiazem, an L-type Ca2+ channel blocker, and ω-conotoxin, an N-type Ca2+ blocker, were used (30). Both Ca2+ channel blockers, 100 μM diltiazem (Fig. 5A) and 1 μM ω-conotoxin (Fig. 5B), strongly inhibited PC12 cell neurite outgrowth induced by MSH-AP but did not have any obvious effects on NGF-induced neurite outgrowth. These findings suggest that Ca2+ influx via N- and L-type Ca2+ channels is involved in the signal transduction of MSH-induced neurite outgrowth but not NGF. We next tested whether deprivation of extracellular Ca2+ affects MAPK activation induced by MSH-AP or NGF (Fig. 5C). MSH-AP-induced MAPK activation was markedly inhibited by EGTA treatment, whereas NGF-induced activity was not affected, suggesting that 1 mM extracellular Ca2+ is required for MSH-induced activation of MAPK but not for NGF.

To determine whether extracellular Ca2+ influx is induced in response to MSH-AP stimulation, fluorescent ratios of F340/F380 were measured to indicate intracellular Ca2+ levels by a real time image processor using Fura 2-AM following MSH-AP stimulation. The addition of KCl, a positive control, caused a quick and transient increase in Ca2+ levels (Fig. 6A). Ca2+ levels also transiently increased 1–2 min after adding MSH-AP (Fig. 6B), whereas Ca2+ levels did not increase after adding AP (Fig. 6C). These findings indicate that extracellular Ca2+ influx in the early stage of the signaling pathway triggers MSH action on PC12 cells.

Is MSH Action on PC12 Cells Mediated by Neuropilin Receptors?—To investigate whether MSH action on PC12 cells occurs via neuropilin-1, neutralizing neuropilin-1 antibodies for the extracellular domain of neuropilin-1 were used. Neuropilin-1 neutralizing antibodies, which blocked MSH collapsing action on sensory growth cones, did not inhibit MSH action on PC12 cells (data not shown). We also used a large excess of MSE to compete with MSH for binding to neuropilins, because MSE have no effect on PC12 cells but binds to both neuropilin-1 and 2 (31) (data not shown). A 40-fold concentration of MSE did not inhibit the action of MSH on PC12 cells (data not shown), suggesting that MSH probably acts on PC12 cells through unknown receptor distinct from neuropilins.

DISCUSSION

In the present study we have shown that MSH can induce neurite outgrowth of PC12 cells in a dose-dependent manner. Nearly all cells showed morphological differentiation with long neurites in response to MSH. This morphological differentiation appeared to be similar to that by NGF. Then we analyzed
the cytoplasmic signaling pathway of MSH action comparing with that of NGF action. The major finding obtained about the signal pathway of the action of MSH can be summarized as follows: (a) MSH-induced neurite outgrowth of PC12 cells was mediated by the sustained activation of MAPK in a similar fashion for NGF. (b) Ras activation was also critical to MSH-induced morphological differentiation of PC12 cells. (c) MSH-induced signaling pathway was independent on the activation of Trks which has been known as NGF receptors (Fig. 4). (d) MSH- but not NGF-induced morphological differentiation of the cells were blocked by two types of calcium blockers. (e) MSH- but not NGF-induced MAPK activation was inhibited by deprivation of extracellular Ca$^{2+}$. (f) MSH induced a transient Ca$^{2+}$ influx of PC12 cells. These results imply that the Ras-MAPK signaling pathway was shared between MSH and NGF, but the upstream signaling is apparently distinct between the two ligands.

So far several studies have reported that Ca$^{2+}$ influx links extracellular signals to Ras-MAPK signaling pathway (29, 32–36). In some cases, Ca$^{2+}$ influx activates Pyk2 and c-Src and results in activation of Shc (SH2/collagen protein) and MAPK (29, 37, 38). In other cases, increase of intracellular Ca$^{2+}$ activates several Ras guanyl-nucleotide-exchange factor (Ras-GEF) molecules, Ras-GRF1 (32, 33), and RasGRP/rbc7 (36) and result in activation of Ras-MAPK signaling system. This study indicates that Ca$^{2+}$ influx is necessary for MSH-induced neurite outgrowth of PC12 cells independently of Trk tyrosine kinase receptors. Thus, it is probable that binding of MSH to its receptor appears to cause Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels followed by activation of Ras-MAPK cascades to induce neurite outgrowth in PC12 cells, although it is unknown what the receptor is for MSH and what links Ca$^{2+}$ influx to Ras-MAPK. A lag in Ca$^{2+}$ increase of about 1 min after adding MSH-AP may suggest that any chemical reactions such as phosphorylation reactions are involved in the upstream of Ca$^{2+}$ and Ras-MAPK signaling cascades of MSH action. Such a lag in Ca$^{2+}$ level of about 1 min has been also shown when PC12 cells were stimulated with NGF (39), of which signaling includes tyrosine and serine/threonin phosphorylations (11–13, 26). Our findings using PKC inhibitors suggest that PKC activation is also involved in MSH-induced neurite outgrowth in PC12 cells as well as NGF-induced neurite outgrowth (26). This result supports a significant relationship between MSH signaling and Ca$^{2+}$ influx.

Recently, it has been reported that neuropilins are receptors for secreted semaphorin molecules. MSH acts as a chemorepellent on sensory and sympathetic axons via neuropilin-1 receptor, and MSE similarly acts as a chemorepellent on sympathetic axons via neuropilin-2 receptor (31). We recently showed that MSH also acted as a chemorepellent on sensory axons via neuropilin-1 receptors (9). Song and colleagues (15) have reported that cGMP analogues could convert the repulsive action of MSD to attractive via neuropilin-1 receptor. Therefore, MSH can act as a chemotactant via neuropilin-1 receptors on PC12 cells. The action of MSH may depend on cell types or cell states modulated by second messengers such as cyclic nucleotides. Otherwise, MSH-induced neurite outgrowth occurs via an unknown receptor that is distinct from neuropilins. This hypothesis is supported by the following results: (a) Neutralizing neuropilin-1 antibodies did not inhibit MSH action on PC12 cells. (b) A large excess of MSE, a competitive ligand for neuropilins (31), did not inhibit MSH-induced neurite outgrowing action. However, these observations do not exclusively rule out a possibility that MSH action on PC12 cells is mediated by neuropilins, because these do not completely refute the possibility that MSH and MSE would bind separate domains of neuropilins. On the other hand, it has been reported that chemorepellent activity of MSD, which is known to act via neuropilin-1 receptor, is independent on extracellular Ca$^{2+}$ level (15, 40). Our results showing the dependence of MSH-mediated signaling on extracellular Ca$^{2+}$ concentration may support the idea that MSH-induced neurite outgrowth of PC12 cells is signaled via a receptor separate from neuropilins.

The present study indicates a possible physiological role for MSH in effecting changes in cell phenotype through cell-cell interactions.

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**REFERENCES**

1. Bentley, D., and O'Connor, T. P. (1994) *Curr. Opin. Neurobiol.* 4, 43–48
2. Tessier-Lavigne, M., and Goodman, C. S. (1996) *Science* 274, 1123–1133
3. Luo, Y., Raible, D., and Raper, J. A. (1993) *Cell* 75, 217–227
4. Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1993) *Cell* 75, 1389–1399
5. Kobayashi, H., Koppel, A. M., Luo, Y., and Raper, J. A. (1997) *J. Neurosci.* 17, 8339–8352
M-SemaH Induces PC12 Cell Neurite Outgrowth via Ca$^{2+}$ Influx