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Cyclic GMP-dependent neurite outgrowth by genipin and nerve growth factor in PC12h cells

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Abstract

We have demonstrated previously that a natural iridoid compound, genipin, induced neuritogenesis through activation of nitric oxide synthase (NOS) and mitogen-activated protein kinase (MAPK) in PC12h cells. In this paper, we investigated whether cyclic GMP (cGMP) and cGMP-dependent protein kinase (PKG) are involved in the neuritogenesis as a result of NOS activation. Furthermore, we also investigated the relationship between cGMP and MAPK activation in the signaling pathway. The genipin-induced neuritogenesis accompanied by induction of neurofilament was significantly inhibited by 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) and KT5823, inhibitors of soluble guanylate cyclase and PKG, respectively. Genipin-induced MAPK phosphorylation was also abolished by ODQ. These inhibitory effects of ODQ were similar to those observed for nerve growth factor (NGF)-induced neurite outgrowth and MAPK phosphorylation. The membrane-permeable cGMP analog, 8-Bromo-cGMP, had prominent neuritogenic activity, which was completely inhibited by a MAPK kinase inhibitor, PD98059. These results suggest that the soluble guanylate cyclase-PKG signaling pathway is important for MAPK activation by genipin as well as NGF during neuritogenesis in PC12h cells.

Keywords: PC12h cell; Neurite outgrowth; Genipin; Nerve growth factor; Cyclic GMP; Mitogen-activated protein kinase

1. Introduction

It has been reported that nerve growth factor (NGF) and other neurotrophic factors induce neurite outgrowth in sensitive types of cells (Greene and Tischler, 1976) and play pivotal roles in the survival and growth of neurons in the central nervous system (Lindholm et al., 1996; Kalcheim et al., 1992). Thus, neurotrophic factors are expected to have a regenerative action in the injured tissues of neurodegenerative diseases such as Alzheimer's disease and Parkinsonism. However, because of their inability to cross the blood–brain barrier and vulnerability to hydrolytic enzymes, they cannot be used as a medical treatment. Therefore, we have been screening natural lipophilic compounds in plants traditionally used for anti-amnestic medications as substitutes for neurotrophins.

PC12 cells, a cell line derived from rat pheochromocytoma, exhibit a variety of neuronal properties resembling those of sympathetic adrenal neurons in response to NGF (Greene and Tischler, 1976) and are considered to be a useful model for studying neuronal differentiation. PC12h cells (Hatanaka, 1981), a subclone of PC12 cells, also have some NGF-responsive cellular events, including neurite outgrowth and induction of tyrosine hydroxylase activity (Hatanaka, 1983). In a previous paper we proposed that genipin, a natural iridoid compound, has an ability to induce neurite outgrowth through activation of several protein kinases including extracellular signal-regulated kinase (ERK), which is a member of mitogen-activated protein kinase (MAPK) family, and activation of nitric oxide synthase (NOS) in PC12h cells (Yamazaki et al., 2001). The neuritogenic activity of NGF has also been to be mediated by the activations of NOS and ERK in PC12h cells (Yamazaki et al., 2001).

Nitric oxide (NO) has been recently proved to be capable of influencing synaptic plasticity, neurotransmitter release and excitotoxicity in ischemia (Schuman and Madison,

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1991; Hirsch et al., 1993; Huang et al., 1994). It has been reported by several groups that NO or NOS activation is required for the induction of neuronal differentiation in response to NGF in PC12 cells (Peunova and Enikolopov, 1995; Poluha et al., 1997; Phung et al., 1999) and in response to depolarization by high concentrations of KCl in subcloned PC12K cells (Nakagawa et al., 2000). Hindley et al. (1997) found that NO donors enhanced NGF-induced neurite outgrowth through activation of soluble guanylate cyclase and cGMP-dependent protein kinase (PKG), while an NO donor alone did not induce neurite outgrowth in the absence of NGF in PC12 cells. Furthermore, Phung et al. (1999) stated that NO played an important role in NGF-induced neurite outgrowth without activation of soluble guanylate cyclase activity. Thus, the participation of cGMP in neurite outgrowth is so far disputable as a regulator in the NO-mediated mechanism.

In PC12 cells, sustained phosphorylation of ERK by MAPK/ERK kinase (MEK) is necessary and sufficient for neuronal differentiation (Cowley et al., 1994; Fukuda et al., 1995). However, the relationship between activation of ERK and the production of NO and/or cGMP has not yet been clearly elucidated.

In this study, we have attempted to determine the contribution of soluble guanylate cyclase and PKG to neurite outgrowth as well as the relationship between cGMP and ERK phosphorylation in the signaling pathway utilized by genipin and NGF in PC12h cells.

2. Materials and methods

2.1. Materials

Genipin was purchased from Wako (Japan) and dissolved in distilled water. NGF (7S, isolated from mouse submandibular gland) and 8-bromoguanosine-3′, 5′-cyclophosphate sodium salt (8-Br-cGMP) were obtained from Sigma (USA) and dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and PBS, respectively. 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (USA) and dissolved in dimethylsulfoxide (DMSO). *N*-Methyl-(8*R*, 9*S*, 11*S*)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-3,10-dihydro-8-11-epoxy-1H, 8H, 11H-2.7b,11*a*-triazadibenzo(a,g)cycloocta(cde) trinden-1-one (KT5823) and 2′-amino-3′-methoxyflavone (PD98059) were purchased from Biomol Research Laboratories (USA) and dissolved in DMSO. Protease inhibitor cocktail (general use), phosphatase inhibitor cocktail 1 and 2, and sodium EDTA were obtained from Sigma.

2.2. Cell culture

PC12h cells were grown as previously reported (Yamazaki et al., 2001). Briefly, cells were plated onto 35 mm plastic culture dishes coated with collagen at a density of 2–4×10^3 cells/cm^2 and cultured for 24 h in Dulbecco's modified Eagle's medium (DMEM) with serum. For Western blot analysis, cells were plated onto 100 mm plastic culture dishes at subconfluent density. The culture medium was then replaced with serum-free DMEM/Ham's F-12 (1:1) to treat the cells with the agents as specified in the results.

2.3. Evaluation of neurite outgrowth

Neurite outgrowth of PC12h cells was evaluated by measuring the length of the longest neurite in individual cells as previously reported (Yamazaki et al., 2001). The neurite length of 100 cells in 10 random fields from two sister culture dishes was averaged to evaluate the neurite outgrowth for each treatment. The data shown in each figure was typical of several separate experiments.

2.4. Western blot analysis for neurofilaments

PC12h cells in one dish (100 mm) were washed with ice-cold PBS and harvested by scraping with a cell scraper in protease inhibitor cocktail dissolved in distilled water after treatment as specified in the results. The cells were disrupted by being passed 10 times through a 25-gauge needle and then centrifuged at $1500 \times g$ for 10 min at 4 °C. Protein

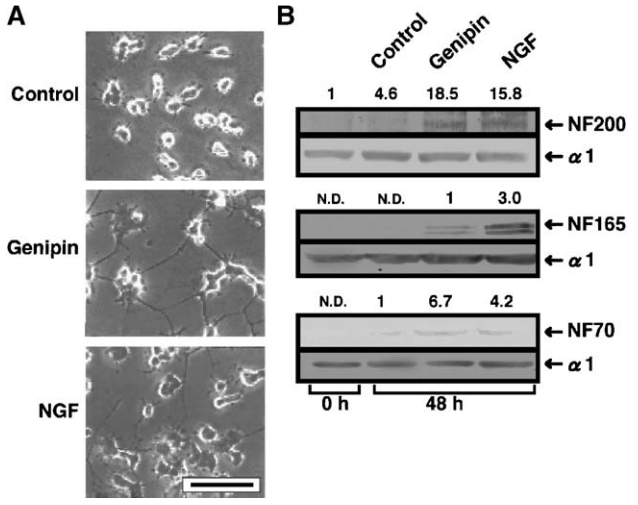

Fig. 1. Genipin- and NGF-induced neuronal differentiation in PC12h cells. (A) Morphological changes induced by genipin and NGF in PC12h cells. Cells were treated with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) for 48 h. Scale bar = 100 μm. (B) Expression of neurofilament subunits in PC12h cells. Cells were treated with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) for 48 h. Cell extracts corresponding to 20 μg (for NF70) or 100 μg (for NF165 and 200) of protein were subjected to 6% SDS-PAGE for each sample. The immunoreactive signal of Na^+, K^+-ATPase α1 subunit (α1) is for assessment of quality of protein loading. The immunoreactive bands of NF70, 165 and 200 were detected using mouse anti-neurofilament monoclonal antibody against subtypes, 70, 165 and 200 kDa, respectively. Values shown above the panels are relative amount of neurofilament subunits normalized for Na+, K+-ATPase α1 subunit protein. N.D. means no detection. Results are representative of three separate experiments.

content in the supernatant was determined using a Bio-Rad protein assay kit according to the method of Bradford with bovine serum albumin as the standard. The supernatant was dissolved in sodium dodecylsulfate (SDS)-sample buffer according to the method of Laemmli without boiling (Laemmli, 1970).

The neurofilament subunits were separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrically transferred to an Immobilon-P polyvinylidene difluoride (PVDF) transfer membrane (Nihon Millipore, Japan). The membrane was treated with methanol for 1 h at room temperature to avoid background noise, completely dried, and incubated to detect the subunits of the neurofilament with a mouse anti-neurofilament 70 kDa (NF70) monoclonal antibody (1:2500 dilution, Chemicon International, USA), a mouse anti-neurofilament 165 kDa (NF165) monoclonal antibody (clone 2H3, 1:500 dilution, American Research Products, USA), or a mouse anti-neurofilament 200 kDa (NF200) monoclonal antibody (clone NE14, 1:1000 dilution, Sigma) for 1 h at room temperature. The membrane was incubated with a mouse anti-Na^+/K^+-ATPase α1 subunit monoclonal antibody (clone C464.6, 1:10,000 dilution, Upstate Biotechnology, USA) to assess the quality of protein loading. The membrane was then incubated with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Ig G) antiserum (1:10,000 dilution, Sigma) as a secondary antiserum for 1 h at room temperature.

The immunoreactive signals were detected with a BCIP/NBT Phosphatase Substrate System detection kit (Kirkegaard and Perry Laboratories, USA). The intensity of the immunoreactive signals was quantified by densitometry as previously reported (Yamazaki et al., 2001).

2.5. Western blot analysis for ERK 1 and 2

Each sample for SDS-PAGE was prepared from PC12h cells in one dish (100 mm) for each treatment as previously reported (Yamazaki et al., 2001). A 10 μg of protein from the cytoplasmic extracts was subjected to 10% SDS-PAGE and then to electric transfer to the Immobilon-P PVDF membrane. The membrane was blocked and completely dried as described above. Immunoreaction, detection of the immunoreactive signals, and its quantitative analysis were performed as previously reported (Yamazaki et al., 2001).

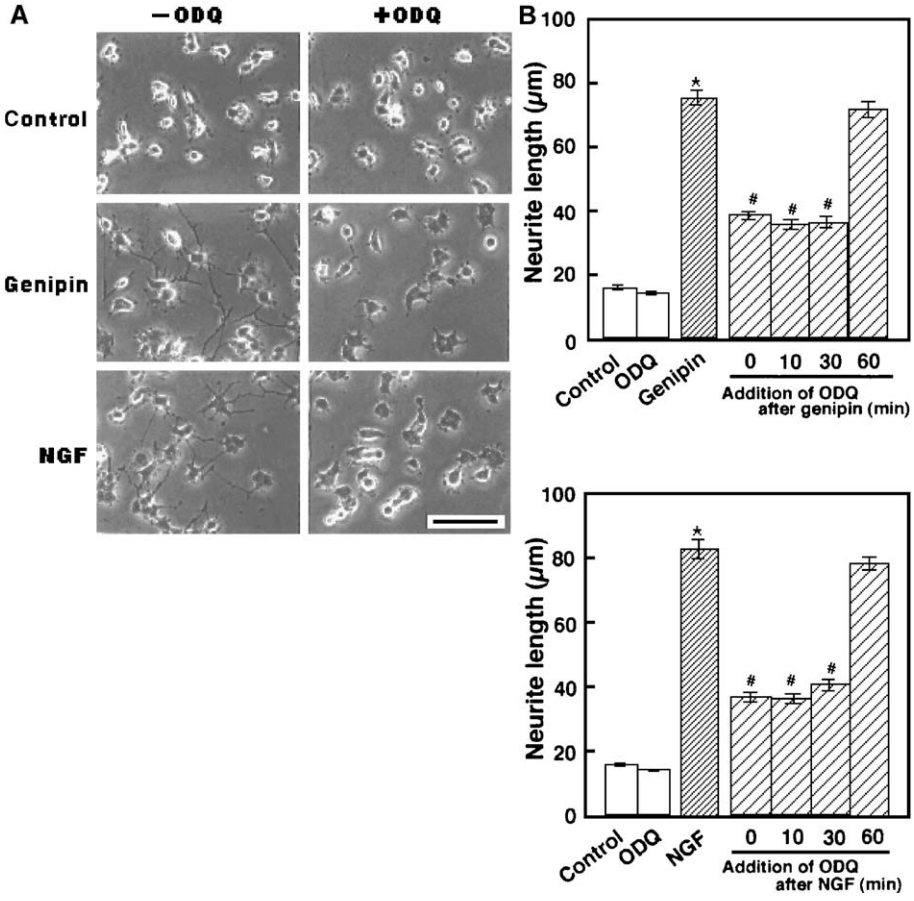

Fig. 2. Commitment point for the inhibitory effect of soluble guanylate cyclase inhibitor on genipin- and NGF-induced neurite outgrowth in PC12h cells. (A) ODQ (50 μM) or its vehicle (Control) was added to the medium 60 min before treatment with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) for 48 h. Scale bar = 100 μm. (B) Cells were treated with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) and then ODQ (50 μM) was added to the medium at various time points after treatment with genipin or NGF as indicated. After 48 h, the neurite length was measured as described in the Materials and methods. Each value is expressed as the mean ± S.E.M. of 100 cells. $^*P < 0.01$ vs. control, $^{\#}P < 0.01$ vs. treatment with genipin or NGF alone.

2.6. Measurement of intracellular cGMP

PC12h cells (10^6 cells) were pretreated with 100 μM 3-isobutyl-1-methylxanthine (IBMX) (Wako), a non-selective inhibitor of PDEs, for 30 min and then added 5 μg/ml genipin or 1 ng/ml NGF was added. Intracellular cGMP concentration was determined with a cGMP enzyme-immunoassay kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to the manufacturer's protocol. The protein contents of the samples were determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.7. Statistical analysis

The statistical significance of the differences between mean values of groups was analyzed using analysis of variance and Scheffe's test.

3. Results

3.1. Neuronal differentiation induced by genipin and NGF in PC12h cells

Genipin (5 μg/ml) and NGF (1 ng/ml) induced comparable neurite outgrowth after 48 h-treatment of PC12h cells as shown in Fig. 1A. It was confirmed in this experiment that all three subunits (NF70, NF165, and NF200) of neurofilament, which are specifically found in neurons and increases with maturing of the axon and neurite (Jacobs and Stevens, 1986), were increased by genipin- and NGF-treated cells by Western blot analysis (Fig. 1B). The amount of growth-associated protein 43, a growth cone-associated protein (Goslin et al., 1988), was also increased by the treatment with genipin for 48 h (data not shown). These results suggest that not only NGF but also genipin can induce neuronal differentiation, evoking functional neurites in PC12h cells.

3.2. Inhibition of neuritogenesis by soluble guanylate cyclase inhibitor

It has been previously reported that NO directly activates soluble guanylate cyclase and increases intracellular cGMP levels (Knowles et al., 1989). In a previous report, we showed that neurite outgrowth induced by both genipin and NGF was inhibited by ODQ, a specific inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995), in a concentration-dependent manner (5–100 μM) in PC12h cells (Yamazaki et al., 2001). We therefore examined the amount of time required for soluble guanylate cyclase activation to affect neurite outgrowth induced by genipin and NGF in PC12h cells. The neurite outgrowth induced by 48 h-treatment with either genipin or NGF was almost completely inhibited by pretreatment with 50 μM ODQ for 60 min (Fig. 2A). Interestingly, it

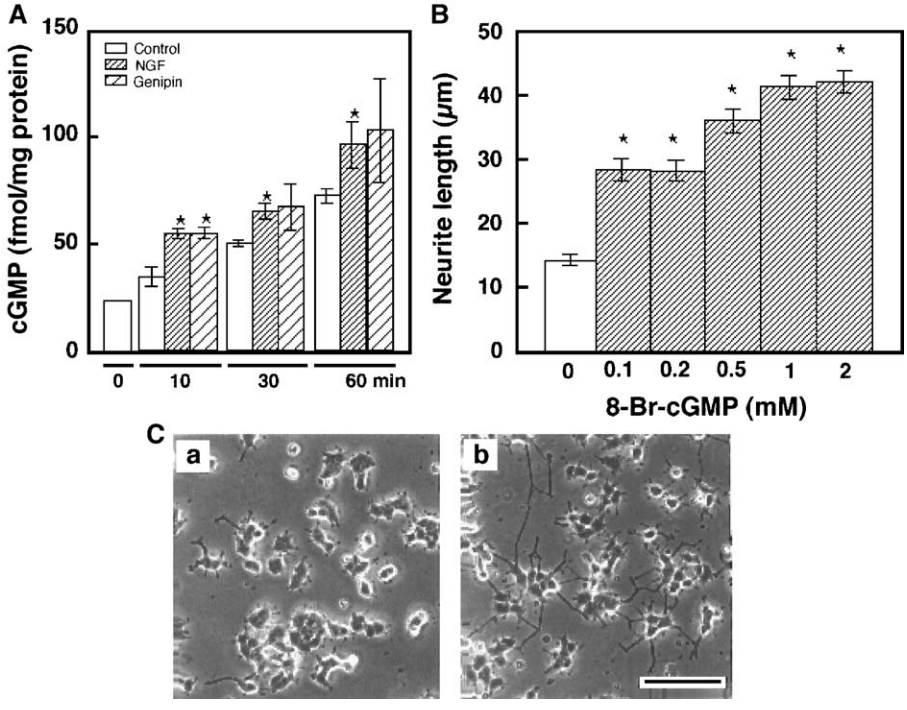

Fig. 3. Neuritogenesis induced by 8-Br-cGMP in PC12h cells. (A) Cells were pretreated with 100 μM IBMX for 30 min, and then 5 μg/ml genipin, 1 ng/ml NGF or their vehicle (Control) was added to the culture for indicated periods. Intracellular cGMP content was determined with a cGMP enzyme immunoassay kit. Each value is expressed as the mean ± S.E.M. from four independent experiments. *$P < 0.05$ vs. corresponding controls. (B) Cells were treated with 8-Br-cGMP (0.1–2 mM) or its vehicle (0) for 48 h. Neurite length was measured as described in the Materials and methods. Each value is expressed as the mean ± S.E.M. of 100 cells. *$P < 0.01$ vs. treatment with vehicle alone. (C) The morphological change induced by 8-Br-cGMP in PC12h cells. Cells were treated with (b) or without (a) 2 mM 8-Br-cGMP for 48 h. Scale bar = 100 μm.

was found in this experiment that ODQ could not inhibit the neurite outgrowth when it was added 60 min after the treatment with genipin or NGF (Fig. 2B, 60 min). ODQ did, however, exert a significant inhibitory effect when it was added 30 min or less after treatment with these stimulators (Fig. 2B, 0–30 min). Furthermore, genipin- and NGF-induced significant elevation of intracellular cGMP production induced by genipin and NGF could be observed within 30 min (Fig. 3A). Therefore, it appears that the induction of neuritogenesis requires a soluble guanylate cyclase-dependent initiation time that is less than 60 min, but more than 30 min, after the addition of genipin and NGF in PC12h cells.

A membrane-permeable cGMP analog, 8-Br-cGMP, induced the neurite outgrowth activity in a concentration-dependent manner (Fig. 3B and C). Therefore, it may be reasonable to conclude that the elevation of intracellular cGMP level evoked by soluble guanylate cyclase activation could induce neurite elongation in PC12h cells treated with genipin and NGF.

3.3. Inhibition of neuritogenesis by a PKG inhibitor

It is known that cGMP has various cellular targets such as PKG, several ion channels, and phosphodiesterase (Schmidt et al., 1993). Therefore, to determine the participation of PKG in the mechanism of neurite outgrowth induced by genipin and NGF in PC12h cells, we tested the effect of KT5823, a selective inhibitor of PKG (Ito and Karachot, 1990), on neurite outgrowth in the presence of these stimulators.

The specificity of the inhibitory effect of KT5823 on PKG was demonstrated by the fact that this inhibitor completely abolished 8-Br-cGMP-induced neurite outgrowth in the concentration range from 0.2 to 1 μM (Fig. 4A, left). Moreover, it had no effect even at a concentration as high as 5 μM on neurite outgrowth induced by dibutyryl cyclic AMP (dbcAMP) which is known to activate cyclic AMP (cAMP)-dependent protein kinase (PKA) (Fig. 4A, right).

The neuritogenic activities of genipin (Fig. 4B) and NGF (Fig. 4C) were partially, but significantly, inhibited by KT5823 dose-dependently, which indicated a partial role, at least in the activation of PKG, in the neuritogenesis by genipin and NGF in PC12h cells.

3.4. Relation between the NO–cGMP–PKG pathway and MAPK cascade

We have previously shown that ERK phosphorylation induced by genipin and NGF was greatly inhibited by a

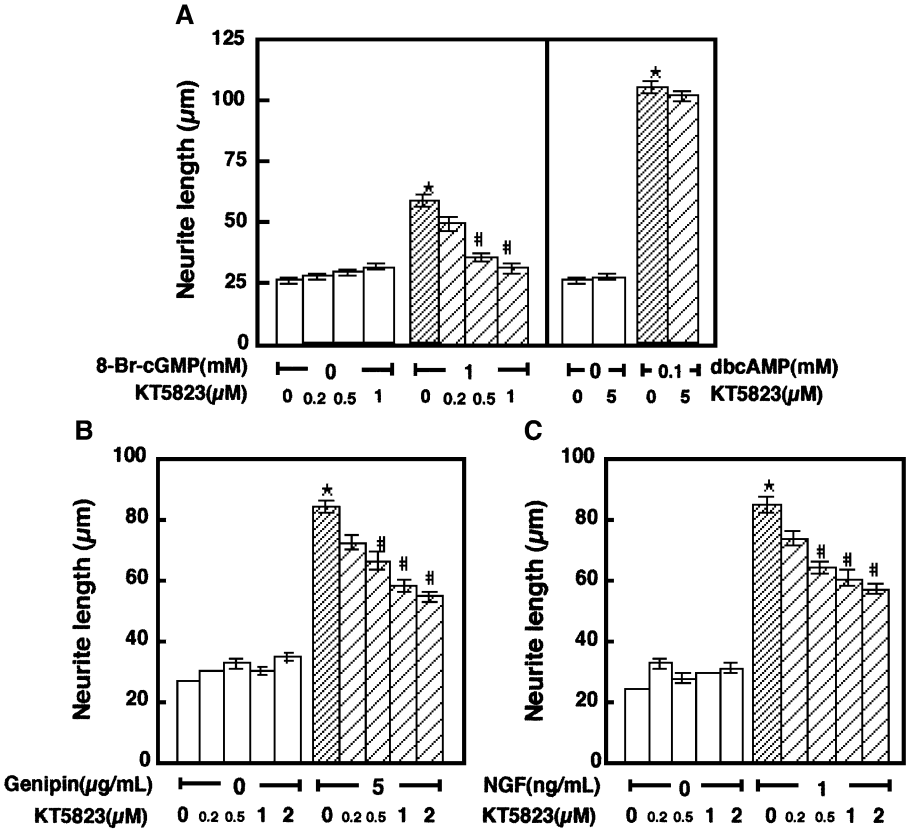

Fig. 4. Effect of PKG inhibitor on neuritogenesis induced by several stimuli in PC12h cells. KT5823 (0.2–5 μM) or its vehicle (0) was added to the medium 30 min before treatment with 8-Br-cGMP (1 mM, A left), dbcAMP (0.1 mM, A, right), genipin (5 μg/ml, B), NGF (1 ng/ml, C) or their vehicle (0) for 48 h. Neurite length was measured as described in the Materials and methods. Each value is expressed as the mean ± S.E.M. of 100 cells. *$P<0.01$ vs. treatment with vehicle alone, $^{\#}P<0.01$ treatment with 8-Br-cGMP, dbcAMP, NGF, or genipin alone.

NOS inhibitor in PC12h cells (Yamazaki et al., 2001), indicating that the MAPK cascade is downstream of NOS activation in the signaling pathway leading to the neurite outgrowth induced by both genipin and NGF. We then investigated the mutual relationship between the production of cGMP and phosphorylation of ERK in the signaling pathway.

Both genipin and NGF induced remarkable and sustained ERK phosphorylation (Fig. 5A, ODQ −). These phosphorylations were maximal within 10 min of treatment and were followed by gradual declines over the next 50 min of incubation. In contrast, ERK phosphorylation in the control cells was hardly detected during the time periods experimented. In the presence of 50 μM ODQ, the genipin- and NGF-induced ERK phosphorylations were gradually inhibited (Fig. 5A, ODQ+). The inhibitory effect of ODQ reached 87% and 98%, in the cells treated with genipin and

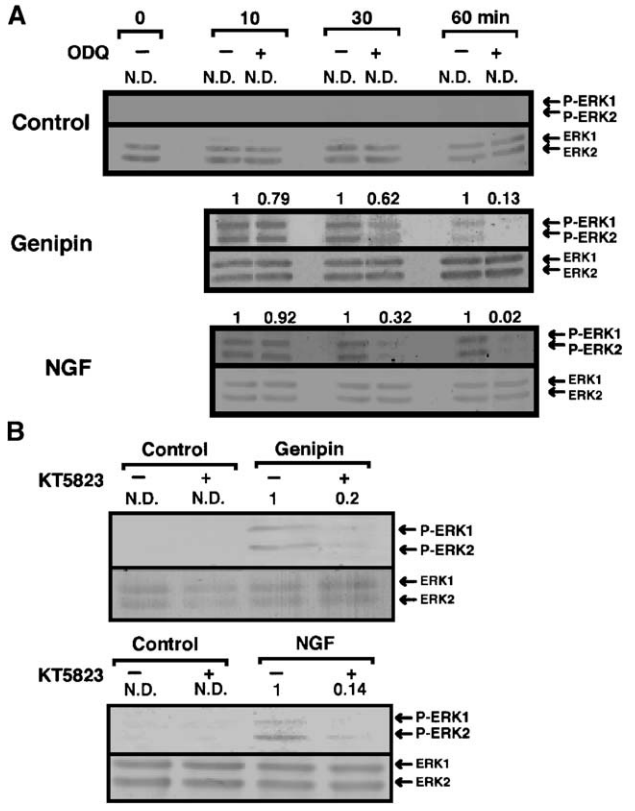

Fig. 5. Western blot analysis for inhibitory effect of soluble guanylate cyclase and PKG inhibitors on genipin- and NGF-induced ERK phosphorylation in PC12h cells. (A) ODQ (50 μM, +) or its vehicle (−) was added to the medium 60 min before treatment with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) for various times. (B) KT5823 (2 μM, +) or its vehicle (−) was added to the medium 15 min before treatment with genipin (5 μg/ml), NGF (1 ng/ml) or their vehicle (Control) for 60 min. Cell extracts corresponding to 10 μg of proteins were subjected to 10% SDS-PAGE for each sample. The immunoreactive bands of total ERKs and phosphorylated ERKs were detected using each specific antiserum. Values shown above the panels are relative phosphorylated ERKs levels normalized for total ERKs levels in corresponding samples. N.D. means no detection. Results are representative of five separate experiments.

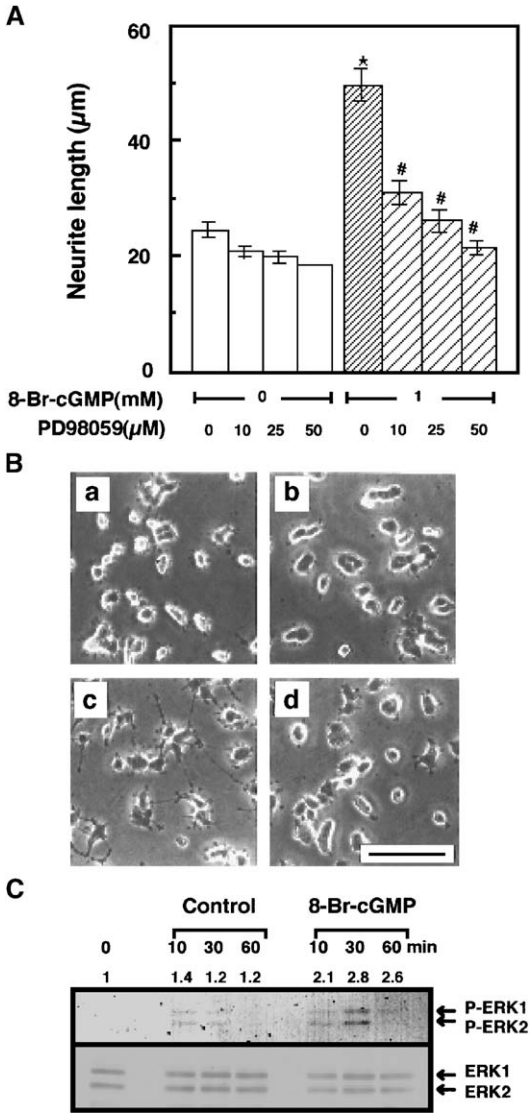

Fig. 6. Involvement of ERK phosphorylation in 8-Br-cGMP-induced neurite outgrowth in PC12h cells. (A and B) PD98059 (10–50 μM) or its vehicle (0) was added to the medium 60 min before treatment with 8-Br-cGMP (1 mM) or its vehicle (0) for 48 h. (A) Neurite length was measured as described in the Materials and methods. Values are expressed as the mean ± S.E.M. of 100 cells. $^{*}P<0.01$ vs. treatment with vehicle alone, $^{\#}P<0.01$ vs. treatment with 8-Br-cGMP alone. (B) Morphological changes. (a) Vehicle, (b) 50 μM PD98059, (c) 1 mM 8-Br-cGMP and (d) 8-Br-cGMP + PD98059. Scale bar = 100 μm. (C) Western blot analysis for 8-Br-cGMP-induced ERK phosphorylation in PC12h cells. Cells were treated with 8-Br-cGMP (1 mM) or its vehicle (Control) for various times. Cell extracts corresponding to 10 μg proteins were subjected to 10% SDS-PAGE for each sample. The immunoreactive bands of total ERKs and phosphorylated ERKs were detected using each specific anti serum. Values shown above the panels are relative phosphorylated ERKs levels normalized for total ERKs levels in corresponding samples. Results are representative of three separate experiments.

NGF for 60 min, respectively. Similarly, KT5823 markedly inhibited the ERK phosphorylation by 80% and 86% in the cells treated with genipin and NGF for 60 min, respectively. The phosphorylation of ERK therefore would follow activation of the NO–cGMP–PKG signaling pathway utilized

by genipin and NGF in PC12h cells. Interestingly, in agreement with the results in Fig. 2, it seems that the participation of cGMP in the signaling pathway of genipin and NGF exerts its effect between 30 and 60 min after treatment with these stimulators.

To confirm the importance of cGMP to neuronal differentiation, we examined the effect of a MEK inhibitor on cGMP-induced neurite outgrowth and the ability of cGMP to induce ERK phosphorylation. Pretreatment of PC12h cells for 60 min with PD98059 inhibited the 8-Br-cGMP-induced neurite outgrowth in a concentration-dependent manner (Fig. 6A). This inhibition was complete at a concentration of 50 μM (Fig. 6A and B). Fig. 6C shows that 8-Br-cGMP is capable of inducing ERK phosphorylation.

4. Discussion

We have demonstrated here that genipin- and NGF-induced neurite outgrowth could be inhibited by early treatment with a soluble guanylate cyclase inhibitor (Fig. 2) and that a cGMP analog, 8-Br-cGMP, alone could induce neurite outgrowth in PC12h cells (Fig. 3). Furthermore, cGMP analog-induced neurite outgrowth was also observed in Neuro 2a cells (unpublished data). Zwiller et al. (1977) reported that a cGMP analog alone or together with a cAMP analog induced morphological differentiation in mouse cultured neuroblastoma cells. In PC12 cells, physiological roles for the NO–cGMP–PKG pathway have been reported, such as the regulation of ryanodine receptor activity (Clementi et al., 1996) and protection against apoptosis (Kim et al., 1999). However, in contrast to PC12h cells, Phung et al. (1999) have previously reported that NO but not cGMP was required for NGF-induced PC12 cell differentiation and that the addition of 8-Br-cGMP alone could not induce neurite outgrowth in this cell line. Moreover, Hindley et al. (1997) indicated that NGF-induced neurite outgrowth was not inhibited by the addition of a soluble guanylate cyclase inhibitor in PC12 cells. The difference in responsiveness to cGMP in neuritogenesis may be related to the content of NOS protein. Some investigators have shown that untreated-PC12 cells do not express any type of NOS (Sheehy et al., 1997; Schonhoff et al., 2001), while we have confirmed by Western blotting that untreated-PC12h (Yamazaki et al., 2001) and Neuro2a cells (unpublished data) actually express NOS protein. Therefore, we believe that neuronal cells endogenously expressing NOS could utilize the NO–cGMP–PKG signaling pathway for neuritogenesis. Thus, the responsiveness to cGMP in neuritogenesis is raised in PC12h and Neuro2a cells. In PC12h cells, we strongly believe that cGMP plays an important role as a major mediator in the induction of neurite outgrowth by genipin as well as by NGF.

In the present study, KT5823, a PKG inhibitor, completely inhibited 8-Br-cGMP-induced neurite outgrowth (Fig. 4A). The inhibitory effect of KT5823 on the NGF- and genipin-induced neurite outgrowth was incomplete yet significant in PC12h cells (Fig. 4B and C). In NGF-induced neuritogenesis of PC12h cells, it appears that the TrkA–Ras–ERK pathway plays a major role. In genipin-induced neuritogenesis of PC12h cells, it appears that cGMP activates not only PKG but also PKA through inhibition of phosphodiesterase type I and/or III (Schmidt et al., 1993) to evoke neurite outgrowth. In fact, we previously reported that the genipin-induced neurite outgrowth was inhibited in the presence of a PKA inhibitor, H-89, in PC12h cells (Yamazaki et al., 1996). In contrast, no inhibition of the NGF-induced neurite outgrowth was detected in the presence of H-89 in PC12h cells (data not shown), which is in agreement with the report that NGF could induce neuritogenesis in a cAMP-insensitive variant of PC12 cell line (Damon et al., 1990).

Previous reports have pointed out the importance of NO in NGF-induced neuronal differentiation in PC12 cells (Peunova and Enikolopov, 1995; Poluha et al., 1997; Phung et al., 1999), but they did not discuss the possibility of NO-cGMP-induced phosphorylation/activation of ERKs. In this report, we have shown that the ERK phosphorylation induced by treatment with genipin and NGF was greatly inhibited by the addition of ODQ and KT5823 in a time-dependent manner (Fig. 5). This inhibition was almost perfect 60 min after treatment with genipin and NGF. The results of two time course experiments (Figs. 2 and 5) strongly suggest that the activation of soluble guanylate cyclase occurred between 30 and 60 min and is important to the neuritogenic activities of genipin and NGF. Moreover, we previously reported that NOS activity was elevated 30 min after treatment with genipin or NGF, its elevation was sustained for 48 h, and that a NOS inhibitor diminished the ERK phosphorylation induced by treatment with these stimulators for 30 min (Yamazaki et al., 2001). Thus, our above results indicate that phosphorylation of ERKs follows activation of the NO–cGMP–PKG pathway in genipin- and NGF-treated PC12h cells.

On the other hand, since both genipin- and NGF-induced early (10 min) ERK phosphorylations were only slightly inhibited by ODQ (Fig. 5A), they might be mediated by some other mechanisms. For example, as widely established, NGF-induced ERK phosphorylation is dependent on Ras activation in PC12 cells (Kaplan and Stephens, 1994). In regard to genipin, although we do not have any experimental evidence, it has been suggested that NO directly activates Ras by its nitrosylation (Lander et al., 1996; Teng et al., 1999). Therefore, we speculate that nitrosylated Ras could induce the early activation of ERK in genipin-treated PC12h cells. However, our results suggest that the step of initial stimulation of NOS activity is considerably important for starting the neuritogenesis by genipin in PC12h cells. Although the mechanism of genipin-induced NOS activation is not clear at this time, our preliminary experiments suggest that genipin directly binds to neuronal NOS and activates it in vitro (Ohkubo et al., in press).

In the near future, we hope to determine whether and/or how PKG stimulates the MAPK cascade to induce neurite outgrowth by genipin and NGF in PC12h cells. Since it has been previously reported that PKG directly activates several components of the MAPK cascade (Hood and Granger, 1998; Soh et al., 2001), it seems that activation of the MAPK cascade could be induced by PKG in genipin-treated PC12h cells.

In summary, the data presented here clearly demonstrate that: (1) in PC12h cells, genipin and NGF induce neurite outgrowth involving the NO–cGMP–PKG pathway; and (2) the NO–cGMP–PKG pathway induces ERK phosphorylation in PC12h cells.

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