Induction of Neurite Outgrowth in PC12 Cells by α-Phenyl-N-tert-butylnitron through Activation of Protein Kinase C and the Ras-Extracellular Signal-regulated Kinase Pathway

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The spin trap α-phenyl-N-tert-butylnitron (PBN) is widely used for studies of the biological effects of free radicals. We previously reported the protective effects of PBN against ischemia-reperfusion injury in gerbil hippocampus by its activation of extracellular signal-regulated kinase (ERK) and suppression of both stress-activated protein kinase and p38 mitogen-activated protein kinase. In the present study, we found that PBN induced neurite outgrowth accompanied by ERK activation in PC12 cells in a dose-dependent manner. The induction of neurite outgrowth was inhibited significantly not only by transient transfection of PC12 cells with dominant negative Ras, but also by treatment with mitogen-activated protein kinase/ERK kinase inhibitor PD98059. The activation of receptor tyrosine kinase TrkA was not involved in PBN-induced neurite outgrowth. A protein kinase C (PKC) inhibitor, GF109203X, was found to inhibit PBN neurite outgrowth. The activation of PKCε was observed after PBN stimulation. PBN-induced neurite outgrowth and ERK activation were counteracted by the thiol-based antioxidant N-acetylcysteine. From these results, it was concluded that PBN induced neurite outgrowth in PC12 cells through activation of the Ras-ERK pathway and PKC.

Rat pheochromocytoma cell line PC12 is a well-established model for the investigation of signal transduction pathways in neuronal differentiation. Neurotrophic factors such as nerve growth factor (NGF) lead the PC12 cells to differentiate into neuronal-like cells with neurites (1–5). The mechanism of neurite outgrowth induced by NGF has been well investigated. It induces cell differentiation and neurite outgrowth by binding with and activating the TrkA receptor tyrosine kinase (6, 7).

This work was supported in part by Grants-in-aid for Basic Science Research 12660266 (to O. I.), 13014292 (to O. I.), 13218003 (to O. I.), 12460135 (to M. K.), and 13876069 (to M. K.) from the Ministry of Education, Science, Sports and Culture of Japan, by Sapporo Industrial Machinery Co. Ltd., and by a grand-in-aid to cooperative research in Rakuno-Gakuen University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: NGF, nerve growth factor; PLCγ, phospholipase Cγ; PEβ-kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; PBN, α-phenyl-N-tert-butylnitron; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetylcysteine; DTT, dithiothreitol; PKC, protein kinase C; PKA, protein kinase A; rsGFP, red shift green fluorescent protein; BAPTA-AM, 1,2-bis(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxyethyl)ester.

This article induces phosphorylation of Shc (8, 9), phospholipase Cγ (PLCγ) (10), and phosphatidylinositol 3-kinase (PI3-kinase) (11), followed by activation of the Ras-extracellular signal-regulated kinase (ERK) cascade (12–14), PKC, and Akt, respectively. In addition, it was demonstrated that the Ras-ERK cascade was necessary and sufficient for NGF-induced neuronal differentiation of PC12 cells (13–16).

α-Phenyl-N-tert-butylnitron (PBN) is an agent used most widely for investigating free radicals in biological systems and is known to be able to prevent oxidative injury without significant toxicity. PBN attenuates the age-related protein oxidation, decrease of enzyme activity, and loss of memory in gerbils (17) and protects hippocampal cells from ischemia-reperfusion injury (18). It also protects LEC rats from copper-induced fulminant hepatitis (19). PBN is thought to exert these biological effects by blocking oxidative stress-induced free radical reactions. Recently, other biological effects of PBN were reported. PBN has the ability to interfere with inflammatory cytokines (20), increase anti-inflammatory cytokine (21), and inhibit the induction of inducible nitric oxide synthase (22) in rats or mice given lipopolysaccharide. These effects may be considered as the interruption of the inflammatory signaling pathways by PBN.

We previously investigated the neuroprotective effect of PBN on ischemia-reperfusion injury in the gerbil hippocampus. Intraperitoneal administration of PBN to gerbils enhances the activation of ERK, suppresses the activation of stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (p38), and protects hippocampal cells from ischemia-reperfusion injury (23). Furthermore, PBN induced the heat shock proteins HSP70 and HSP27 in the gerbil hippocampus. These data suggested that PBN had the ability not only to trap free radicals as its inherent activity but also to regulate the mitogen-activated protein kinase (MAPK) pathway.

In the present study, we determined the ability of PBN to induce neurite outgrowth and ERK activation in PC12 cells to clarify further aspects of PBN. We showed that PBN induced neurite outgrowth in PC12 cells through activation of the Ras-ERK pathway and PKC.

EXPERIMENTAL PROCEDURES

Materials—Rat pheochromocytoma PC12 cells were obtained from the RIKEN Cell Bank. NGF, pUSEamp, H-Ras (dominant negative) in pUSEamp, and antibodies to Shc, Grb2, phosphotyrosine (4G10), TrkA, PLCγ, PKCε, and phospho-PKCε were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to ERK, phospho-ERK, SAPK, phospho-SAPK, p38, phospho-p38, phospho-TrkA, Akt, phospho-Akt, and PD98059 were from New England Biolabs (Beverly, MA). PBN, N-acetylcysteine (NAC), dithiothreitol (DTT), 2-mercaptoethanol, GF109203X, poly-L-lysine hydrobromide, and the protease inhibitor mixture were from Sigma-Aldrich. R252a, wortmannin, and H89 were from Calbiochem, Inc. LipofectAMINE 2000 was from Life Technolo-
Induction of Neurite Outgrowth in PC12 Cells by PBN

Neurite Outgrowth—PC12 cells were seeded in poly-L-lysine-coated 6-well plates at a density of $1 \times 10^5$ cells/well with complete medium, and after 16–24 h, cells were treated with or without 10 μM PBN or 50 ng/ml NGF for 72 h. For experiments combining PBN (or NGF) and inhibitors, each inhibitor (the TrkA tyrosine kinase inhibitor K252a, the MAPK/ERK kinase inhibitor PD98059, the PI3-kinase inhibitor wortmannin, the PKC inhibitor GF109203X, and the protein kinase A (PKA) inhibitor H89) was added 60 min before stimulation. The effects of antioxidants (NAC, DTT, and 2-mercaptoethanol) were also examined in a similar manner. The percentage of cells with neurites extending at least 2 diameters of the cell body was counted.

Transient Transfection—PC12 cells were transiently transfected with pUSEamp expression vector containing a cDNA construct encoding dominant negative H-Ras (DNRas) mutated serine to asparagine at position 17. For visualization, cells were co-transfected with the pQBI25 vector encoding red shift green fluorescent protein (rsGFP).

RESULTS

Induction of Neurite Outgrowth in PC12 Cells by Treatment with PBN—PC12 cells were incubated with 10 μM PBN, 50 ng/ml NGF, or 10 μM NAC for 72 h. As shown in Fig. 1A, no neurite outgrowth was found in control or NAC-treated cells. By contrast, morphological changes to neuronal differentiated cells occurred in PBN- or NGF-treated cells. The percentages of cells showing neurite outgrowth are presented in Fig. 1B. PBN-induced neurite outgrowth in a dose-dependent manner. Ten μM PBN induced neurite outgrowth in 68.9 ± 2.7% of PC12 cells, whereas NGF did so in 98.8 ± 0.8% of PC12 cells. Morphologically, the majority of neurites induced by PBN were bipolar in contrast to the multipolar neurites observed in NGF-treated cells. Although both PBN and NAC are antioxidants, NAC induced no neurite outgrowth. This meant that PBN-induced neurite outgrowth could not be explained by the ability of PBN to scavenge free radicals.

Induction of Selective Activation of ERK in MAPK Signaling Pathways by PBN—It has been reported that the MAPK pathway is crucial for NGF-induced neuronal differentiation (15, 24). To investigate the effects of PBN on the activation of MAPKs, we examined the phosphorylation of ERK, SAPK, and...
PBN (10 mM) for the indicated times or with NGF (50 ng/ml) for 5 min. Western low-serum medium for 16 h. Cells were then stimulated with PBN (10 mM) for the indicated times or with NGF (50 ng/ml) for 5 min. Western blots were performed using anti-phospho-ERK, anti-phospho-SAPK, and anti-phospho-p38 antibodies. Blots were then stripped and reprobed with anti-ERK, anti-SAPK, and anti-p38 antibodies, respectively, to verify that equal amounts of proteins were present in each sample. Similar results were obtained from at least three independent experiments.

p38 in PC12 cells treated with PBN. Cells were serum-starved by incubation with low-serum medium for 16 h and then treated with PBN or NGF. PBN induced only the phosphorylation of ERK with a peak at 5 min, and the amount of phosphorylated ERK decreased to the control level after 60 min. By contrast, NGF activated all MAPKs examined (Fig. 2).

**Induction of Neurite Outgrowth Induced by PBN in PC12 Cells Is Dependent on the Ras-ERK Pathway but Independent of TrkA**—The ERK cascade is usually initiated by the interaction of NGF with TrkA receptor tyrosine kinase, which results in the autophosphorylation of the receptor. Phosphorylated TrkA activates Ras through the tyrosine phosphorylation of Shc and the subsequent association of phospho-Shc with adapter proteins Grb2 and SOS (6–9). To investigate the participation of the TrkA pathway in the induction of neurite outgrowth by PBN, we examined the effects of a specific inhibitor of TrkA tyrosine kinase, PD98059 (26), on neurite outgrowth induced by PBN in PC12 cells. PD98059 caused significant inhibition of neurite outgrowth induced by PBN, but K252a did not, although both significantly inhibited neurite outgrowth induced by NGF (Fig. 3A). PD98059 also inhibited the phosphorylation of ERK, but K252a did not so at 5 min after the PBN treatment (Fig. 3B). When we examined the phosphorylation of TrkA using an antibody specific to TrkA phosphorylated at Tyr-490, it was found that the phosphorylation of TrkA tyrosine kinase was not induced by PBN, whereas it was induced by NGF at 5 min after stimulation (Fig. 3C). These results suggested that the phosphorylation of TrkA was not involved in PBN-induced neurite outgrowth but that the activation of ERK was necessary for neurite outgrowth caused by PBN and NGF. The phosphorylation of TrkA causes the tyrosine phosphorylation of Shc and its association with Grb2 and SOS. The phosphorylation of PLCγ, which hydrolyzes phosphatidylinositol-4,5-diphosphate into diacylglycerol and inositol-1,4,5-triphosphate, and the activation of PI3-kinase followed by the phosphorylation of Akt are also caused by the phosphorylation of TrkA. The treatment of cells with PBN for 5 min did not cause the phosphorylation of Shc and the subsequent binding to Grb2 (Fig. 4A), the phosphorylation of PLCγ (Fig. 4B) or the phosphorylation of Akt resulting from the activation of PI3-kinase (Fig. 4C), whereas phosphorylation of NGF activated all of these proteins. Furthermore, we examined the effect of a short treatment of cells with PBN on these signaling molecules because the PBN-induced activation of ERK occurred quickly (within 5 min), as shown in Fig. 2. However, treatment with PBN for 0.5, 1, and 2.5 min did not cause phosphorylation of Shc and the subsequent binding to Grb2 and phosphorylation of PLCγ and Akt (data not shown).

Because the Ras-ERK pathway is necessary for neurite outgrowth induced by NGF, we further examined the requirement for Ras activation in PBN-induced neurite outgrowth. PC12 cells were transiently transfected with a construct encoding DNras. Cells were co-transfected with a pQB125 vector encoding rsGFP at 20:1 (DNras/rsGFP) to allow the identification of cells transfected with the DNras construct. By observing only rsGFP-positive cells, the induction of neurite outgrowth in cells transfected with an empty vector and an rsGFP vector stimulated with PBN and NGF was confirmed to be 62.2 ± 2.7% and 98.7 ± 0.5%, respectively (Figs. 5, A and B), values similar to those seen in nontransfected cells (Fig. 1B). By contrast, neurite outgrowths from PBN- and NGF-stimulated cells transfected with a DNras construct and an rsGFP vector were significantly decreased to 9.2 ± 1.3% and 24.9 ± 2.5%, respectively (Fig. 5, A and B). These results suggested that neurite outgrowth induced by PBN was dependent on the Ras-ERK pathway but independent of TrkA tyrosine kinase.

**Activation of PKCε by PBN**—There are several mediators that induce GDP-GTP exchange to Ras and thereby activate the Ras-ERK pathway. It has been reported that the Ras-ERK pathway is activated by PLCγ (27), PKC (14), and extracellular calcium influx (3). On the other hand, the activation of PKA induces Ras-independent activation of B-raf and leads to ERK activation (28). We therefore examined other mediators that are necessary for PBN-induced neurite outgrowth using various inhibitors. Fig. 6A shows the effects of various inhibitors on PBN-induced neurite outgrowth in PC12 cells. Inhibitors were added 1 h before PBN treatment. The percentage of neurite-induced cells was significantly decreased by PKC inhibitor GF109203X, whereas BAPTA-AM (an intracellular calcium chelator), wortmannin (a PI3-kinase inhibitor), and H89 (a PKA inhibitor) had no effects. However, GF109203X caused a modest reduction in PBN-dependent ERK phosphorylation (71% and 75% inhibition in p42 and p44, respectively) (Fig. 6B). These data suggested that PKC partly regulated the activation of ERK by PBN. PKC is a family of serine-threonine kinases classified by its sensitivity to calcium, diacylglycerol, and phorbol esters (29). Because intracellular calcium chelator BAPTA-AM had no effect on either neurite outgrowth or ERK phosphorylation by PBN (Fig. 6, A and B), we further examined the activation of PKCε, which is classified as a calcium-independent PKC. Stimulation by PBN as well as NGF induced marked phosphorylation of PKCε within 5 min, and it continued for at least 120 min (Fig. 7). These results suggested that the activation of PKCε played a role in neurite outgrowth.
used potent antioxidants and investigated the effects on neurite outgrowth and ERK activation induced by PBN. NAC inhibited PBN-induced neurite outgrowth in a dose-dependent manner, and the percentage of cells inducing the neurite outgrowth decreased from 68.9 ± 2.7% to 4.4 ± 2.1%, whereas the inhibitory effect of NAC on the neurite outgrowth induced by NGF was small (ranging from 98.7 ± 1.1% to 67.3 ± 2.9%) compared with that of PBN (Fig. 8A). ERK phosphorylation was also decreased by NAC. In addition, other antioxidants, DTT and 2-mercaptoethanol, inhibited ERK phosphorylation. These results led us to conclude that PBN might induce neurite outgrowth in PC12 cells via activation of the Ras-ERK signaling pathway and activation of PKCe by modification of the intracellular redox state.

**DISCUSSION**

The spin trap PBN has been widely used for the detection of free radicals. Moreover, it has been reported that PBN has a wide range of pharmacological effects, including the inhibition of age-related protein oxidation (17), protection against ischemia-reperfusion injury in the gerbil hippocampus (18), protection of LEC rats from copper-induced fulminant hepatitis (19), and inhibition of anti-inflammatory effects in lipopolysaccharide-treated rats or mice (20, 21). Our previous studies demonstrated that administration of PBN to gerbils protected the hippocampal cells from ischemia-reperfusion injury, enhanced the activation of ERK, and suppressed the activation of SAPK and p38. Furthermore, PBN administration induced HSP70 and HSP27 (23). Because we observed another pharmacological effect of PBN, induction of neurite outgrowth in PC12 cells, in the present experiments we investigated the mechanism of this effect with special emphasis on the signal transduction pathway involved in neurite outgrowth of PC12 cells.

Recently, several substances with the ability to induce neurites in PC12 cells were reported. For example, the mouse semaphorin H molecule induces neurite outgrowth through extracellular calcium influx and the Ras-ERK pathway (3), and the 38-amino acid isoform of pituitary adenylate cyclase-activating peptide (PACAP38) induces it via activation of PKC and ERK, but not via activation of PKA, TrkA, Ras, or Sre (4). The fungal protein p15 (5), a synthetic peptide ligand (sequence, ASKKPKRNIKA) of the neural cell adhesion molecule (31), and
bone morphogenetic protein-2 (1) are reported to induce neurite outgrowth in PC12 cells. Because all these substances are proteins or peptides, PBN is thought to be a unique substance with regard to induction of neurite outgrowth in PC12 cells.

Numerous studies on NGF-induced neuronal differentiation in PC12 cells have revealed that the association of NGF with TrkA receptor tyrosine kinase is an initial step (6, 7). The activation of TrkA induces the phosphorylation of PLC/PI3-kinase, and Shc (10, 11). Subsequently, the association of the phosphorylated Shc with adapter proteins Grb2 and SOS causes GDP-GTP exchange of Ras (8, 9). The activation of Ras causes activation of the Ras-ERK cascade, resulting in neurite outgrowth. In the present study, PBN also induced neurite outgrowth in about 70% of the PC12 cells (Fig. 1, A and B) and activated ERK (Fig. 2), which was necessary to induce neurites (Fig. 3, A and B). However, unlike NGF, it induced no phosphorylation of TrkA and PLC/PI3-kinase (Fig. 3 C), no phosphorylation of Akt accompanied by PI3-kinase phosphorylation, and no phosphorylation of Shc followed by association with Grb2 (Fig. 4).

NGF induces the continuous activation of ERK in PC12 cells, whereas epidermal growth factor activates ERK transiently and cannot induce neurite outgrowth in PC12 cells (32). However, by overexpression of the epidermal growth factor receptor or activated form of MAPK/ERK kinase, ERK is continuously activated, resulting in the induction of neurite outgrowth (16, 33). These reports demonstrate that the continuous activation of ERK is necessary for neurite outgrowth. In the present

**Fig. 5.** Ras dependence of the induction of neurite outgrowth by PBN in PC12 cells. A, neurite outgrowth was assessed in PC12 cells transfected with DNRas and treated with PBN or NGF. PC12 cells were transiently transfected with an empty vector and rsGFP vector (top panels) or a DNRas (S17N) cDNA construct and rsGFP vector (bottom panels) at a 20:1 ratio. Transfected cells were stimulated with PBN (10 mM) or NGF (50 ng/ml) for 72 h. Micrographs show PC12 cells visualized by rsGFP fluorescence. B, inhibition of PBN- or NGF-induced neurite outgrowth by transfection of DNRas. PC12 cells were transfected and stimulated under the same conditions described in A. At 72 h after stimulation, the percentage of rsGFP-positive cells with neurites was determined. Each value is the mean ± S.D. of about 100 cells obtained from three independent experiments. **, p < 0.001, Student’s t test.

**Fig. 6.** The effects of various inhibitors on neurite outgrowth and on the phosphorylation of ERK induced by PBN. A, PC12 cells grown at a density of 1 × 10⁵ cells/cm² were pretreated with BAPTA-AM (50 μM), wortmannin (10 μM), GF109203X (5 μM), and H89 (1 μM) for 1 h and then stimulated with PBN (10 mM). The percentage of cells with neurites was determined at 72 h after stimulation. Each value is the mean ± S.D. of about 200 cells obtained from three independent experiments. **, p < 0.001, Student’s t test. B, PC12 cells grown at a density of 2 × 10⁴ cells/cm² were serum-starved by incubation with low-serum medium for 16 h. Cells were then treated with PBN (10 mM) for the indicated times or with NGF (50 ng/ml) for 5 min. In the inhibitory experiment, GF109203X (5 μM) was added 1 h before stimulation. Cells were lysed, and Western blots were performed using an anti-phospho-ERK antibody. Blots were then stripped and reprobed with an anti-PKCε antibody to verify that equal amounts of protein were present in each sample. Similar results were obtained from at least three independent experiments.

**Fig. 7.** Activation of PKCe by PBN in PC12 cells. PC12 cells grown at a density of 2 × 10⁵ cells/cm² were serum-starved by incubation with low-serum medium for 16 h. Cells were then treated with PBN (10 mM) or NGF (50 ng/ml) for the indicated times. In the inhibitory experiment, GF109203X (5 μM) was added 1 h before stimulation. Cells were lysed, and Western blots were performed using an anti-phospho-PKCε antibody. Blots were then stripped and reprobed with an anti-PKCε antibody to verify that equal amounts of protein were present in each sample. Similar results were obtained from at least three independent experiments.
Induction of Neurite Outgrowth in PC12 Cells by PBN

PC12 cells grown at a density of 2 × 10^5 cells/cm² were pretreated with various concentrations of NAC for 1 h and then stimulated with PBN (10 mM) or NGF (50 ng/ml). The percentage of cells with neurites was determined at 72 h after stimulation. Each value is the mean ± S.D. of about 200 cells obtained from three independent experiments. *p < 0.01 vs. NAC-treated and NGF-treated cells, respectively, by Student’s t test. Data were analyzed using a one-way ANOVA followed by a Dunnett’s multiple-comparison test. 

Fig. 8. Inhibition of PBN-induced neurite outgrowth and phosphorylation of ERK by antioxidants. A, effects of NAC on neurite outgrowth induced by PBN or NGF. PC12 cells grown at a density of 1 × 10^5 cells/cm² were pretreated with various concentrations of NAC for 1 h and then stimulated with PBN (10 mM) or NGF (50 ng/ml). The percentage of cells with neurites was determined at 72 h after stimulation. Each value is the mean ± S.D. of about 200 cells obtained from three independent experiments. *p < 0.01 vs. NAC-untreated and NGF-treated cells, respectively, by Student’s t test. B, Western blots were performed using an anti-phospho-ERK antibody. Blots were then stripped and reprobed with an anti-ERK antibody to verify that equal amounts of protein were present in each sample. Similar results were obtained from at least three independent experiments.

results, neurites from PC12 cells treated with PBN were bipolar in a different fashion than those from cells treated with NGF. Considering our results together with the reports cited above, it is conceivable that this difference is attributable to the transient and lesser activation of ERK by PBN as compared with NGF. Furthermore, NGF induces both neurite outgrowth and cell proliferation via ERK and PI3-kinase cascades, respectively. In NGF-stimulated PC12 cells, blockade of the ERK pathway inhibited neurite outgrowth but not proliferation (data not shown). The other hand, PI3-kinase signaling is reported to be necessary for PC12 cell proliferation, although the blockade of this pathway did not affect neurite outgrowth (11), an observation that was again confirmed by our results shown in Fig. 6, A and B. In our experiments, the number of PC12 cells cultured with NGF for 72 h greatly increased in comparison to the number of untreated cells, whereas PBN did not induce cell death and did not influence the cell growth rate (data not shown). This observation supports the hypothesis that PBN can activate the ERK pathway to induce neurite outgrowth but cannot activate the PI3-kinase-Akt pathway to promote proliferation.

It is known that several cascades activate ERK in PC12 cells. PKC activates ERK via Ras activation (13, 14), whereas the Ras-independent mechanism preferentially activates ERK through direct activation of Raf in PC12 cells (34). The extracellular calcium influx induced by membrane depolarization mediates PKA-dependent ERK activation through the formation of Rap1 (a small GTPase of the Ras family)/B-Raf (an isotype of Raf-1) signaling complex in PC12 cells (28). By performing experiments using various inhibitors, we demonstrated that a PKC inhibitor, GFI09203X, inhibited PBN-induced neurite outgrowth (Fig. 6A), whereas it had a moderate inhibitory effect on the activation of ERK at 5 min after PBN stimulation. These data suggested that PKC-dependent ERK activation may be partly involved in PBN-induced neurite outgrowth.

The PKC family is a family of lipid-regulated serine-threonine kinases that phosphorylates a variety of cellular proteins and plays an essential role in the signal transduction mechanism. PKC isozymes have been grouped into three subclasses according to their regulatory properties. The classic PKCs include PKCα, PKCβ, and PKCγ, which can be activated by calcium and/or by diacylglycerol and phorbol esters. The second group is the novel PKCs, including PKCδ, PKCε, PKCθ, and PKCγ, which can be activated by diacylglycerol and phorbol esters, but not by calcium. The third group is the atypical PKCs, including PKCζ and PKCλ, which are unresponsive to calcium and diacylglycerol/phorbol esters (35, 36). In NGF-induced neurite outgrowth, the activation of PKC by bryostatin or by a phorbol ester such as phorbol 12-myristate 13-acetate enhances neurite outgrowth (37, 38). Moreover, the PKCδ and PKCε isotypes of PKC play a role in NGF-induced neurite outgrowth (38, 39), and activation of PKCε is more effective in enhancing neurite outgrowth (40, 41).

In the present experiments, PBN-induced neurite outgrowth was not inhibited by the intracellular calcium chelator BAPTA-AM (Fig. 6, A and B), indicating that the PKC involved in PBN-induced neurite outgrowth was independent of calcium. Based on this result and the properties of PKCs described above, we examined the activation of PKCε by PBN. PBN activated PKCε within 5 min, like NGF, and this activation continued for at least 120 min (Fig. 7). However, although activation of PKC is necessary to induce neurite outgrowth by PBN, activation of PKCε alone is not sufficient to do so because treatment with phorbol 12-myristate 13-acetate, a PKC activator, alone caused no neurite outgrowth in PC12 cells (42, 43).

The present study showed that PBN induced neurite outgrowth in PC12 cells but that NAC did not (Fig. 1, A and B), although both have an antioxidative capability. This result indicated that their antioxidative activities were not involved in neurite outgrowth in PC12 cells. On the contrary, NAC greatly counteracted PBN-induced neurite outgrowth and counteracted NGF-induced neurite outgrowth to a lesser extent (Fig. 8A). Furthermore, the phosphorylation of ERK was prominently inhibited not only by NAC but also by other thiol-based antioxidants, DTT and 2-mercaptoethanol (Fig. 8B). Because of the fact that treatment with NAC, DTT, or 2-mercaptoethanol changed the intracellular redox state to a more reduced condition, it was assumed that PBN acted as an oxidizing agent. However, because no neurites were induced by treatment with oxidizing agents such as H₂O₂ (data not shown), it was suggested that PBN-induced neurite outgrowth did not occur as a result of changing the intracellular redox state to the oxidative condition. Although NAC has been reported to suppress the
signal transduction pathway of NGF-induced neurite outgrowth by activating transcription factor AP-1 and suppressing a MAPK kinase kinase (44), it inhibited PBN-induced neurite outgrowth more potently than did NGF (Fig. 8A). This suggested that NAC suppressed another pathway in PBN-induced neurite outgrowth.

How does PBN induce neurite outgrowth? One hypothesis is proposed from an inherent property of PBN. PBN is a potent spin-trapping agent that interacts not only with oxygen radicals but also with carbon-, nitrogen-, or sulfur-centered radicals. Recent reports showed that tryptophan, tyrosyl, cysteinyl, and glycol radicals were detected by various spin traps using the electron spin resonance method in vitro and in vivo (45–48). Because reactive oxygen species such as hydrogen peroxide and superoxide are generated by various oxidative stresses, enzyme reactions (49), and growth factors such as NGF (50), epidermal growth factor (51, 52), fibroblast growth factor (53, 54) and transforming growth factor β1 (53), it is generally accepted that reactive radicals of amino acid such as cysteinyl radicals generated by intracellular reactive oxygen species after various stimuli exist in cells. Recently, the importance of a cysteine-rich region for the activation of several kinases was proposed. Modification of the cysteine-rich regions of serine-threonine kinases such as Raf-1 and PKC regulates the redox activation of these kinases (55), and the cysteine-rich region of Raf-1 is important for its activation (56). Thus, it is conceivable that PBN forms spin adducts with the reactive radicals of cysteine in proteins, causing conformational changes in proteins and consequently affecting the signal transduction pathway.

Another possibility, the generation of NO from spin traps, including PBN, has been reported (57). This report showed that NO was generated under the oxidative condition from PBN and induced nitrite. We carried out similar experiments according to the method of Saito et al. (57) and confirmed NO production in the reaction of PBN with OH radical. The reduction of NO production by NAC was observed (data not shown). Furthermore, NO was reported to bind to the cysteine residue (Cys-118) of Ras and lead to GDP-GTP exchange, activating the ERK cascade (58). Thus, it is likely that PBN indirectly activates Ras through NO generation.

In conclusion, we demonstrated here that PBN, a spin trap, could induce neurite outgrowth of PC12 cells in a dose-dependent manner. PBN-induced neurite outgrowth was mediated by the activation of ERK. The activation of Ras was critical, but unlike NGF, PBN did not cause activation of the TrkA receptor tyrosine kinase and subsequent activation of Shc, PI3-kinase, and PLCγ. PBN activated PKC. The activation of PKC was necessary for neurite outgrowth induced by PBN, although the activation of PKC was not involved in ERK activation. A thiold-based antioxidant, NAC, inhibited PBN-induced neurite outgrowth in a dose-dependent manner, and not only NAC but also other SH-reducing agents could inhibit PBN-induced phosphorylation of ERK. These data suggest that PBN induces neurite outgrowth through activation of the Ras-ERK pathway and PKC.
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J. Biol. Chem. 2001, 276:32779-32785.
doi: 10.1074/jbc.M101403200 originally published online July 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101403200

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