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Potentiation of nerve growth factor-action by picrosides I and II, natural iridoids, in PC12D cells

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Abstract

Natural iridoid, picroside I (β-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-hydroxy-1a-(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl, 6-(3-phenyl-2-propenoate)) or II (β-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxy]-1a-(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl) alone did not exhibit neuritogenic activity, but caused a concentration-dependent (> 0.1 μM) enhancement of nerve growth factor (NGF, 2 ng/ml)-induced neurite outgrowth from PC12D cells. The picroside-induced enhancing action of NGF was abolished by GF109203X (2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide) (0.1 μM), a protein kinase C inhibitor. Furthermore, PD98059 (2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one) (20 μM), a potent mitogen-activated protein (MAP) kinase kinase inhibitor, completely blocked the picroside-induced enhancement of neurite outgrowth in the presence of NGF (2 ng/ml), suggesting that picrosides activate the MAP kinase-dependent signaling pathway. Interestingly, no increase in the expression of phosphorylated MAP kinase was observed in picroside-treated (60 μM) PC12D cells in the presence of NGF (2 ng/ml). These results suggest that picroside I or II enhances NGF-induced neurite outgrowth from PC12D cells, probably by amplifying a down-stream step of MAP kinase in the NGF receptor-mediated intracellular MAP kinase-dependent signaling pathway.

Keywords: Picroside I; Picroside II; Neurite outgrowth activity; Nerve growth factor (NGF); Mitogen-activated protein (MAP) kinase; PC12D cell

1. Introduction

Dementia is one of the most common health problems of aged populations. It is a dehumanizing condition for the patient, whose intellectual capacity deteriorates to the extent that the performance of routine daily activities is impaired. Several forms of dementia have been distinguished, the most common being Alzheimer's disease and cerebrovascular dementia (Berchtold and Cotman, 1998). It has been reported that nerve growth factor (NGF) stimulates the outgrowth of neurites in neuronal cells and plays an important role in the survival and maintenance of neurons in the central nervous system (Woodruff and Franklin, 1997; Patrick et al., 1996). Thus, NGF may have therapeutic efficacy in the treatment of neurodegenerative diseases, including Alzheimer's disease and cerebrovascular dementia. However, NGF can be used for medical treatment only when directly injected into the brain, since it is a polypeptide of high molecular weight, does not cross the blood–brain barrier, and is easily metabolized by peptidases when administrated peripherally. A useful strategy for addressing the drug delivery problem is to administer drugs that either enhance the action of NGF or increase the expression of NGF in the appropriate cell population (Brinton and Yamazaki, 1998).

Recently, several synthetic compounds such as AIT-082 (4-[[3-(1,6-dihydro-6-oxo-9-purin-9-yl)-1-oxopropyl]amino]benzoic acid) (Middlemiss et al., 1995), SR57746 (1-2-(naphth-2-yl)ethyl]-4-(3-trifluoromethylphenyl)-1, 2,5, 6-etrahydropyridine hydrochloride) (Pradines et al., 1995) and Aroclor 1254 (a mixture of polychlorinated biphenyls) (Angus and Contreras, 1995) have been found to enhance the action of NGF on neurite outgrowth from PC12 cells. However, there have been a few reports of natural compounds possessing the ability to potentiate the action of NGF on the outgrowth of neurites from neuronal cells (Ito

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et al., 1999, Li et al., 1999). Therefore, in the course of our studies on pharmacologically active substances in medicinal plants, much attention has been given to the occurrence of natural products which are not peptides and which have NGF-potentiating activity, since these substances may contribute to the basic study and the medical treatment of dementia. In this paper, we present the first report on the detailed pharmacological properties of picrosides I (β-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-hydroxy-1a-(hydroxymethyl)-oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl, 6-(3-phenyl-2-propenoate)) and II (β-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxy]-1a-(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl), unique natural products which potentiate NGF-mediated neurite outgrowth from PC12D cells.

2. Materials and methods

2.1. Materials

We isolated picrosides I and II as enhancers of NGF from *Picrorhiza scrophulariiflora* by evaluating the NGF-potentiating activity of each chromatographed fraction as follows. The dried roots of *P. scrophulariiflora* (40 g) were extracted with methanol. The methanol extract was partitioned between ethyl acetate and water. NGF-potentiating activity was observed in the ethyl acetate fraction. This fraction was chromatographed over silica gel to give several fractions of which the NGF-potentiating activity was evaluated. Chromatography of the active fraction and examination of the NGF-potentiating activity of the chromatographed fractions were repeatedly performed to yield two active compounds. These compounds were elucidated to be picroside I (120 mg) and picroside II (100 mg) (Fig. 1) on the basis of physicochemical data such as nuclear magnetic resonance, mass and infrared spectra (Kitagawa et al., 1971; Weinges et al., 1975). For all experiments, picrosides were dissolved in dimethyl sulfoxide (DMSO); the concentration of DMSO was less than 0.1%. At this concentration, DMSO had no effect on the cells.

7S NGF and poly-L-lysine were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (high glucose; DMEM) was from Gibco RBL (Grand Island, NY, USA). Fetal calf serum and horse serum were from Cell Culture Laboratory (Cleveland, OH, USA) and ICN Biochemical (Costa Mesa, CA, USA), respectively. Calyculin A and potassium bisperoxo(1, 10-phenanthroline)oxovanadate (V) were from Calbiochem. Anti-active mitogen-activated protein (MAP) kinase 1/2 antibody and anti-MAP kinase 1/2 antibody were purchased from Promega. Goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase was from New England BioLabs. Glutaraldehyde was purchased from Wako Pure Chemical (Tokyo, Japan). All other reagents or drugs were of analytical grade.

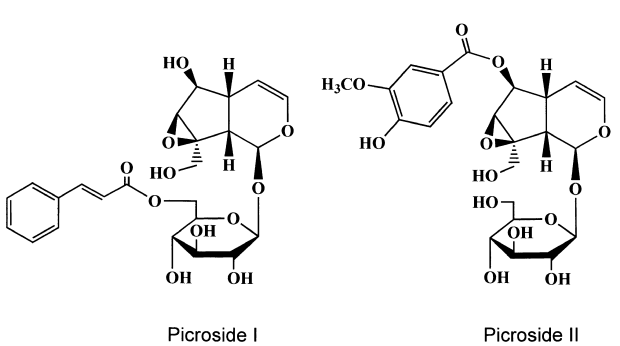

Fig. 1. Chemical structures of picrosides I and II.

2.2. PC12D cell culture

PC12D cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 5% fetal calf serum, 10% horse serum and 2 mM glutamine (Li et al., 1999).

2.3. Measurement of NGF-potentiating activity and morphological observation

Measurement of NGF-potentiating activity and morphological observation of PC12D cells were performed as previously reported (Li et al., 1999). Cells were dissociated by incubation with 1 mM EGTA (*O,O′*-bis(2-aminoethyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid) in phosphate-buffered saline (PBS) for 1 h and were seeded in 24-well culture plates (2×10^4 cells/well) coated with poly-L-lysine (Sano and Kitajima, 1998). After 24 h, the medium was changed to an appropriate test medium containing various concentrations of NGF, 1% fetal calf serum and 2% horse serum. After 48 h, each culture was fixed with 2% glutaraldehyde in PBS and stored in PBS solution. The neurite outgrowth from PC12D cells was monitored under a phase-contrast microscope. Processes with a length equivalent to one or more diameters of the cell body were scored as neurites. The potentiation of NGF was assessed by examining the proportion of neurite-bearing cells to total cells (at least 100 cells) in randomly selected areas. The effects of drugs were always compared with those of NGF treatment (30 ng/ml) as a positive control in the same experiment.

2.4. Cell lysate preparation and Western blot analysis of activated MAP kinase 1 / 2

For Western blot analysis, PC12D cells were plated at a cell density of 1×10^6 cells/35-mm culture dish, and cultured in the culture medium overnight. Cells were treated with or without picroside I or II (60 μM) in the presence or absence of 7S NGF for 10 min. After the cells were washed with ice-cold PBS, they were lysed by directly adding 90 μl of lysis buffer [10 mM HEPES (2-[4-(2-hy-

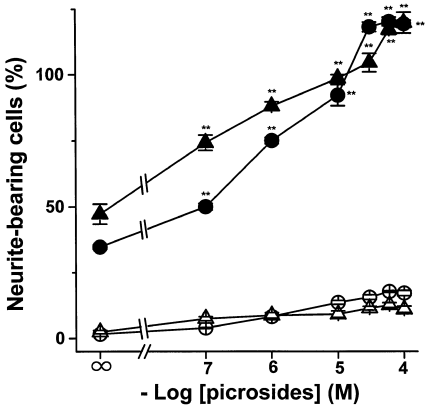

Fig. 2. The concentration–response curve for picroside I or II in the potentiation of neurite outgrowth from PC12D cells. ○, Picroside I; ●, picroside I plus 2 ng/ml NGF; △, picroside II; ▲, picroside II plus 2 ng/ml NGF. The number of neurite-bearing cells is expressed as a percentage of the maximum number in response to NGF (30 ng/ml, 100%) in the absence of picrosides I and II. Each point represents the mean ± S.E. from four experiments. Statistically significant difference from the response to 2 ng/ml NGF in the absence of picrosides I or II is indicated in the figure (ANOVA): $^{*}P < 0.05$, $^{**}P < 0.01$.

droxyethyl)-1-piperazinyl]ethanesulfonic acid)-NaOH (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA, disodium salt), 1% sodium dodecyl sulfate (SDS), 320 nM okadaic acid, 10 nM calyculin A, 10 mM NaF, 1 mM sodium orthovanadate, 5 μM potassium bisperoxo(1, 10-phenanthroline)oxovanadate (V), 1 mM *p*-aminophenyl methanesulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml phosphoramidon] to the culture dishes and were scraped from the culture dishes. Cell lysates were boiled for 5 min and subsequently sonicated for 3 min. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Bio-Rad) as previously reported (Yamakuni et al., 1998). Blots were blocked at room temperature for 1 h in 2% bovine serum albumin in Tris-buffered saline/Tween buffer [10 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 0.2% (v/v) Tween 20]. For detection of phosphorylated MAP kinase 1/2, blots were incubated with anti-active MAP kinase 1/2 antibody (1:5000 dilution in 2% bovine serum albumin/Tris-buffered saline/Tween buffer) for 2 h at room temperature and washed with Tris-buffered saline/Tween buffer, followed by a 1-h incubation with goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase (1:10000 dilution in Tris-buffered saline/Tween buffer) and subsequently washed with Tris-buffered saline/Tween buffer. Immunoreactive signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Blots were then stripped by incubating them in stripping buffer [62.5 mM Tris–HCl (pH 7.5), 2% SDS and 100 mM β-mercaptoethanol] in a 50°C-water bath. They were then reprobed with anti-MAP kinase 1/2 antibody according to the immuno-detection procedure described above, except that anti-MAP kinase 1/2 antibody was used as the primary antibody. Protein contents of cell lysates were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as a standard. The density of the bands corresponding to MAP kinase 1 and MAP kinase 2 was analyzed by densitometry (Advanced American Biotechnology, Fullerton, CA, USA). Data are expressed relative to control.

2.5. Statistical analysis of the data

The data are expressed as the means ± S.E. Statistically significant differences were determined by one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

3. Results

3.1. Effects of picrosides I and II on NGF-mediated neurite outgrowth from PC12D cells

The effects of picrosides on the neurite outgrowth from PC12D cells were examined in the absence or presence of NGF. Picroside I or II even at a high concentration (60 μM) failed to induce neurite outgrowth from PC12D cells but caused a concentration-dependent (> 0.1 μM) enhancement of NGF-induced (2 ng/ml) neurite outgrowth from PC12D cells (Fig. 2). The concentration–response curve of NGF for the proportion of neurite-bearing cells was shifted to the left by picroside I or II (60 μM) and the maximum response was markedly increased (Fig. 3). The

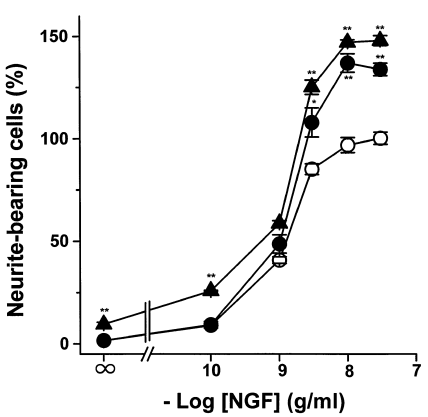

Fig. 3. Effects of picroside I or II on the concentration–response curve for NGF in the potentiation of neurite outgrowth from PC12D cells. ○, Control; ●, 60 μM picroside I; ▲, 60 μM picroside II. The number of neurite-bearing cells is expressed as a percentage of the maximum number in response to NGF (30 ng/ml, 100%) in the absence of picroside I and II. Each point represents the mean ± S.E. from four experiments. Statistically significant difference from the control is indicated in the figure (ANOVA): $^{*}P < 0.05$, $^{**}P < 0.01$.

increase in neurite-bearing cells was maintained throughout the culture period, when the neurites were stabilized (data not shown). In addition, the number of neurites per neurite-bearing cell was increased from 1 to 1.9 ± 0.20 and 3.1 ± 0.40 after treatment with NGF at 2 ng/ml and 30 ng/ml, respectively. Picroside I or II alone had no effect on the number of neurites, while in the presence of NGF (2 ng/ml) picroside II but not picroside I enhanced the number of neurites (2.8 ± 0.36).

3.2. Morphological observation

The effects of picroside I or II on neurite outgrowth from PC12D cells were examined in the presence of NGF. When cultured in the presence of NGF (2 ng/ml) for 48 h, a few PC12D cells had neurites greater than one cell diameter (Fig. 4(A)). NGF (30 ng/ml) markedly induced neurite outgrowth from PC12D cells (Fig. 4(B)). Addition of picroside I or II (60 μM) together with NGF (2 ng/ml) significantly increased the proportion of neurite-bearing cells (Fig. 4(C),(D)) compared to that in cultures treated with NGF (2 ng/ml) alone (Fig. 4(A)).

3.3. Effects of PD98059 (2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one) and GF109203X (2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide) on picroside-induced enhancement of neurite outgrowth

The picroside-induced (60 μM) enhancement of NGF-mediated (2 ng/ml) neurite outgrowth was inhibited by PD98059, a MAP kinase kinase inhibitor, and by GF109203X, a protein kinase C inhibitor, in a concentration-dependent manner (data not shown). PD98059 (20 μM) and GF109203X (100 nM) completely blocked the picroside-induced (60 μM) enhancement of the outgrowth of neurites from PC12D cells in the presence of NGF (2 ng/ml) (Fig. 5), suggesting an involvement of MAP kinase and protein kinase C in the picroside-induced enhancement of neurite outgrowth.

3.4. Effects of picrosides on the phosphorylation of MAP kinase 1/2

NGF increased the expression of biphosphorylated MAP kinase 1/2 in a concentration-dependent manner without

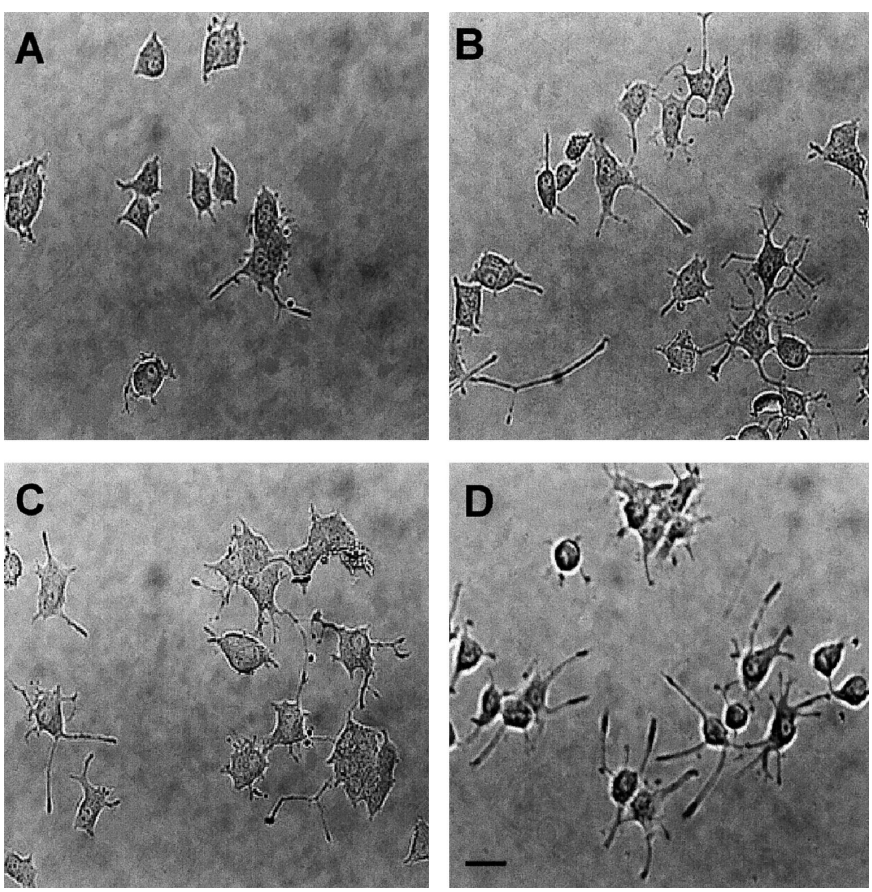

Fig. 4. The morphological change induced by picroside I or II in PC12D cells. (A) 2 ng/ml NGF, (B) 30 ng/ml NGF, (C) 60 μM picroside I plus 2 ng/ml NGF, (D) 60 μM picroside II plus 2 ng/ml NGF. Scale bar, 50 μm.

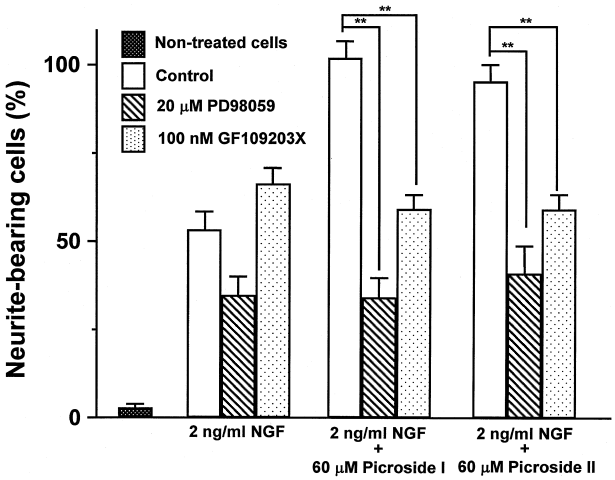

Fig. 5. Effects of PD98059 and GF109203X on the picroside-induced enhancement of neurite outgrowth by NGF. The number of neurite-bearing cells is expressed as a percentage of the maximum number in response to NGF (30 ng/ml, 100%) in the absence of picrosides I and II. Each point represents the mean ± S.E. from four experiments. Statistically significant difference from the control in the presence of picroside I or II is indicated in the figure (ANOVA): $^{**}P < 0.01$.

affecting the expression of total MAP kinase 1/2 protein (Fig. 6(A)–(C), lanes 1,2 and 7). The picrosides did not potentiate the upregulation of the expression of phosphorylated MAP kinase 1/2 induced by NGF (Figs. 6(A)–(C), lanes 3 and 4), and did not upregulate the expression of phosphorylated MAP kinase 1/2 in the absence of NGF (Figs. 6(A)–(C), lanes 5 and 6).

4. Discussion

PC12 cells have been extensively used as a useful model system to study the mechanism of action of NGF on sympathetic neurons. PC12D cells, a subline of PC12 cells, resemble cultured sympathetic neurons (Sano and Kitajima, 1998). Picroside I or II alone, even at a high concentration, did not cause neurite elongation from PC12D cells but markedly increased the number of neurite-bearing cells in cultures supplemented with NGF. Furthermore, a larger proportion of cells elaborated neurites after treatment with a saturating concentration of NGF plus picroside I or II than when treated with NGF alone. These results suggest that simultaneous activation by NGF and picroside I or II leads to a complementary effect on the neurite outgrowth of PC12D cells.

NGF activates the MAP kinase kinase-MAP kinase signaling pathway to cause neurite outgrowth from PC12 cells via phosphorylation of MAP kinase kinase and MAP kinase 1/2 (Goto et al., 1990; Gomez and Cohen, 1991). Phosphorylation of both the tyrosine and threonine residues

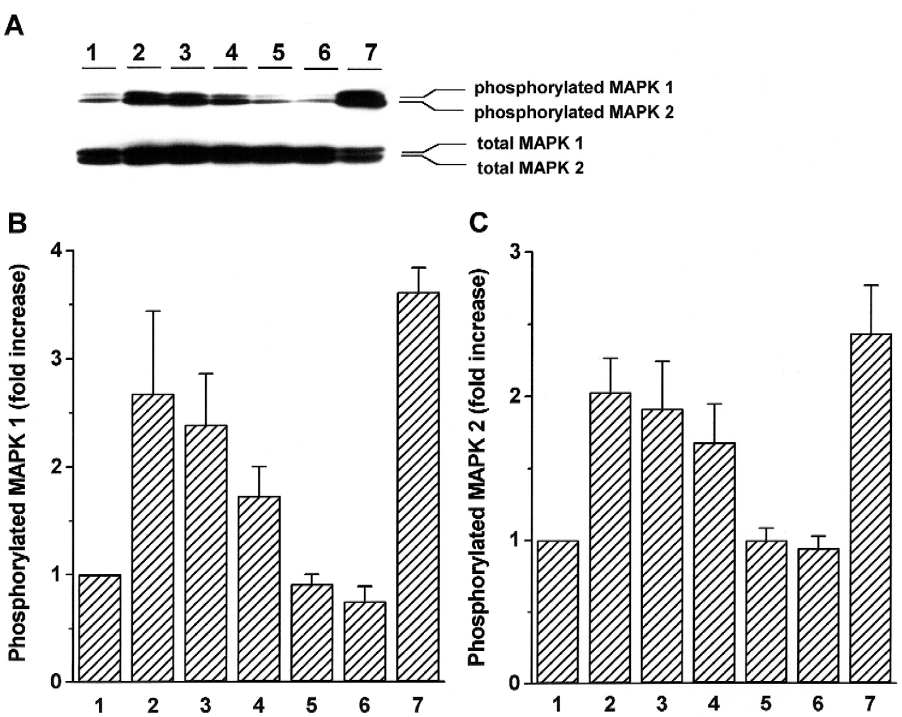

Fig. 6. Biphosphorylation of MAP kinase 1/2 in response to NGF and/or picroside I or II. (A) MAP kinase 1/2 was detected by Western blot analysis with anti-biphosphorylated MAP kinase 1/2 antibody (top) or anti-total MAP kinase 1/2 antibody (bottom). Total cell lysate samples (16 μg of protein/lane) were separated on 12.5% SDS-PAGE gels. Each photograph is representative of two independent Western blot analyses, the density of the bands corresponding to MAP kinase 1 (B) or MAP kinase 2 (C). The results are expressed as fold of control (lane 1). For panel (A), (B) and (C), cells were untreated (control, lane 1), or treated for 10 min with 2 ng/ml NGF (lane 2), 60 μM picroside I plus 2 ng/ml NGF (lane 3), 60 μM picroside II plus 2 ng/ml NGF (lane 4), 60 μM picroside I (lane 5), 60 μM picroside II (lane 6) and 30 ng/ml NGF (lane 7), respectively.

of MAP kinase 1/2 by MAP kinase kinase is required for full enzymatic activation (Payne et al., 1991; Seger and Krebs, 1995). Recently, it has been reported that MAP kinase-dependent and -independent pathways are involved in NGF-induced formation of processes in PC12 cells (Kita et al., 1998). More recently, PKC has been shown to play an important role in neurite outgrowth from PC12 cells via activation of the MAP kinase pathway (Brodie et al., 1999). In this experiment, NGF-induced neurite outgrowth was only partially blocked by PD98059, a representative MAP kinase kinase inhibitor, and GF109203X, a protein kinase C inhibitor. A possible interpretation of this observation is that the MAP kinase-dependent signaling pathway plays only a partial role in the neurotrophic effect of NGF. It is of great interest that PD98059 completely inhibited the picroside-induced enhancement of the NGF-mediated outgrowth of neurites from PC12D cells, suggesting a MAP kinase-dependent action of picrosides. Western blot analysis was done in order to elucidate whether the site of action of picrosides is up- or down-stream step of MAP kinase in the MAP kinase-dependent signaling pathway. This analysis indicated that these compounds did not stimulate NGF-induced MAP kinase activation. These observations suggest that picrosides potentiate NGF-induced neurite outgrowth from PC12D cells probably by amplifying a down-stream step of MAP kinase in the NGF receptor-mediated intracellular MAP kinase-dependent signaling pathway. Picrosides I and II may be useful pharmacological tools to clarify the mechanism of action of NGF in neurite outgrowth from PC12D cells.

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