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Journal of Cellular Biochemistry 105:414–423 (2008)

Journal of Cellular Biochemistry

Pregabalin and Gabapentin Inhibit Substance P-Induced NF-κB Activation in Neuroblastoma and Glioma Cells

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ABSTRACT

Pregabalin and gabapentin are lipophilic amino acid derivatives of γ-amino butyric acid that show anticonvulsant and analgesic activity against neuropathic pain. In this study, we investigated their actions on substance P-induced NF-κB activation in human neuroblastoma and rat glioma cells. Pregabalin and gabapentin decreased substance P-induced NF-κB activation in these cells. These drugs also inhibited NF-κB activation in rat spinal dorsal root ganglia cells pre-treated in vitro with substance P. These results suggest a previously undefined role of pregabalin and gabapentin in the regulation of inflammation-related intracellular signaling in both neuronal and glial cells. J. Cell. Biochem. 105: 414–423, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: PREGABALIN; GABAPENTIN; SUBSTANCE P; NF-KB; NEUROBLASTOMA; GLIOMA

Gabapentin (GBP) and pregabalin (PGB) are lipophilic structural analogues of γ-amino butyric acid (GABA) that were developed as potential anticonvulsants [Bryans and Wustrow, 1999]. Many studies have shown that the drugs do not act as GABA agonists and indeed have little demonstrable effect on any aspect of GABA transmission [Taylor et al., 1998; Maneuf et al., 2003]. It is still not certain what are the important actions underlying their anticonvulsant effects. A recent study has added clarity to the current understanding of GBP pharmacology by concluding that it is not an inhibitor of any conventional subtype of voltage-gated calcium channel, but rather a selective blocker of calcium channels that contain the α2δ-1 subunit, with pharmacodynamic effects and a cellular-specificity which mirror the presence, structure and biochemical state of the α2δ-1 protein [Brown and Randall, 2005]. Blockade of voltage-gated calcium channels that contain the α2δ-1 subunit is believed to be the predominant pharmacological mechanism of both GBP and PGB [Graeme, 2006]. GBP is an effective therapeutic agent when given systemically in the treatment of some forms of neuropathic or postsurgical pain [Rosenberg et al., 1997; Backonja et al., 1998; Rowbotham et al., 1998; Dirks et al., 2002; Serpell and Neuropathic Pain Study Group, 2002], and it also reduces experimental pain in humans after sensitization of the skin with capsaicin and heat [Werner et al., 2001]. Furthermore, GBP and another derivative of GABA pregabalin, reduce nociceptive behaviors in animal models of neuropathic pain or inflammation such as nerve ligation, injection of immune antigens, herpes infection, arthritis, diabetes, postoperative pain, and thermal injury [Xiao and Bennett, 1996; Field et al., 1997; Houghton et al., 1998; Partridge et al., 1998; Taylor et al., 1998; Chen et al., 2001; Takasaki et al., 2001]. In contrast, neither GBP nor PGB alters acute nociceptive responses [Hunter et al., 1997; Stanfa et al., 1997], suggesting that the antinociceptive action of these drugs is dependent on alterations that occur specifically in neuropathic or inflammatory conditions.

Abbreviations used: BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; DMEM, Dulbeco's modified Eagle's Medium; DRG, dorsal root ganglia; DTT, dithiothreitol; EMSA, electrophoresis mobility shift assay; EPSCs, excitatory postsynaptic currents; FBS, fetal bovine serum; GABA, γ-amino butyric acid substance; GBP, gabapentin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IκB-α, inhibitory κB-α; MEM, minimum essential medium; NF-κB, nuclear factor-κB; NK, neurokinin; PBS, phosphate buffered saline; PGB, pregabalin; PKC, protein kinsase C; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate; SP, substance P.

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Received 29 February 2008; Accepted 19 May 2008 • DOI 10.1002/jcb.21837 • 2008 Wiley-Liss, Inc.
Published online 9 July 2008 in Wiley InterScience (www.interscience.wiley.com).

In animal models of neuropathic pain or inflammation, there is enhanced excitability in response to activation of synaptic inputs from primary sensory neurons in the dorsal spinal cord [Yashpal et al., 1982; Ryu et al., 1988; Dubner and Ruda, 1992]. Indeed, numerous studies have demonstrated that there is an increase in the content and release of the peptidergic neurotransmitters, substance P (SP) and calcitonin gene-related peptide, from small-diameter sensory neurons during inflammation or in neuropathic pain models [Oku et al., 1987; Nanayama et al., 1989; Southall et al., 1998]. SP is a member of peptides called tachykinins that are involved in the regulation of several biologic processes. SP is synthesized in dorsal root ganglia and C-fibers of sensory neurons, where it functions as a neurotransmitter [McGillis et al., 1990]. SP is also axonally transported to peripheral nerve endings, where it is released in response to traumatic stimuli and induces various biological effects [Oku et al., 1987; McGillis et al., 1990]. The neuropeptide has been implicated in neurogenic control of inflammation. SP can stimulate T cell proliferation, B cell differentiation, macrophage respiratory burst and mast cell degranulation [McGillis et al., 1990]. SP also induces the synthesis of proinflammatory cytokines in neuroglial and lymphoid cells [McGillis et al., 1990]. In astrocytes and microglia, SP has been reported to induce the secretion of cytokines, such as IL-1 and IL-6 [Martin et al., 1992; Gitter et al., 1994]. Since proinflammatory cytokines have been involved in several neuropathies, SP could initiate or exacerbate inflammatory processes observed in these diseases [Barker, 1991; Mrak et al., 1995]. Molecular studies have demonstrated that SP exerts its biologic activities upon binding to a G-protein-coupled receptor of the neurokinin (NK) receptor family [Macdonald and Boyd, 1989; Regoli et al., 1989]. Intracellularly, receptor-mediated signal is followed by phosphoinositide hydrolysis, calcium mobilization, a rise of cAMP levels, mitogen-activated protein kinase activation, transcriptional activation of NF-κB and novel inflammatory gene expression [Martin et al., 1992; Bordey et al., 1994; Kavelaars et al., 1994; Lieb et al., 1997]. Thus, we investigated whether SP exerts its biological activity on the transcriptional activation of NF-κB in SP-reactive neuronal and glial cells. Additionally, we intended to investigate whether anticonvulsant drugs such as PGB and GBP affect SP-mediated inflammatory events.

In this study, we investigated the effects of SP on NF-κB activation in the human neuroblastoma SH-SY5Y and rat glioma C6 cell lines. We found that micromolar concentrations of SP activated NF-κB and that PGB and GBP decreased the SP-induced NF-κB activation in these cells. These drugs also inhibited NF-κB activation in rat spinal dorsal root ganglia (DRG) pre-treated in vitro with substance P.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

The human neuroblastoma cell line SH-SY5Y was obtained from the Korean Cell Line Bank (KCLB: Seoul, Korea) and grown in media [1:1, modified Eagle's medium (MEM: Gibco-BRL, Rockville, MD, USA) and F12 (Gibco-BRL)] supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Gibco-BRL). The rat glioma cell line C6 was also purchased from KCLB and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). All cell types were incubated in a 5% CO_2 humidified incubator at 37°C. SP and GBP were purchased from Sigma Chemical Co. (Deisenhofen, Germany). PGB was kindly provided by Pfizer Pharmaceutical Korea Co. (Seoul, Korea).

Cells were treated for 2 h with concentration of 5 μM or 10 μM SP. If needed, the cells were pre-treated for 2 h with 5 μM SP, followed by the treatment with the indicated concentration of PGB or GBP for 2 h.

PREPARATION OF RAT SPINAL DRG AND TREATMENT

Postnatal day 15 Sprague Dawley rats (CrjBgi: CD (SD) IGS) were purchased from the Facility for Animal Research of Yonsei University College of Medicine. The animal surgery were reviewed and approved by the Institutional Animal Care Committee at Yonsei University. For dissection, rats were anesthetized using ether. The dorsal root ganglia lie along the vertebral column by the spine. Thus, the spinal column was dissected with filament roots close to DRG hanged. Under a binocular microscope, ganglia were cut using forceps from spinal cord and transferred to 60 mm culture dish filled with PBS (about 50 ganglia per dish). A ganglion was cut into three or four slices. The DRG slices were incubated for 15 min in PBS containing collagenase I (0.15%) and for 15 min in trypsin (0.025%) and BSA (0.4%) at 37°C followed by trituration in culture medium containing F12 (50%) and DMEM (50%). The slices were plated and submerged in a culture medium containing F12 (50%) and DMEM (50%), supplemented with glutamine, penicillin, streptomycin and serum substitute, containing 0.35% bovine serum albumin [Paveliev et al., 2004]. The tissue slices were pre-treated for 2 h with 10 μM SP, followed by the treatment with the indicated concentration of PGB or GBP for 2 h.

PREPARATION OF NUCLEAR AND CYTOSOLIC EXTRACTS FROM CELLS AND TISSUE SLICES

Cells (1×10^7) were lysed by incubation at 4°C for 10 min in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Tissue slices were lysed using glass pestle in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM PMSF. The lysates were centrifuged and the supernatant stored at −70°C as a cytosolic extract. The pellet was resuspended in ice-cold buffer consisting of 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF. After incubation at 4°C for 20 min, the extract was centrifuged for 6 min, and the supernatant was collected, aliquoted, and stored at −70°C as a nuclear extract. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol from Bio-Rad (Richmond, CA).

ELECTROPHORESIS MOBILITY SHIFT ASSAY (EMSA)

The NF-κB-binding oligonucleotide was labeled with $[^{32}P]$-γATP by T4 polynucleotide kinase and purified on a Nick column (Pharmacia Biotech., Uppsala, Sweden). The binding reaction was carried out in

25 μl of mixture containing 5 μl of incubation buffer (10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA), 10 μg of nuclear extract, and 100,000 cpm of the labeled probe. A 50-fold excess of unlabeled NF-κB oligonucleotide as a competitor was added where necessary for competition assay. 5 or 10 μg of antibody for p50, p52, c-Rel or p65 was added where necessary for super-shift assay. After 30 min of incubation at room temperature, samples were electrophoresed through a 6% nondenaturing polyacrylamide gel.

TOTAL CELL LYSATE EXTRACTION AND WESTERN BLOT ANALYSIS

Cells (5×10^6) were harvested and suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng PMSF per ml, 0.03% aprotinin, and 1 μM sodium orthovanadate) and incubated on ice for 30 min. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol (Bio-Rad, Richmond, CA). Total cell lysates were used for measuring COX-2 levels. Nuclear and cytosolic extracts were used for measuring p65 and IκB-α levels, respectively. The extracts were subjected to 12% SDS-polyacrylamide gel electrophoresis. After a 3-h transfer of the gel to PVDF membrane (Amersham Life Sciences, Arlington Heights, IL), the blots were blocked with 5% fat-free dry milk in PBS containing 0.1% Tween-20 for 2 h at room temperature and then washed in the same buffer. Each protein level was detected with each corresponding antibody (Santa Cruz Biotech., Santa Cruz, CA). The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL).

DETECTION OF IL-6 PRODUCTION

DRG tissue slices were treated with 10 μM SP and indicated concentration of PGB or GBP for 24 h. Supernatants were then harvested, centrifuged for 30 min at 10,000*g* and assayed for the presence of IL-6 using an IL-6-specific ELISA kit (Amersham), according to the manufacturer's manual. Experiments were conducted three times in triplicate each.

QUANTITATIVE ANALYSIS

For quantitative analysis, protein–DNA complex was excised and quantified by liquid scintillation counting. In all experiments, statistical significance was calculated for the data from three or four independent experiments using one-way ANOVA (Excel, Microsoft). The error bars represent the standard deviations of the mean.

RESULTS

SP INDUCES NF-κB ACTIVATION IN NEURONAL AND GLIAL CELLS

The effect of the neuropeptide SP on the activation of NF-κB was investigated in the human neuroblastoma SH-SY5Y and rat glioma C6 cells. The SH-SY5Y cells have been reported previously to

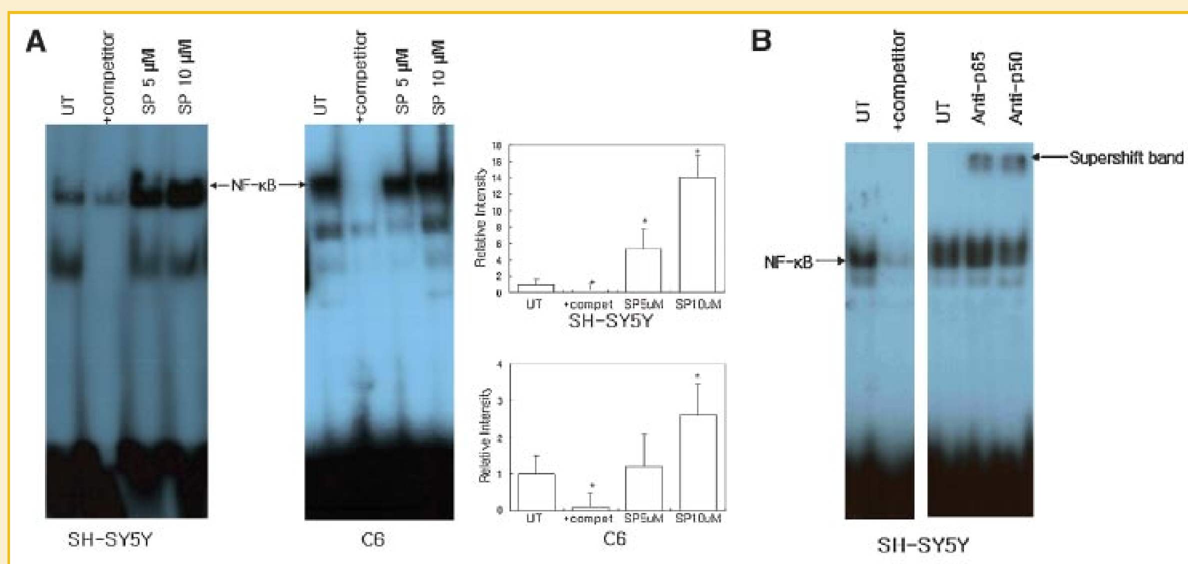

Fig. 1. (A) Dose-dependent NF-κB activation by SP. SH-SY5Y and C6 cells were treated for 2 h with the indicated concentrations of SP. Nuclear extracts were prepared from untreated control (lane1), SP-treated cells (lane 3, 4), and analyzed by EMSA using a ^{32}P-labeled NF-κB-binding oligonucleotide as described in Materials and Methods Section. Nuclear extracts from cells were incubated with a 50-fold excess of unlabeled NF-κB-binding oligonucleotide as a competitor (lane 2). The NF-κB/DNA complex is indicated by arrows. Quantitative analysis was performed and results were expressed as relative activity to untreated control. Asterisks indicate statistically significant difference between treatment and untreated control condition ($^{*}P < 0.01$, $^{**}P < 0.05$). (UT: nuclear extracts from untreated cells). (B) Specificity of SP-induced NF-κB activation in SH-SY5Y cells. Nuclear extracts from cells were incubated with a 50-fold excess of unlabeled NF-κB-binding oligonucleotide as a competitor (lane 2). Nuclear extracts of SP-stimulated SH-SY5Y cells were prepared and either left untreated (lane 3) or incubated with specific antibodies against the NF-κB subunits p65 (lane 4), p50 (lane 5). Both antibodies shifted the band to a higher molecular weight, thus indicating that the SP-activated complex consisted of p50 and p65 subunits. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

respond to NK-1 activation [Beattie et al., 1995]. The C6 cells also have been reported previously to express functional NK-1 receptors [van Ginkel and Pascual, 1996]. Cells were treated for 2 h with concentrations of 5 and 10 μM SP. Subsequently, nuclear extracts were prepared and analyzed for DNA-binding activity to a ^{32}P-labeled NF-κB-specific oligonucleotide. As shown in Figure 1A, SP caused the protein–DNA complex to be intensified in a concentration-dependent manner.

To ascertain the specificity of NF-κB in SH-SY5Y and C6 cells, EMSA was conducted with excess amount of unlabeled NF-κB oligonucleotide. Incubation of unstimulated nuclear extracts with 50-fold excess unlabeled NF-κB oligonucleotide before EMSA abolished the activation of NF-κB/DNA complex (Fig. 1A, B), indicating that the retarded band observed by EMSA was due to indeed NF-κB.

To investigate whether the retarded band observed by EMSA in SH-SY5Y cells was indeed a complex of the p50 and p65 subunits of NF-κB, we incubated nuclear extracts from the cells with antibodies to the p50 and the p65 subunit of NF-κB. Both antibodies shifted the band to a higher molecular weight (Fig. 1B), thus indicating that the SP-activated complex consisted of p50 and p65 subunits.

PGB AND GBP INHIBIT SP-INDUCED NF-κB ACTIVATION IN HUMAN NEUROBLASTOMA AND GLIOMA CELLS

SH-SY5Y and C6 cells were treated with a concentration of 5 μM of SP and then treated with different concentrations of PGB and GBP. PGB and GBP, which have never been reported previously to antagonize NF-κB-binding activity, reduced SP-induced NF-κB activation in both cell lines. NF-κB activation induced by 5 μM SP was abrogated in a dose-dependent manner by PGB and GBP (Fig. 2). When we tested the pre- or post-incubation required for PGB and GBP to block SP-induced NF-κB activation, the treatment of

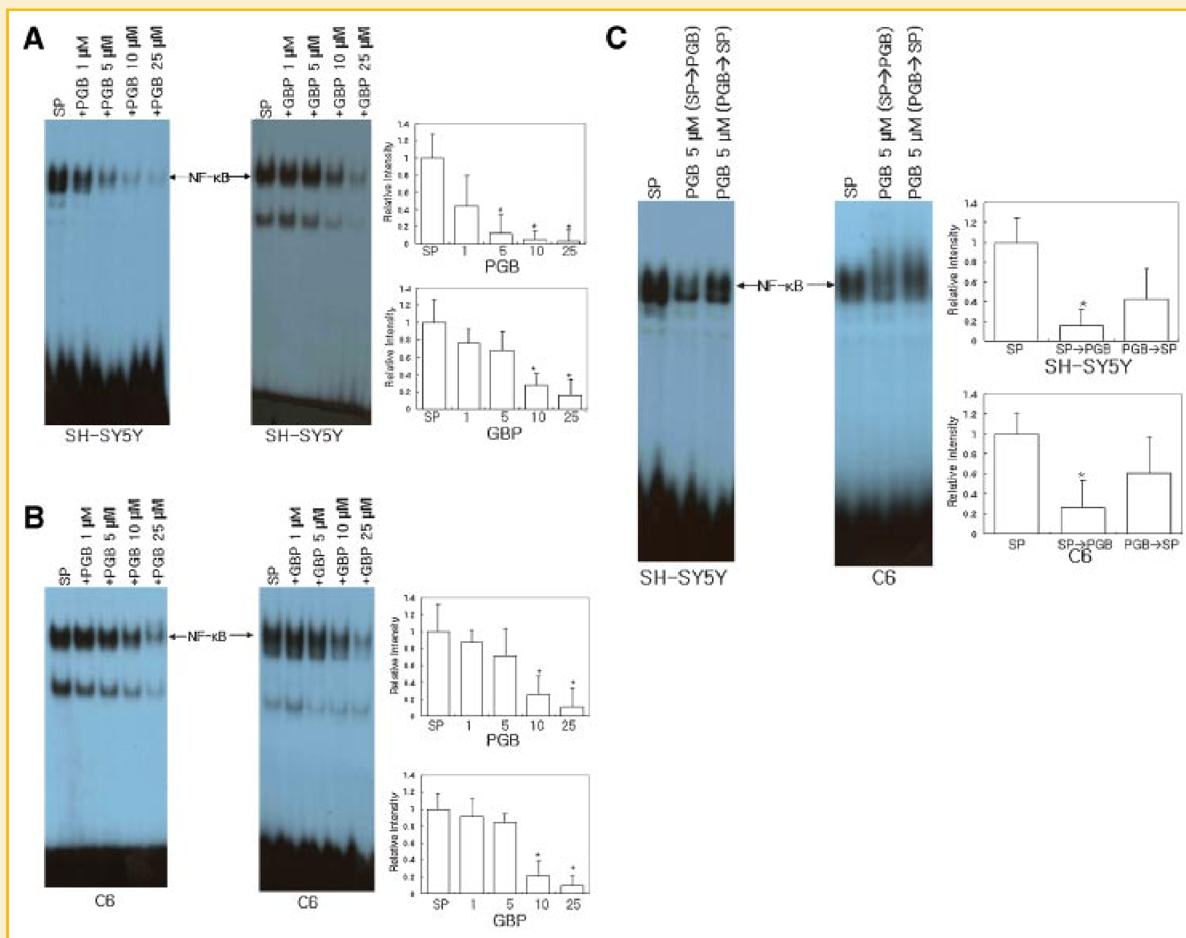

Fig. 2. Inhibition of SP-induced NF-κB activation by PGB and GBP in SH-SY5Y cells (A) and C6 cells (B). Asterisks indicate statistically significant difference between drug treatment and SP-induced control ($^{*}P<0.01$, $^{**}P<0.05$). SH-SY5Y and C6 cells were treated for 2 h with 5 μM concentration of SP and then treated with the indicated concentration of PGB and GBP for 2 h. Nuclear extracts were prepared from cells and analyzed by EMSA. The NF-κB/DNA complex is indicated by arrows. Quantitative analysis was performed and results were expressed as relative activity to SP-induced control. (C) SH-SY5Y and C6 cells were treated for 2 h with 5 μM of SP and then treated with 5 μM of PGB (lane 2s). The cells were pre-treated for 1 h with 5 μM PGB, followed by the treatment with SP for 2 h (lane 3s). Each data point represents a mean of three individual values and standard deviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PGB and GBP following SP showed the inhibition effect on NF-κB activation apparently (Fig. 2C). The treatment of PGB and GBP followed by SP showed slight inhibitory activity. Thus, it seems that the inhibition of PGB and GBP against NF-κB is due to the interference of SP-induced signaling.

It has been shown that *N*-tosyl-phenylalanine chloromethyl ketone and herbimycin A down-regulate NF-κB activation by chemically modifying the NF-κB subunits and thus preventing direct binding of NF-κB to DNA [Finco et al., 1994; Mahon and O'Neill, 1995; Shishodia et al., 2004]. To determine whether PGB and GBP also suppress NF-κB activation by directly modifying NF-κB proteins, we isolated nuclear extracts from SP-treated cells and incubated with enough concentrations of PGB or GBP for 2 h at room temperature. Then DNA-binding activity was detected using EMSA. As shown in Figure 3, PGB and GBP did not interfere the binding of DNA with NF-κB proteins prepared by treatment with SP. We concluded that PGB and GBP inhibited NF-κB activation through a mechanism different from that by which *N*-tosyl-phenylalanine chloromethyl ketone and herbimycin A inhibit NF-κB activation.

PGB AND GBP INHIBIT NF-κB ACTIVATION IN RAT SPINAL CORD DRG PRE-TREATED IN VITRO WITH SP

We further investigated the specificity of the PGB and GBP effects in experiments with rat tissue DRG pre-treated in vitro with 10 μM SP. Inhibition was also observed upon incubation with PGB and GBP (Fig. 4). These results indicate that the inhibitory effects of PGB and GBP on SP-induced NF-κB activation are evident not only in cultured cells but also in DRG tissues.

PGB AND GBP INHIBIT SP-INDUCED NUCLEAR LOCALIZATION OF p65

In an attempt to understand the mechanism underlying the inhibitory effects of PGB and GBP on NF-κB activation, SH-SY5Y cells were treated with PGB and GBP at concentrations that resulted in the inhibition of NF-κB activation. Western blot analysis was performed to examine the degradation of the inhibitory factor IκB-α and the nuclear localization of the functionally active subunit p65. In this study, SP was shown to induce the translocation of p65 to the nucleus, which is required for NF-κB activation. As shown in Figure 5, SP induced the degradation of IκB-α and the localization of p65 to the nucleus, and PGB and GBP treatment suppressed IκB-α degradation and p65 translocation.

PGB AND GBP INHIBIT EXPRESSION OF COX-2

Our results indicated that SP activates NF-κB through increasing nuclear localization of p65 in SH-SY5Y cells. Because COX-2 is a NF-κB-regulated gene product, we investigated whether suppression of SP-induced NF-κB activation by PGB and GBP abrogates induction of this target gene. SH-SY5Y cells, either untreated or pre-treated with SP, were exposed to PGB and GBP for different concentrations. Whole cell extracts were prepared and analyzed by Western blotting. As shown in Figure 6, PGB and GBP inhibited

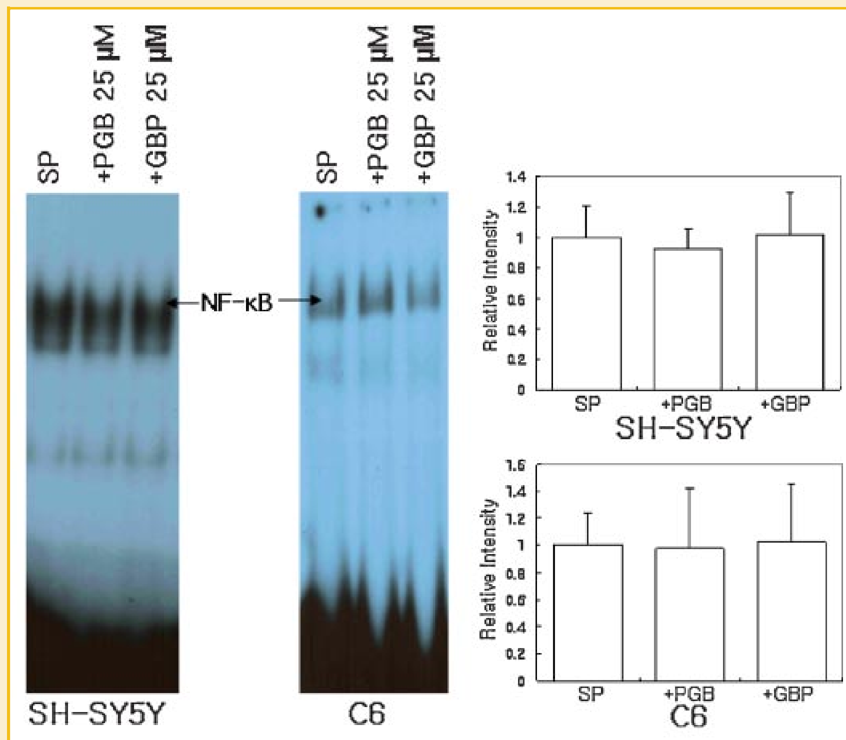

Fig. 3. PGB and GBP inhibit SP-induced NF-κB activation not by direct-interruption of NF-κB binding to their consensus sequences. Nuclear extracts (10 μg) from SH-SY5Y and C6 cells treated with 5 μM SP for 2 h were treated with 25 μM of PGB or GBP for 2 h at room temperature and then assayed for DNA binding by EMSA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

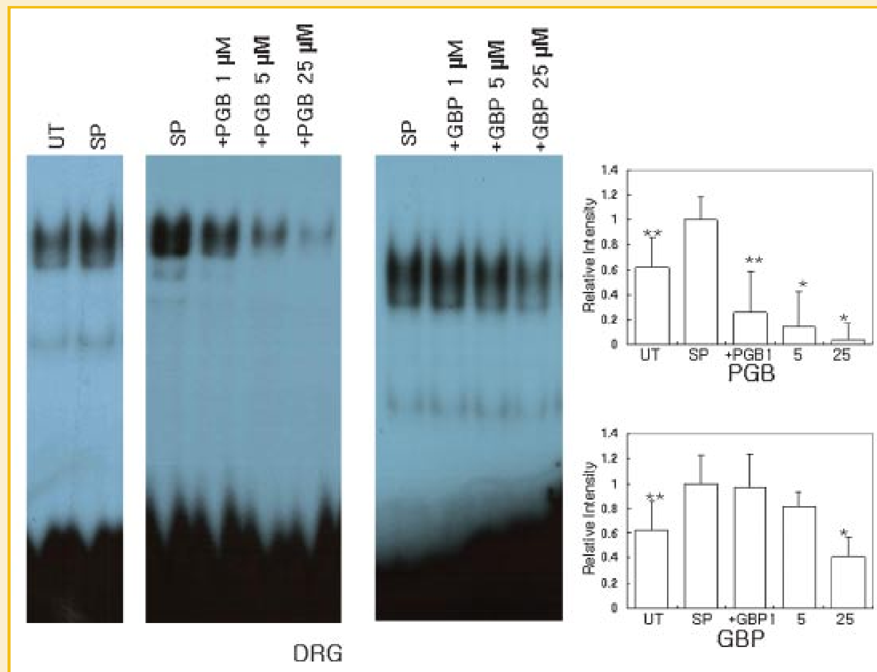

Fig. 4. Inhibition of SP-induced NF-κB activation by PGB and GBP in DRG tissues. The DRG slices were pre-treated for 2 h with 10 μM SP, followed by the treatment with the indicated concentrations of PGB or GBP for 2 h. Nuclear extracts were analyzed by EMSA. Quantitative analysis was performed and the results were expressed as relative activity to the SP-activated control. Asterisks indicate statistically significant difference between treatment and each control condition ($^{*}P < 0.01$, $^{**}P < 0.05$). Each data point represents a mean of three individual values and standard deviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the expression of COX-2, which contains consensus element for NF-κB-binding site in a concentration dependent manner.

PGB AND GBP INHIBIT SP-INDUCED IL-6 SECRETION

Since secretion of the cytokine IL-6 has been shown to be controlled by SP [McGillis et al., 1990; Martin et al., 1992; Gitter et al., 1994], the effect of GBP and PGB on IL-6 secretion was investigated using DRG tissue slices. After the treatment of SP with GBP and PGB, the secretion of IL-6 protein was analyzed by an ELISA technique. As shown in Figure 7, the secretion of IL-6 protein was induced by SP and abrogated by GBP and PGB.

DISCUSSION

SP has been known to play an important role in neurogenic inflammation and to exacerbate inflammatory processes within the central nervous system [Barker, 1991]. An important aspect in SP action is the induction and modulation of secretion of proinflammatory cytokines in glial cells [Lieb et al., 1997]. The mechanism of SP-induced signal transduction is rather incompletely understood. It has been suggested that the activation of NF-κB transcription factors could be involved in SP-induced cytokine expression [Lieb et al., 1997]. We also showed effects of SP in a neuronal cell line, a glial cell line and rat DRG on the activation of NF-κB, an important transcriptional activator of genes encoding proinflammatory cytokines. SH-SY5Y cells which were originated from human neuroblast and C6 cells from rat glia were used as cell system. These cell lines were selected because they were known to respond to NK-1 activation [Beattie et al., 1995; van Ginkel and Pascual, 1996].

The goal of this study was to investigate the effect of PGB and GBP on the activation of NF-κB induced by SP. It was suggested that PGB and GBP did attenuate the stimulated release of neuropeptides from spinal cord slices taken from the side ipsilateral to inflammation [Fehrenbacher et al., 2003]. These findings suggest that PGB and GBP act on cellular mechanisms that are not active under resting conditions, but play a role in presynaptic transmitter release during pathological conditions such as inflammation. Evidence supporting this idea was observed previously by electrophysiological experiments demonstrating that GBP had no consistent effect on excitatory or inhibitory currents in lamina II of spinal slices from noninflamed rats in response to stimulation of either high- or low-threshold primary afferents [Patel et al., 2000; Moore et al., 2002]. Additionally, Patel et al. found that while application of GBP did not change excitatory postsynaptic currents (EPSCs) in dorsal horn neurons of substantia gelatinosa taken from non-inflamed rats, the same application to dorsal horn neurons taken from rats with diabetes induced by streptozocin reduced the magnitude of EPSCs by 75% in 15 of 20 neurons [Bahrami and Mohammadi, 2006]. We found that PGB and GBP suppressed NF-κB activation induced by SP, inflammatory neuropeptide through inhibition of p65 nuclear localization. PGB and GBP suppressed inducible NF-κB in both neuronal SH-SY5Y and glial C6 cell lines without specificity. NF-κB-regulated gene products COX-2 expression was also suppressed by PGB and GBP. These data first suggest

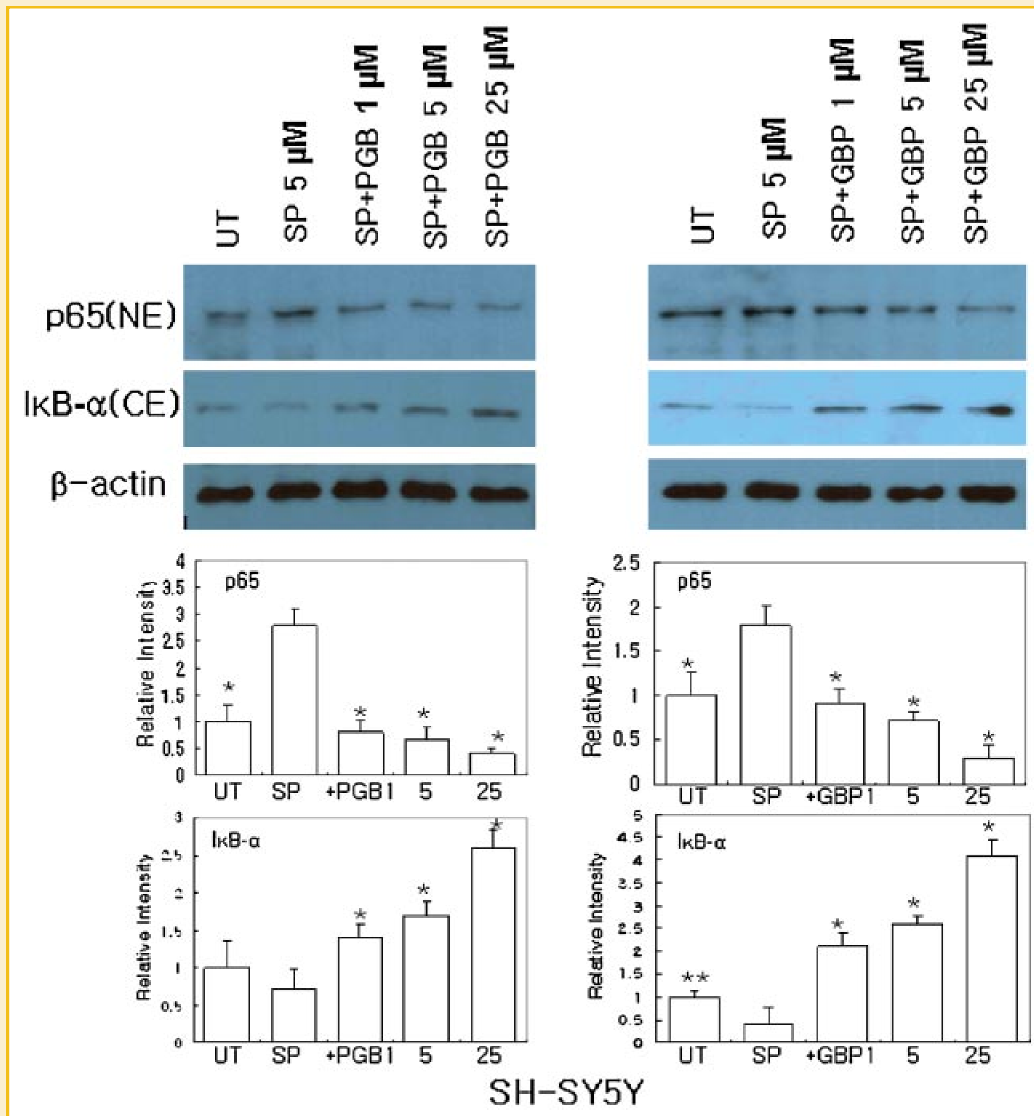

Fig. 5. PGB and GBP inhibit SP-induced NF-κB activation by preventing the degradation of IκB-α and the nuclear localization of p65 subunit. SH-SY5Y cells were treated for 2 h with 5 μM SP and then treated with the indicated concentrations of PGB and GBP for 2 h. Nuclear and cytoplasmic extracts were prepared from and analyzed by Western blot using p65 and IκB-α antibodies, respectively. NE, nuclear extract; CE, cytoplasmic extract. Results from one of three experiments are provided. Densitometric analysis was performed on the autoradiograph for quantitative analysis using the TotalLab Image Analysis software (Nonlinear Dynamics Ltd., UK). Signal intensities are expressed in arbitrary units. β-Actin was used to normalize the protein level. Values are expressed as relative to SP-treated control. Each data point represents a mean of three individual values and standard deviations. Asterisks indicate statistically significant difference between drug treatment (or UT) and SP-induced control ($^{*}P < 0.01$, $^{**}P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that PGB and GBP exert inhibitory activity on NF-κB which is an essential mediator of SP-induced cytokine synthesis. PGB and GBP may therefore target the NF-κB pathway and interfere with SP-driven neurologic disorders. The approach in these respects can provide a molecular explanation on how PGB and GBP could selectively attenuate the enhancement of neurotransmitter release and be effective in alleviating symptoms of inflammatory and neuropathic pain without altering acute nociception. Since inflammation and neuropathic pain cause an augmentation of stimulated neuropeptide release and because GBP and PGB are antinociceptive only in states of inflammation or neuropathy, we suggest an explanation that these drugs could block the signal augmented by transmitter release and produced by inflammation. Altogether these results, the suppression of SP-induced NF-κB activation by PGB and GBP might partially explain a reason why PGB and GBP play roles under inflammatory condition.

According to pharmacokinetic study [Bahrami and Mohammadi, 2006; Jalalizadeh et al., 2007], the maximum plasma concentration of GBP after administration of a single 400 mg neurontin tablet to 12 volunteers was 3.33 ± 1.19 μg/ml. The observed value was comparable to the concentration of 19.5 μM. Thus, the concentrations used in this study for GBP and PGB are clinically relevant.

We examined the mechanism by which PGB and GBP repressed NF-κB DNA-binding activity. PGB and GBP inhibited the

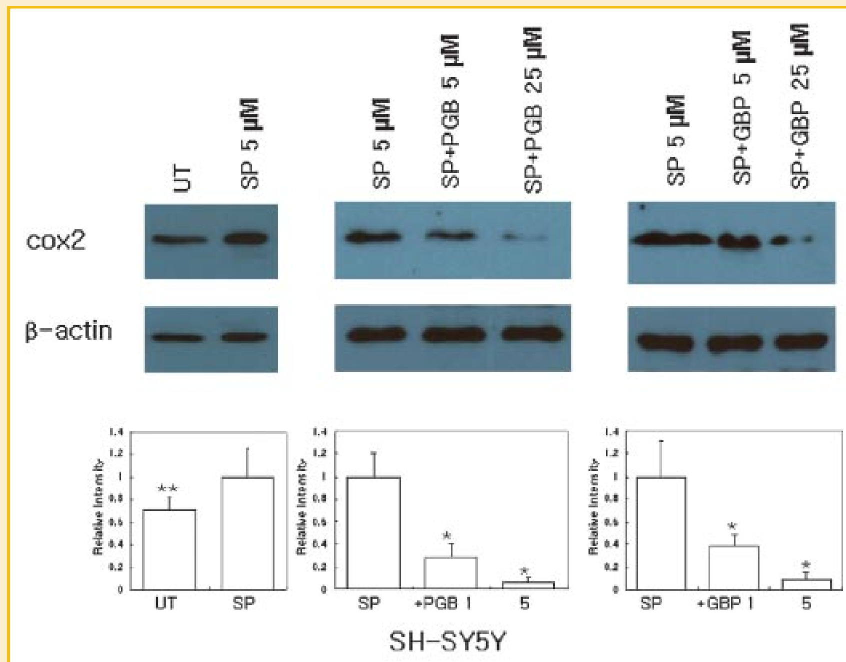

Fig. 6. PGB and GBP repress expression of NF-κB target gene, COX-2 in a dose-dependent manner. SH-SY5Y cells were treated for 2 h with 5 µM SP and then treated with the indicated concentrations of PGB and GBP for 2 h. Cell extracts were prepared from and analyzed by Western blot using COX-2 antibody. Results from one of three experiments are provided. Densitometric analysis was performed on the autoradiograph for quantitative analysis using the TotalLab Image Analysis software (Nonlinear Dynamics Ltd., UK). Signal intensities are expressed in arbitrary units. β-Actin was used to normalize the protein level. Values are expressed as relative to SP-treated control. Each data point represents a mean of three individual values and standard deviations. Asterisks indicate statistically significant difference between drug treatment (or UT) and SP-induced control ($^{*}P < 0.01$, $^{**}P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degradation of IkB-α and the translocation of p65 to the nucleus in a concentration-dependent manner in SH-SY5Y cells (Fig. 5). This result is consistent with other reports that the repression of NF-κB activation is mediated through blockade of IkB-a degradation, which in turn prevents translocation of p65 to the nucleus [Thanos and Maniatis, 1995; Han et al., 2004]. However, PGB and GBP did not induce a direct disruption of NF-κB binding to its consensus sequence (Fig. 3). Based on these findings, we conclude that induced NF-κB activation of SH-SY5Y cells is suppressed by PGB and GBP via inhibition of nuclear localization of p65 resulting from the blocking of IkB-α degradation, but without direct disruption of NF-κB DNA-binding activity. While the treatment of PGB and GBP following SP showed an apparent inhibition effect on NF-κB activation (Fig. 2C), the treatment of PGB and GBP followed by SP showed slight inhibitory activity. Nevertheless, it does not necessarily mean that the inhibition of PGB and GBP against NF-κB is not due to the interference of SP-induced signaling. It is because that PGB and GBP lasted and could influence on late onset of SP-induced signaling.

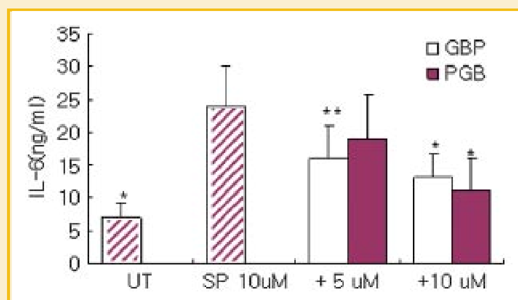

Fig. 7. PGB and GBP inhibit SP-induced IL-6 secretion. DRG tissues were treated for 24 h with 10 µM SP and 25 µM PGB or GBP. Supernatants were then harvested and analyzed using an IL-6-specific ELISA kit. Each data point represents a mean of nine individual values and standard deviations. Asterisks indicate statistically significant difference between drug treatment (or UT) and SP-induced control ($^{*}P < 0.01$, $^{**}P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Because phosphorylation of IkB-α and p65 is needed for NF-κB activation [Ghosh and Karin, 2002], it is possible that PGB and GBP inhibit the kinase involved in their phosphorylation. Whilst it was suggested that PGB and GBP reduced the facilitatory effect of PKC and adenylyl cyclase activation on glutamate release in trigeminal nucleus slices and this might be related to a reduced phosphorylation of voltage-gated calcium channel [Maneuf and McKnight, 2001], it needs to be further investigated which kinases are involved in PGB and GBP-mediated mechanism.

ACKNOWLEDGMENTS

This research was supported by a grant from Dongduk Women's University.

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