Mechanism of Neurotrophic Action of Nobiletin in PC12D Cells†

Hiroyuki Nagase,‡ Tohru Yamakuni,‡ Kentaro Matsuzaki,‡ Yuji Maruyama,‡ Jiro Kasahara,§ Yoshimi Hinohara,|| Shunzo Kondo,|| Yoshihiro Mimaki,⊥ Yutaka Sashida,⊥ A. William Tank,# Kohji Fukunaga,§ and Yasushi Ohizumi*,‡

Departments of Pharmaceutical Molecular Biology and Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan, Mitsubishi Kagaku Institute of Life Sciences, Minamiooya, Machida, Tokyo 194-8511, Japan, Laboratory of Medicinal Plant Science, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan, and Department of Pharmacology and Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642

Received April 6, 2005; Revised Manuscript Received August 4, 2005

ABSTRACT: Nobiletin is a nonpeptide compound with a low molecular weight from a citrus fruit and has the activity to rescue bulbectomy-induced memory impairment. Here we describe that nobiletin itself induces neurite outgrowth in PC12D cells, a rat pheochromocytoma cell line, like NGF, and the molecular mechanism of its neurotrophic action. As cultured in the presence of nobiletin or NGF for 48 h and then assayed using a scanning electron microscope, PC12D cells treated with nobiletin showed morphology with flatter and larger cell bodies than the cells cultured with NGF. Nobiletin-induced neurite outgrowth was inhibited by PD98059 and U0126 but not K252a. Consistently, nobiletin caused a concentration-dependent enhancement of Erk/MAP kinase phosphorylation and a sustained increment of phosphorylation of MEK and Erk/MAP kinase, resulting in a stimulation of CREB phosphorylation and CRE-mediated transcription. This compound also increased intracellular cAMP and CRE-mediated transcription in the presence of forskolin and enhanced PKA activity to stimulate phosphorylation of multiple PKA substrates in PC12D cells. Furthermore, nobiletin preferentially inhibited Ca^{2+}/CaM-dependent phosphodiesterase in vitro. This compound failed to stimulate phosphorylation of Erk5, which is known to be induced by NGF/TrkA signaling. These results suggest that nobiletin induces neurite outgrowth by activating a cAMP/PKA/MEK/Erk/MAP kinase-dependent but not TrkA-dependent signaling pathway coupling with CRE-mediated gene transcription and may thus become a novel type of biochemical probe for elucidation of the molecular mechanism of neuronal differentiation.

It is well-known that nerve growth factor (NGF)[1] promotes the differentiation and survival of the basal forebrain cholinergic neurons (BFCNs) during development and adulthood (*1*, *2*). BFCNs play a critical role in learning and memory (*3*, *4*). Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive and memory deterioration, with a devastating impact on the whole society (*5*). In patients with AD, cholinergic neuronal loss is particularly noticeable in the neocortex and hippocampus (*3*, *4*, *6*, *7*). Such a loss of BFCNs underlies the behavioral and cognitive deficits observed in AD (*4*, *8*, *9*). Recent studies on the efficacy and function of NGF in the BFCNs have suggested that NGF is used as a potential therapeutic agent to prevent the degeneration of cholinergic neurons in patients with AD (*10*, *11*). In support of this idea, it has been also demonstrated that exogenous NGF ameliorates early signs of neurodegeneration in transgenic mice in which anti-NGF antibody is produced and secreted by neuronal and glial cells to neutralize NGF activity in the extracellular space (*12*), providing further evidence that NGF exerts significant protection against the degeneration of cholinergic neurons. Numerous efforts have been made to apply NGF to treatment of AD (*2*). Nevertheless, infusions of NGF into the cerebroventricular system led to a variety of undesirable effects (*2*, *13*). It is also an important problem that, on the peripheral administration of NGF, this neurotrophic factor cannot cross the blood−brain barrier and is easily cleaved by peptidases. Such molecular properties of NGF actually restrict the application to the treatment (*10*), although it has been shown that a noninvasive olfactory route is adopted to facilitate the delivery and the access of NGF to the brain (*14*).

† This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Y.O. and T.Y.), Sankyo Foundation of Life Science (T.Y.), and Brain Science Foundation (T.Y.). Preliminary data on the pharmacological action of nobiletin alternatively called F-1 have been reported previously (Y. Ohizumi, T. Yamakuni, and Y. Li, Proceedings of the 2004 Annual Meeting of the Korea Society of Applied Pharmacology, Seoul, Korea, pp 53−57).

* Corresponding author. Tel: +81-22-217-6851. Fax: +81-22-217-6850. E-mail: ohizumi@mail.pharm.tohoku.ac.jp.

‡ Department of Pharmaceutical Molecular Biology, Tohoku University.

§ Department of Pharmacology, Tohoku University.

|| Mitsubishi Kagaku Institute of Life Sciences.

⊥ Tokyo University of Pharmacy and Life Science.

University of Rochester Medical Center.

[1] Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; BFCN, basal forebrain cholinergic neuron; BSA, bovine serum albumin; CNS, central nervous system; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular signal-regulated kinase; IBMX, 3-isobutyl-1-methylxanthine; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PDE, phosphodiesterase; PKA, protein kinase A; RME, reference memory error; TBS, Tris-buffered saline; TBST, Tris-buffered saline−Tween 20.

10.1021/bi050643x CCC: $30.25
Published on Web 09/27/2005

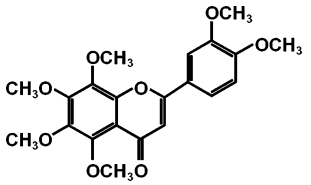

FIGURE 1: Chemical structure of nobiletin.

Large numbers of compounds from natural resources have provided not only useful pharmacological tools (*15*−*17*) but also novel leading compounds for drug development (*18*). In the course of our survey of substances having neurotrophic activity and/or improving action on memory impairment from natural resources, we successfully found nobiletin, a flavonoid from *Citrus depressa* (Figure 1), as a candidate for a biochemical probe with a unique mechanism of neurotrophic action. Here we first describe a detailed mechanism of the neurotrophic action of this compound in PC12D cells.

EXPERIMENTAL PROCEDURES

Materials. The Sumitomo nerve cell culture system, poly-(L-lysine) and cytosine arabinofuranoside (AraC), neurobasal medium, B27 supplement, and horse serum (HS), DMEM, fetal calf serum (FCS), and semi-fetal calf serum were purchased from Sumitomo Bakelite, Sigma Chemical Co., GIBCO, Nissui, ICN Biochemical, Inc., and Mitsubishi Chemical Co., respectively. Anti-phospho-CREB (Ser 133) and anti-phospho-(Ser/Thr) PKA substrate antibodies and horseradish peroxidase- (HRP-) conjugated anti-rabbit IgG, Alexa fluor 546-conjugated anti-rabbit IgG, bovine serum albumin (BSA), normal goat serum and anti-PKA antibody, and SuperSignal West Pico chemiluminescent substrate were obtained from Cell Signaling Technology, Molecular Probes, Inc., Sigma Chemical Co., Santa Cruz Biotechnology (Santa Cruz, CA), and Pierce. A firefly luciferase reporter plasmid containing cyclic AMP response (CRE) element inserted into the upstream of a TATA-like promoter (pTAL) region taken from herpes simplex thymidine kinase promoter, a renilla luciferase vector, phRG-TK, and a dual luciferase assay kit, and LipofectAMINE 2000 were purchased from Clontech, Promega, and Invitrogen. K252a, a TrkA tyrosine kinase inhibitor, PD98059 and U0126, Erk/MAPK kinase inhibitors, wortmannin, a phosphatidylinositol 3 (PI3) kinase inhibitor, GF109203X, a protein kinase C (PKC) inhibitor, H-89, a PKA inhibitor, SQ22536, an adenylate cyclase inhibitor, IBMX, a nonselective phosphodiesterase (PDE) inhibitor, and BAPTA-AM, an intracellular Ca^{2+} chelator, were obtained from Calbiochem. All other reagents or drugs were of analytical grade.

Extraction and Isolation of Nobiletin. The peels of *C. depressa* (dry weight, 500 g) were extracted with hot MeOH (2 L) for 2 h twice. The MeOH extract was concentrated and passed through a Diaion HP-20 (Mitsubishi-Chemical) column eluted successively with H_2O−methanol (7:3, 2:3, 1:4), methanol, ethanol, and finally ethyl acetate (each 2 L). The ethyl acetate eluate fraction (10 g) was chromatographed over silica gel (Fuji-Silysia Chemical) eluted with $CHCl_3$−methanol (19:1) and divided into five fractions (I−V). Fraction III (1.63 g) was chromatographed over silica gel eluted with hexane−acetone (2: 1) to obtain a purified compound (725 mg). On the basis of physicochemical data such as nuclear magnetic resonance, mass, and infrared spectra, this compound was identified as nobiletin (*19*). The purity was confirmed to be almost 100% from the ^1H and ^{13}C NMR spectral features and by HPLC analysis of the isolated compound under the following conditions: a Tosoh HPLC system (pump, CCPM; detector, UV-8000), a Capcell Pak C18 UG120 column (5 μm, 4.6 mm i.d. × 250 mm; Shiseido, Tokyo, Japan), MeOH−H_2O−H_3PO_4 solvent (65:35:0.05 v/v/v), 1.0 mL/min flow rate, and UV 340 nm detection.

Cell Culture. PC12D cells were cultured in high-glucose DMEM supplemented with 10% heat-inactivated HS/5% heat-inactivated FCS in a 5% CO_2 incubator at 37 °C as described previously (*20*).

Neurite Outgrowth Assay. Morphological assays using phase-contrast and scanning electron microscopies were carried out as described previously (*21*, *22*). To examine the effects of K252a, PD98059 and U0126, wortmannin, and GF109203X on nobiletin-induced neurite outgrowth from PC12D cells, each inhibitor was added to the culture medium at 30 min prior to 48 h treatment with nobiletin.

Transient Transfection and Reporter Gene Assay. Cells were plated on 48-well plates at a cell density of 8.0×10^4 cells per well and cultured for 24 h. Cells were then subjected to transfection. Transfection and reporter gene assay were conducted as described previously (*23*). A firefly luciferase reporter plasmid containing cyclic AMP response (CRE) element inserted into the upstream of a TATA-like promoter (pTAL) region taken from herpes simplex thymidine kinase promoter was purchased from Clontech. A *Renilla* luciferase control vector, phRG-TK (Promega), was used as an internal control to normalize the difference in transfection efficiency. PC12D cells were transfected by lipofection using LipofectAMINE 2000 (Invitrogen). To assess the contribution of PKA to the CRE-dependent transcription, an expression vector for PKA inhibitor (PKI), an endogenous PKA inhibitor (*24*), was cotransfected with the reporter plasmid for 43 h. Following transfection, cells were treated with vehicle, 1 μM forskolin, or 100 μM nobiletin for 5 h. Thereafter, cells were harvested to assay the activities of both firefly and seapansy luciferase by using a dual luciferase assay kit (Promega).

Western Blot Analysis. Cells were plated on 35 cm plates at a cell density of 1×10^6 cells per well and cultured for 48 h. Cells were subjected to treatment with nobiletin and chemical agents. Western blotting was performed as described previously (*22*, *25*). The blotted membrane was blocked in TBST buffer (100 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl, pH 7.5) containing 1% BSA for 2 h at room temperature. The membrane was thereafter incubated successively with either anti-phospho-ERK (Thr 202/Tyr 204) or anti-phospho-CREB (Ser 133) antibodies in 1% BSA/TBST buffer overnight at 4 °C and horseradish peroxidase-(HRP-) conjugated anti-rabbit IgG (Cell Signaling Technology) for 2 h at room temperature. Following stripping of the antibodies, the membranes were reprobed with anti-PKA antibody in 1% BSA/TBST buffer overnight at 4 °C and subsequently incubated with HRP-conjugated anti-rabbit IgG. For Western blot analysis of protein phosphorylation by PKA in nobiletin-treated PC12D cells, cells were treated with 30 μM nobiletin for different times and subjected to Western blot analysis using anti-phospho-(Ser/Thr) PKA substrate antibody. Immunoreactivities were visualized with Super-

Signal West Pico chemiluminescent substrate (Pierce). Protein assay was performed using BCA protein assay reagent (Pierce) as described previously (*25*).

Intracellular cAMP Content Assay. Cells were plated on 35 cm plates at a cell density of 1×10^6 cells per plate and cultured for 48 h. Cells were then subjected to treatment with nobiletin and chemical agents for 10 min. After culture medium was removed, culture dishes were immediately boiled to inactivate the phosphodiesterase activity at 95 °C for 3 min. Following addition of 10% trichloroacetic acid to culture dishes, cells were lysed to prepare samples for assay of intracellular cAMP content. The intracellular cyclic AMP content was measured with a cAMP radioimmunoassay kit (Yamasa Shoyu).

PKA Enzyme Activity Assay. Cells were plated on 35 cm plates at a cell density of 2×10^6 cells per plate and cultured for 24 h. Cells were treated with 30 μM nobiletin or 10 μM forskolin for 15 min. Following washing of the cells, the cells were immediately homogenized in 200 μL of homogenizing buffer (0.15 M NaCl, 30 mM $Na_4P_2O_7$, 0.5% Triton X-100, 10 mM EDTA, 4 mM EGTA, 1 mM dithiothreitol, 100 μM leupeptin, 75 μM pepstatin, 50 μg/mL trypsin inhibitor, 100 nM calyculin A, 50 mM Tris-HCl, pH 7.5). The homogenates were subjected to PKA enzyme activity assay. PKA enzyme activity assay was conducted as described previously (*26*).

Phosphodiesterase (PDE) Assay. The reaction mixture contained 0.06 unit/mL bovine heart PDE, 2.68 unit/mL 5′-nucleotidase, 2 mM $MgCl_2$, and 4 mM Tris-HCl, pH 7.4. After incubation of the reaction mixtures at 30 °C for 5 min, nobiletin and PDE inhibitors were added and incubated for additional 5 min. The reaction was initiated by the addition of cAMP (at a final concentration of 2 mM), stopped by the addition of TCA following 10 min incubation at 30 °C, and chilled on ice. The amount of hydrolyzed cAMP or cGMP was assayed as the amount of inorganic phosphate, which was the decomposition product using the Martin−Doty method (*27*) to assay the bovine heart PDE activity.

Hippocampal Neuron Culture. Under ether anesthesia of a 19-day pregnant Sprague-Dawley rat (Japan SLC), embryos were immediately decapitated. The brain was quickly removed, and hippocampi were collected in ice-cold Dulbecco's phosphate-buffered saline (DPBS) under sterile conditions. The hippocampal neurons were dissociated according to the standard methods with the Sumitomo nerve cell culture system (Sumitomo Bakelite), plated on a poly-(L-lysine)-coated coverslip at a density of 3.8×10^4 cells per well, and cultured at 37 °C in the neurobasal medium containing B27 supplement (GIBCO), 500 μM L-glutamine, and penicillin−streptomycin (25000 units each). After 1 day culture, half the medium was replaced by the medium containing a mitotic inhibitor, cytosine arabinofuranoside (AraC), to minimize glial proliferation. Neurons were subjected to Western blot analysis and immunostaining on days 13−15 after plating.

Immunostaining. Cultured hippocampal neurons were fixed by 1% paraformaldehyde in PBS for 30 min at room temperature, washed, permeabilized with Triton X-100, and soaked in blocking buffer (DPBS containing 5% BSA and 5% normal goat serum) for 30 min at room temperature. Following blocking, immunostaining was conducted by successive incubation with the primary anti-phospho-CREB (Ser 133) and with the secondary Alexa fluor 546-conjugated anti-rabbit IgG (Cell Signaling Technology). The fluorescent images were obtained by an interlined cooled charge-coupled device (CCD) camera.

Statistical Analyses. For data from the experiments described above, statistical comparisons were made by using Student's t test. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Nobiletin Induces Neurite Outgrowth from PC12D Cells. A rat pheochromocytoma cell line, PC12 cells, is widely used as a suitable cellular model for biochemical studies of neuronal differentiation (*2*, *28*). Therefore, the mechanism of action of nobiletin was studied using a subclone of PC12 cells, PC12D cells (*20*). As assayed using phase-contrast microscopy, nobiletin induced neurite outgrowth, like NGF, in a concentration-dependent manner (Figure 2A,C). Morphological analysis using a scanning electron microscope also showed that PC12D cells treated with 100 μM nobiletin exhibited flatter and larger cell bodies than cells treated with 50 ng/mL NGF (Figure 2B). Moreover, nobiletin-induced neurite outgrowth in PC12D cells was prevented by PD98059 and U0126, inhibitors of MEK or Erk/MAP kinase kinase, but not by wortmannin and GF109203X, with approximately 50% inhibition by U0126 (Figure 2D), suggesting the presence of the Erk/MAP kinase-dependent and -independent mechanisms underlying this neuritegenic action. In addition, K252a did not inhibit the nobiletin-induced neurite outgrowth but rather significantly enhanced it in PC12D cells (Figure 2D).

Nobiletin Triggers Activation of PKA/MEK/Erk/MAP Kinase-Dependent Signaling Accompanied by Stimulation of CREB Phosphorylation and CRE-Mediated Transcription in PC12D Cells. Nobiletin concentration- and time-dependently caused the enhanced phosphorylation of Erk/MAP kinase (Figure 3A,C). Consistent with the morphological observations described above, nobiletin also caused a persistent increase in a MEK phosphorylation leading to a sustained rise in phosphorylation of Erk/MAP kinase that has been demonstrated to be required for NGF-induced neurite outgrowth in PC12 cells (*9*; Figure 3C). Increased phosphorylation of Erk/MAP kinase by nobiletin appeared at 3 min after treatment and remained elevated for at least 1 h. Furthermore, the stimulated phosphorylation of Erk/MAP kinase was accompanied by a transient augmentation of CREB phosphorylation without any effects on PKAα protein expression in a concentration- and time-dependent manner in PC12D cells (Figure 3B,C). It was also shown that the nobiletin-induced increase in CREB phosphorylation was almost abolished by 10 μM H-89 and 10 μM U0126 and markedly inhibited by 500 μM SQ22536, but not 50 μM BAPT-AM, suggesting the involvement of a PKA/MEK/Erk/MAP kinase-dependent and intracellular Ca^{2+}-independent signaling pathway in this stimulation of CREB phosphorylation (Figure 3D).

Nobiletin Does Not Activate Erk5 Phosphorylation in PC12D Cells. As described above, nobiletin was shown to mimic a neuritogenic action of NGF in PC12D cells by activating an Erk/MAP kinase-dependent signaling pathway. We further examined the effects of nobiletin on Erk5

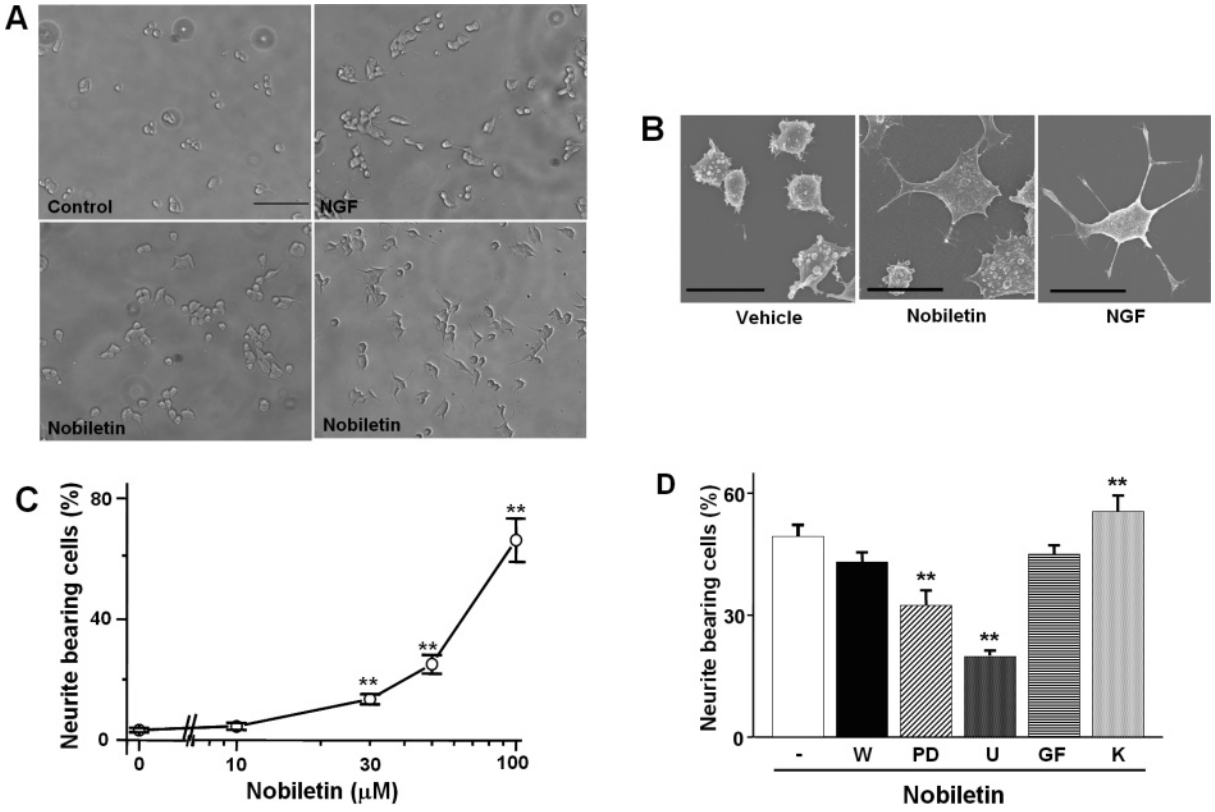

FIGURE 2: Nobiletin induces neurite extension from PC12D cells in a MEK/Erk/MAP kinase-dependent manner. Nobiletin-induced morphological change observed by using phase contrast (A) and scanning electron (B) microscopes. (A) Cells were treated with vehicle, 50 ng/mL NGF, or nobiletin (bottom left, 30 μM; bottom right, 100 μM) for 48 h. Scale bar = 100 μm. (B) Cells treated with 100 μM nobiletin showed flatter and larger cell bodies than cells treated with 50 ng/mL NGF. Scale bar = 20 μm. (C) Concentration-dependent effects of nobiletin on neurite outgrowth. Data are the means ± SEM. **, $P < 0.01$ versus vehicle control. (D) Effects of protein kinase inhibitors on the nobiletin-induced neurite outgrowth. Data are the means ± SEM. **, $P < 0.01$ versus vehicle control. Key: W, 100 nM wortmannin; PD, 20 μM PD98059; U, 50 nM U0126; GF, 1 μM GF109203X; K, 100 nM K252a.

phosphorylation in PC12D cells, since it has been recently reported that Erk5 mediates a retrograde signaling of NGF via TrkA in PC12 cells (*30*). Nobiletin failed to induce Erk5 phosphorylation at all tested time points, whereas NGF appreciably increased the protein phosphorylation at 60 min after treatment (Figure 4), suggesting that the effects of nobiletin described above do not result from its direct agonistic action on TrkA.

Nobiletin Affects Intracellular cAMP Content To Activate PKA-Dependent Signaling and CRE-Mediated Transcription in PC12D Cells. The pairing of 100 μM nobiletin with 10 μM forskolin synergistically increased the intracellular cAMP level at 10 min after the treatment, like the pairing of 1 mM IBMX, a nonselective phosphodiesterase (PDE) inhibitor, with 10 μM forskolin, in PC12D cells, whereas nobiletin at the same concentration alone little affected the intracellular cAMP concentration (Figure 5A). CRE-mediated transcription was also more potently synergistically activated by the pairing of 30 μM nobiletin with forskolin than by the pairing of 100 μM IBMX with forskolin, while nobiletin exerted no enhancing effect on activation of CRE-mediated transcription by 1 mM IBMX even at 100 μM (Figure 5B), suggesting that the action of nobiletin involves an inhibition of PDE. Nobiletin-induced stimulation of CRE-mediated transcription was also abolished by overexpression of a protein kinase inhibitor (PKI), while forskolin-induced stimulation of CRE-mediated transcription was appreciably inhibited by overexpression of PKI, suggesting the involvement of PKA in the enhancing effect of nobiletin on CRE-mediated transcription (Figure 5C). Consistent with this finding, it was actually observed that 30 μM nobiletin appreciably increased the enzyme activity of PKA like forskolin (Figure 5D), to stimulate the phosphorylation of multiple PKA substrates in a different mode from that of the action of NGF (Figure 5E).

Nobiletin Preferentially Inhibits Ca^{2+}/CaM-Dependent PDE Activity in Vitro. The activity of nobiletin to inhibit PDE in vitro was examined in comparison with those of the nonselective PDE inhibitors, IBMX and theophilline. Nobiletin and the PDE inhibitors concentration-dependently inhibited the PDE activity, catalyzing the hydrolysis of cAMP with the following rank order: IBMX > nobiletin > theophilline (Figure 6A). Nobiletin also to lesser degree inhibited the PDE activity to catalyze the hydrolysis of cGMP than IBMX (Figure 6B). As shown in Figure 6C, nobiletin inhibited the activity of Ca^{2+}/CaM-dependent PDE or PDE1 but not Ca^{2+}/CaM-independent PDE, while IBMX prevented the activities of both Ca^{2+}/CaM-dependent and -independent PDEs.

Nobiletin Increases MEK, Erk/MAP Kinase, and CREB Phosphorylations in Cultured Hippocampal Neurons. Furthermore, the effects of nobiletin on CREB phosphorylation in primary neurons were examined. In cultured hippocampal neurons, not only phosphorylation of MEK and Erk/MAP kinase (Figure 7A) but also that of CREB was increased by 100 μM nobiletin within 10 min, as in PC12D cells (Figure 7B). It was also observed that nobiletin enhanced phosphorylation of Erk/MAP kinase in cultured rat hippocampal and

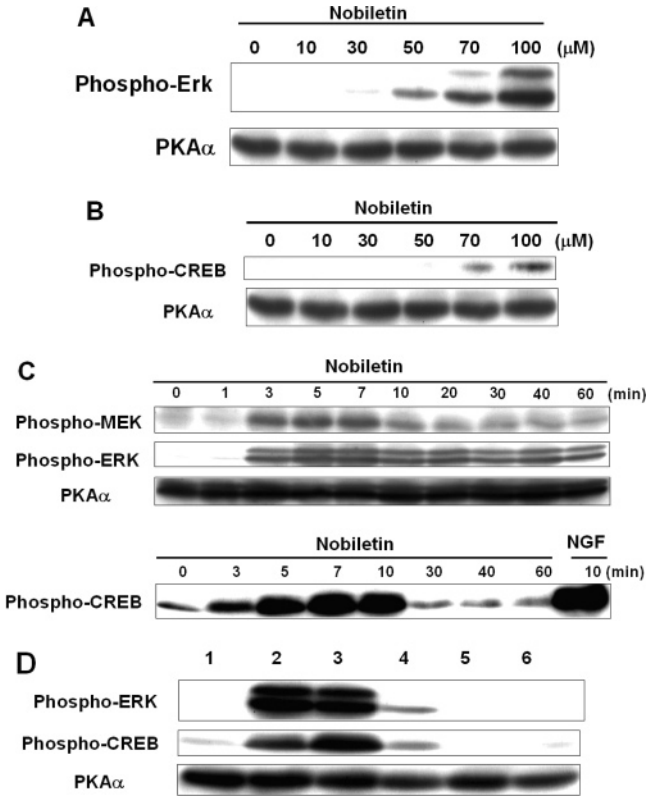

FIGURE 3: Nobiletin activates a PKA/MEK/Erk/MAP kinase-dependent signaling cascade resulting in potentiation of CREB phosphorylation in PC12D cells. Concentration-dependent effects of nobiletin on the phosphorylation of Erk/MAP kinase (A) and CREB (B). (C) Time-dependent effects of nobiletin on the phosphorylation of MEK, Erk/MAP kinase, and CREB. PC12D cells plated at a density of 5×10^5 cells per 35 mm dish were cultured for 48 h. Cells were then stimulated with nobiletin (10−100 μM) for the indicated times or with NGF (50 ng/mL) for 10 min. Western blot analyses were performed using anti-phospho-MEK, anti-phospho-ERK, and anti-phospho-CREB antibodies. Blots were then stripped and reprobed with anti-PKAα antibody, respectively, to verify that equal amounts of proteins were present in each sample. Similar results were obtained from at least three independent experiments. (D) Effects of H-89, U0126, SQ22536, and BAPTA-AM on nobiletin-induced stimulation of ERK and CREB phosphorylation. Lanes: 1, vehicle control; 2, 100 μM nobiletin; 3, 100 μM nobiletin + 50 μM BAPTA-AM; 4, 100 μM nobiletin + 500 μM SQ22536; 5, 100 μM nobiletin + 10 μM H-89; 6, 100 μM nobiletin + 10 μM U0126. Cells were also cultured in the presence of 10 μM H-89, 10 μM U0126, 500 μM SQ22536, or 50 μM BAPTA-AM for 30 min and then treated with vehicle or 100 μM nobiletin for 10 min to examine the effects of these reagents on the action of nobiletin. H-89 at 10 μM has been documented to inhibit PKA activity in PC12D cells (*41*). Western blot analyses were performed as described above. Similar results were obtained from at least three independent experiments.

cortical neurons at 3−100 μM (Matsuzaki et al., data not shown).

DISCUSSION

During recent decades there has been a dramatically growing awareness about the urgency of seeking more effective therapeutic interventions for patients with AD. Numerous efforts have been made for development of fundamental therapeutic drugs for AD. In the course of our survey of substances having neurotrophic activity and/or improving action on memory impairment due to neurodegeneration from natural resources, we successfully found

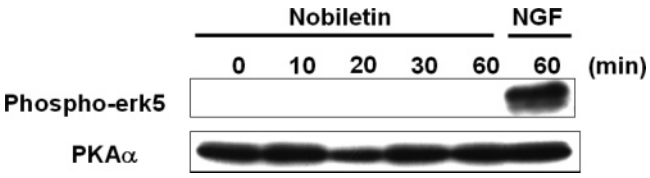

FIGURE 4: Effects of nobiletin on Erk5 phosphorylation in PC12D cells. Cells plated at a density of 5×10^5 cells per 35 mm dish were cultured for 48 h and then stimulated with nobiletin (100 μM) or NGF (50 ng/mL) for the indicated times. Western blots were performed using anti-phospho-Erk5 antibody. Blots were then stripped and reprobed with anti-PKAα antibody, respectively, to verify that equal amounts of proteins were present in each sample. Similar results were obtained from at least two independent experiments.

nobiletin as a novel neurotrophic substance with low molecular weight. In the present study it was demonstrated that nobiletin induced neurite outgrowth accompanied by activation of PKA activity and MEK/Erk/MAP kinase-dependent signaling and enhanced CREB phosphorylation and CRE-dependent transcription in PC12D cells, with preferentially inhibiting Ca^{2+}/CaM-dependent PDE or PDE1 in vitro. This natural compound also increased MEK, Erk/MAP kinase, and CREB phosphorylation in cultured hippocampal neurons. These findings provide the first evidence that nobiletin serves as a novel neurotrophic substance in the cell culture systems.

In this study, we investigated the mechanism by which nobiletin induced neuronal differentiation in PC12D cells. Nobiletin was shown to inhibit the PDE activity to catalyze the hydrolysis of cAMP (*31*) and cGMP in vitro,and was for the first time demonstrated to preferentially prevent the Ca^{2+}/CaM-dependent PDE activity catalyzing the hydrolysis of cAMP in vitro, raising the possibility that this natural compound directly inhibits PDE activity at the local region of cells and thereby increases intracellular cAMP concentration to activate PKA in vivo. In support of this interpretation, it was in fact observed that, in PC12D cells, the pairing of nobiletin with forskolin showed a synergistic effect on the intracellular cAMP level, like the pairing of IBMX with forskolin, although nobiletin alone little affected the intracellular cAMP concentration, and that PKA activity was enhanced by nobiletin to appreciably increase multiple proteins showing immunoreactivity for anti-phospho-(Ser/Thr) PKA substrate antibody in PC12D cells. Also, it was shown that nobiletin alone activated CRE-dependent transcription, and the pairing of nobiletin with a low concentration of forskolin synergistically enhanced CRE-dependent transcription. But nobiletin exhibited no additive effect on activation of CRE-dependent transcription by IBMX. Therefore, it is quite possible that nobiletin directly inhibits PDE activity at least at the intracellular local region to increase the intracellular cAMP concentration and thereby activate PKA in PC12D cells. cAMP activates Rap1, a small GTP-binding protein in the Ras family which serves as a selective activator of B-Raf, in a PKA-dependent manner, to stimulate B-Raf activity leading to activation of MEK, resulting in a sustained activation of Erk/MAP kinase (*32*−*34*) that is associated with neuronal differentiation (*29*), like NGF in PC12 cells. PC12D cells have been documented to express B-Raf as well (*35*), and the expression of this protein was actually verified in the same cell line employed for the

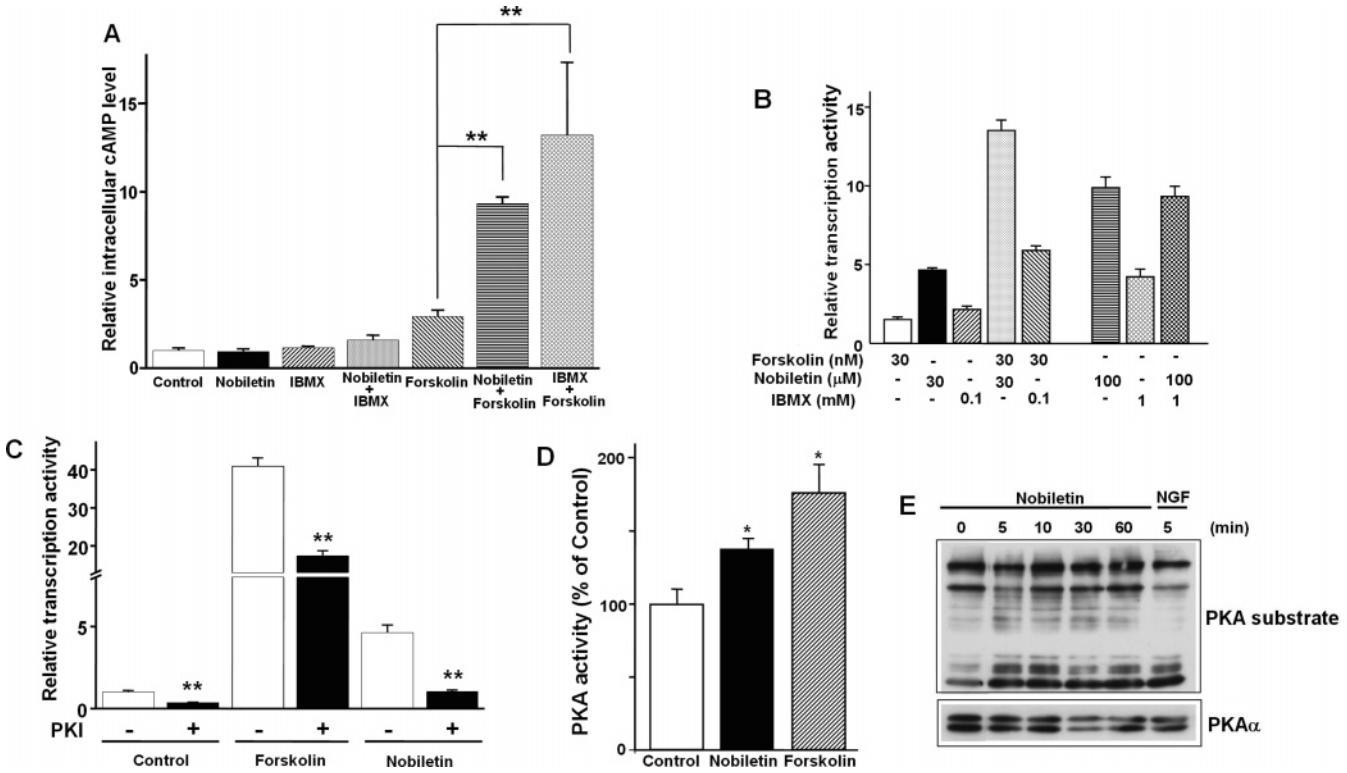

FIGURE 5: Effects of nobiletin, IBMX, a PDE inhibitor, and forskolin on the intracellular cAMP concentration and CRE-mediated gene transcription in PC12D cells. (A) Effects of nobiletin, IBMX, and forskolin on intracellular cAMP in PC12D cells. Cells plated at a density of 1×10^6 cells per 35 mm dish were cultured for 48 h. Cells were then treated with vehicle, 100 μM nobiletin, 1 mM IBMX, and 10 μM forskolin for 10 min. The amount of intracellular cAMP was measured by a radioimmunoassay. Control denotes vehicle control. **, $P < 0.01$ versus cells treated with forskolin. Data are the means ± SEM ($n = 4$). (B) Effects of nobiletin, IBMX, and forskolin on CRE-mediated transcription in PC12D cells. Cells plated at a density of 8×10^4 cells per 48-well plate were cultured for 24 h. Cells were then transfected with a luciferase reporter construct showing CRE-dependent transcription for 19 h. Following transfection, cells were treated with different concentrations of forskolin, nobiletin, or IBMX for 5 h. Data are the means ± SEM ($n = 4$). Similar results were obtained from at least three independent experiments. (C) Effects of PKI on forskolin- and nobiletin-induced CRE-mediated transcription. Following transfection, cells were treated with vehicle, 1 μM forskolin, or 100 μM nobiletin for 5 h. Control denotes vehicle control. Data are the means ± SEM ($n = 4$). **, $P < 0.01$ versus cells transfected without a PKI expression vector. (D) Effects of nobiletin and NGF on protein phosphorylation by PKA in PC12D cells. (E) Effects of nobiletin and NGF on protein phosphorylation by PKA in PC12D cells. Cells were treated with 30 μM nobiletin or NGF for indicated times. Cell lysates were separated on 12.5% SDS−PAGE gel and subjected to Western blot analysis using anti-phospho-(Ser/Thr) PKA substrate antibody followed by reprobing with anti-PKAα antibody. In PC12D cells, the amount of multiple proteins showing immunoreactivity for anti-phospho-(Ser/Thr) PKA substrate antibody was observed to increase from 5 min onward following treatment with nobiletin with a different phosphorylation profile from that of the cells treated with NGF. Similar results were obtained from at least two independent experiments.

present study.[2] It was shown that nobiletin induced a sustained increase in Erk/MAP kinase phosphorylation which was preceded by a prolonged stimulation of MEK phosphorylation. Not only U0126 but also H89 abolished nobiletin-induced increase in Erk/MAP kinase phosphorylation in PC12D cells. An adenylate cyclase inhibitor markedly prevented phosphorylation of Erk/MAP kinase. Therefore, it is plausible to interpret that an inhibition of PDE by nobiletin serves as the upstream event of activation of the Rap1/B-Raf MEK/Erk/MAP kinase-dependent cascade. Furthermore, nobiletin-induced neurite outgrowth in PC12D cells was partially prevented by PD98059 and U0126, but not by wortmannin and GF109203X, suggesting the presence of the MEK/Erk/MAP kinase-dependent and -independent mechanisms underlying this neuritegenic action. These findings thus suggest that nobiletin-induced neurite outgrowth results from a sustained activation of MEK/Erk/MAP kinase via PKA/Rap1/B-Raf in PC12D cells.

It was demonstrated that U0126, the MEK inhibitor, abolished an increase in CREB phosphorylation by nobiletin like the PKA inhibitor, indicating a dependency of the increased CREB phosphorylation on PKA- and MEK/Erk/MAP kinase-dependent signaling. This is supported by the fact that an adenylate cyclase inhibitor markedly prevented an increase in phosphorylation of Erk/MAP kinase and CREB by nobiletin and that stimulation of CRE-dependent transcription by nobiletin was profoundly inhibited by overexpression of PKI. In addition, this natural compound activated transcription from the neuronal gene encoding tyrosin hydroxylase in PC12D cells,[3] demonstrating the activity of nobiletin to modulate neuronal gene expression. Yao et al. have reported reduced Erk nuclear translocation after PKA inhibition (*32*). NGF and forskolin have been demonstrated to activate Erk/MAP kinase to induce the nuclear translocation and nuclear activation of RSK2 which in turn phosphorylates CREB to stimulate CRE-dependent transcription (*37*, *38*). Accordingly, it is suggested that nobiletin enhances CRE-dependent transcription by CREB activation probably via Erk/MAP kinase/RSK2.

[2] Wasaki et al., unpublished observations.

[3] Nagase et al., unpublished observations.

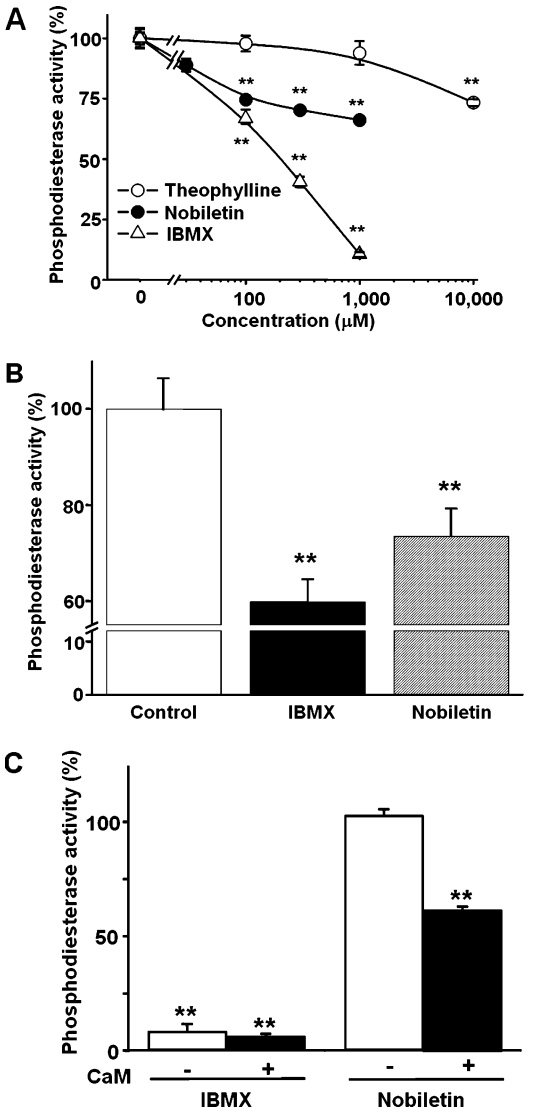

FIGURE 6: Effects of PDE inhibitors and nobiletin on bovine heart PDE activity in vitro. (A) Effects of PDE inhibitors and nobiletin on bovine heart PDE activity to hydrolyze cAMP. Following incubation of PDE with different concentrations of IBMX (triangle), theophylline (open circle), or nobiletin (closed circle) for 10 min, the amount of cAMP hydrolyzed by PDE was assayed using the Martin−Doty method. Data are the means ± SEM ($n = 4$). **, $P < 0.01$ versus vehicle control. (B) Effects of IBMX and nobiletin on the bovine heart PDE activity to hydrolyze cGMP. Following incubation of PDE with vehicle, 1 mM IBMX, or 100 μM nobiletin for 10 min, the amount of hydrolyzed cGMP was assayed using the Martin−Doty method. Data are the means ± SEM ($n = 4$). **, $P < 0.01$ versus vehicle control. (C) Effect of IBMX and nobiletin on the bovine heart PDE activity to hydrolyze cAMP in the absence or presence of Ca^{2+}/calmodulin. Following incubation with vehicle (control), 1 mM IBMX, or 1 mM nobiletin in the absence or presence of 3 μM Ca^{2+} and 10 units/mL calmodulin for 10 min, the amount of cAMP hydrolyzed by PDE was assayed as described above. Data are the means ± SEM ($n = 4$). **, $P < 0.01$ versus each vehicle control.

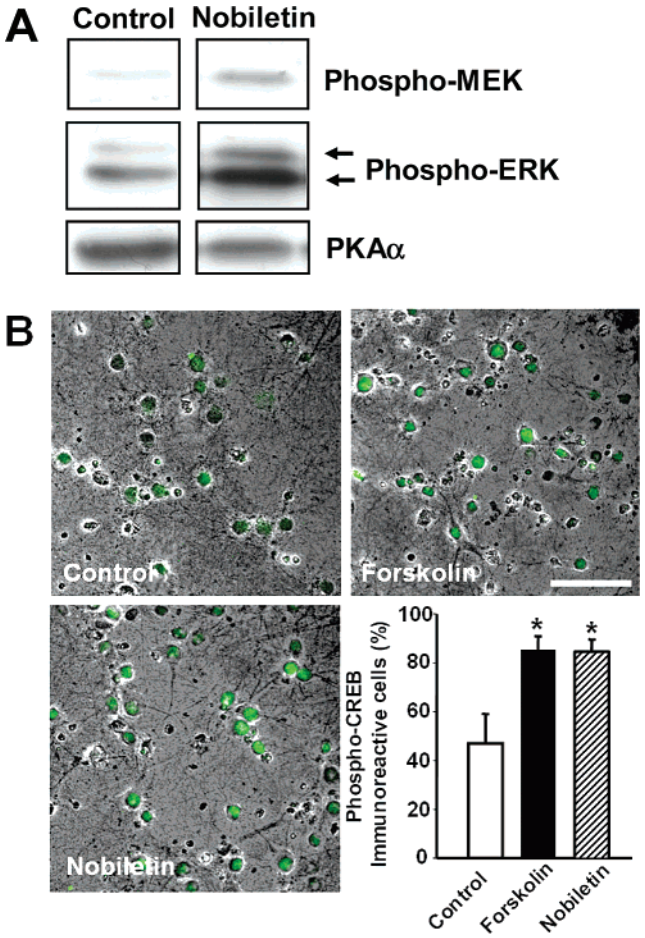

FIGURE 7: Nobiletin potentiates MEK, Erk, and CREB phosphorylation in cultured rat hippocampal neurons. (A) Effects of nobiletin on the phosphorylation of MEK and Erk in cultured rat hippocunpus neurons. Cells were seeded at a density of 1×10^6 cells per 35 mm dish and cultured for 14 days. Cells were then stimulated with nobiletin at 10 min at 100 μM. Western blot analyses were performed using anti-phospho-MEK or anti-phospho-Erk antibodies. Blots were then stripped and reprobed with anti-PKAα antibody to verify that each lane contained equal amounts of proteins. (B) Effects of nobiletin on the phosphorylation of CREB in cultured rat hippocunpus neurons. Cells were treated with 10 μM forskolin or 100 μM nobiletin for 10 min and then subjected to immunostaining. The histogram represents the result of quantitative analysis of the immunofluorescence experiment (bottom right panel). Data are the means ± SEM. *, $P < 0.05$ versus vehicle control.

Erk5 MAP kinase mediates a retrograde signaling of NGF via TrkA in PC12 cells (*30*). The action of nobiletin and NGF shares a common mechanism requiring MEK/Erk/MAP kinase-dependent signaling, as described above. Nevertheless, nobiletin failed to induce Erk5 phosphorylation at all tested time points, unlike NGF. Also, the TrkA inhibitor, K252a, did not inhibit the nobiletin-induced neurite outgrowth but rather significantly enhanced it via an unknown mechanism in PC12D cells. These findings suggest that the actions of nobiletin, including neuritogenic action, appear not to result from TrkA mediation.

Increasing evidence indicates that cAMP plays an important role in the hippocampal long-term potentiation associated with learning and memory (*39*). On the other hand, Aβ has been documented to inhibit the PKA/CREB pathway and long-term potentiation in cultured hippocampal neurons (*40*). The most important finding in this study is that nobiletin triggers neurite outgrowth and CRE-dependent transcription via CREB activation in a cAMP/PKA/MEK/Erk/MAP kinase-dependent manner in PC12D cells. Interestingly, nobiletin induced increments of MEK, Erk/MAP kinase, and CREB phosphorylation in cultured hippocampal neurons as observed in PC12D cells, although it has been shown that TPA-stimulated phsphorylation of MEK1/2 is prevented by this natural compound (*41*). Nobiletin also improved impair-

ment of memory in Aβ-infused rats.[4] Although the mechanism underlying this protective action on Aβ-induced impairment of memory remains to be defined, a cAMP/PKA/MEK/Erk/MAP kinase-dependent signaling by nobiletin might serve as a mechanism required for improvement of impaired memory by this compound in Aβ-infused rats as well.

In conclusion, nobiletin, a citrus flavonoid, induced neurite outgrowth accompanied by a sustained increase in phosphorylation of MEK and Erk/MAP kinase in cultured hippocampal neurons as well as in PC12D cells. In PC12D cells this compound stimulated CREB phosphorylation and CRE-mediated transcription in a MEK/Erk/MAP-kinase-dependent signaling cascade. Also, nobiletin activated PKA activity, and the pairing of nobiletin with forskolin synergistically augumented the intracellular cAMP level and CRE-mediated transcription. Nobiletin showed a preferential inhibition of Ca^{2+}/CaM-dependent PDE in vitro. In cultured hippocampal neurons MEK, Erk/MAP kinase, and CREB phosphorylation was enhanced by nobiletin. These results suggest that nobiletin induces neurite outgrowth and CRE-mediated transcription dependently of a cAMP/PKA/MEK/Erk/MAP-kinase signaling cascade but not TrkA with modulating neuronal gene expression probably by inhibiting PDE activity and may thus become a novel type of biochemical probe for elucidation of the molecular mechanism underlying neuronal differentiation, including neurite outgrowth and neuronal gene transcription.

ACKNOWLEDGMENT

We appreciate Drs. K. Ohashi and K. Mizuno for the excellent technical advice and scientific discussions. We are grateful to Dr. K. Nagamoto-Combs for providing pRSV-PKI plasmid.

REFERENCES

1. Levi-Montalcini, R. (1987) The nerve growth factor 35 years later, *Science 237*, 1154−1162.
2. Sofroniew, M. V., Howe, C. I., and Mobley, W. C. (2001) Nerve growth factor signaling, neuroprotection, and neural repair, *Annu. Rev. Neurosci. 24*, 1217−1281.
3. Kesner, R. P. (1988) Enhancement of the acoustic startle response by stimulation of an excitatory pathway from the central amygdala/basal nucleus of Meynert to the pontine reticular formation, *Neurobiol. Aging 9*, 609−616.
4. Bartus, R. T. (2000) On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis, *Exp. Neurol. 163*, 495−529.
5. Cummings, J. L. (2004) Alzheimer's disease, *N. Engl. J. Med. 351*, 56−67.
6. Kasa, P., Rakonczay, Z., and Guly, K. (1997) The cholinergic system in Alzheimer's disease, *Prog. Neurobiol. 52*, 511−535.
7. Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and Delong, M. R. (1982) Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain, *Science 215*, 1237−1239.
8. Patrick, C. W., Jr., Kukreti, S., and McIntire, L. V. (1996) Quantitative effects of peripheral monocytes and nerve growth factor on CNS neuronal morphometric outgrowth parameters in vivo, *Exp. Neurol. 138*, 277−285.
9. Woodruff, R. H., and Franklin, R. J. (1997) Growth factors and remyelination in the CNS, *Histol. Histopathol. 12*, 459−466.
10. Brinton, R. D., and Yamazaki, R. S. (1998) Advances and challenges in the preventation and treatment of Alzheimer's disease, *Pharmacol. Res. 15*, 386−398.
11. Connor, B., and Dragunow, M. (1998) The role of neuronal growth factors in neurodegenerative disorders of the human brain, *Brain Res. Rev. 27*, 1−39.
12. Capsoni, S., Giannotta, S., and Cattaneo, A. (2002) Nerve growth factor and galantamine ameliorate early signs of neurodegeneration in anti-nerve growth factor mice, *Proc. Natl. Acad. Sci. U.S.A. 99*, 12432−12437.
13. Eriksdotter-Jonhangen, M., et al. (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease, *Dementia Geriatr. Cognit. Disord. 9*, 246−257.
14. Chen, X. Q., Fawcett, J. R., Rahman, Y. E., Ala, T. A., and Frey, W. H., II (1998) Delivery of nerve growth factor to the brain via the olfactory pathway, *J. Alzheimer's Dis. 1*, 35−44.
15. Furukawa, K.-I., Sakai, K., Watanabe, S., Maruyama, K., Murakami, M., Yamaguchi, K., and Ohizumi, Y. (1993) Goniodomin A induces modulation of actomyosin ATPase activity mediated through conformational change of actin, *J. Biol. Chem. 268*, 26026−26031.
16. Obara, Y., Aoki, T., Kusano, M., and Ohizumi, Y. (2002) β-Eudesmol induces neurite outgrowth in rat pheochromocytoma cells accompanied by an activation of mitogen-activated protein kinase, *J. Pharmacol. Exp. Ther. 301*, 803−811.
17. Ohizumi, Y. (1997) Application of physiologically active substances isolated from natural resources to pharmacological studies, *Jpn. J. Pharmacol. 73*, 263−289.
18. Liu, J. (1993) FK506 and cyclosporin: molecular probes for studying intracellular signal transduction, *Trends Pharmacol. Sci. 14*, 182−188.
19. Machida, K., and Osawa, K. (1989) On the flavonoid constituents from the peels of *Citrus hassaku* Hort. ex Tanaka, *Chem. Pharm. Bull. 37*, 1092−1094.
20. Katoh-Semba, R., Kitajima, S., Yamazaki, Y., and Sano, M. (1987) Neuritic growth from a new subline of PC12 pheochromocytoma cells: cyclic AMP mimics the action of nerve growth factor, *J. Neurosci. Res. 17*, 36−44.
21. Li, P., Yamakuni, T., Matsunaga, K., Kondo, S., and Ohizumi, Y. (2003) Nardosinone enhances nerve growth factor-induced neurite outgrowth in a mitogen-activated protein kinase- and protein kinase C-dependent manner in PC12D cells, *J. Pharmacol. Sci. 93*, 122−125.
22. Li, P., Matsunaga, K., Yamakuni, T., and Ohizumi, Y. (2003) Nardosinone, the first enhancer of neurite outgrowth-promoting activity of staurosporine and dibutyryl cyclic AMP in PC12D cells, *Dev. Brain Res. 145*, 177−183.
23. Suzuki, T., Yamakuni, T., Hagiwara, M., and Ichinose, H. (2002) Identification of ATP-2 as a transcriptional regulator for the tyrosine hydroxylase gene, *J. Biol. Chem. 277*, 40768−40774.
24. Piech-Dumas, K. M., et al. (2001) The cAMP responsive element and CREB partially mediate the response of the tyrosine hydroxylase gene to phorbol ester, *J. Neurochem. 76*, 1376−1385.
25. Yamakuni, T., Yamamoto, T., Hoshino, M., Song, S.-Y., Yamamoto, H., Kunikata-Sumitomo, M., Minegishi, A., Kubota, M., Ito, M., and Konishi, S. (1998) A novel protein containing cdc10/SW16 motifs regulates expression of mRNA encoding catecholamine biosynthesizing enzymes, *J. Biol. Chem. 273*, 27051−27054.
26. Arakane, F., Fukunaga, K., Araki, S., Abe, S., Satake, M., Miyazaki, K., Okamura, H., and Miyamoto, E. (1994) Glycolipid isolated from *Aplysia kurodai* can activate cyclic adenosine 3′,5′-monophosphate-dependent protein kinase from rat brain, *J. Neurochem. 62*, 86−93.
27. Martin, J. B., and Doty, D. M. (1949) Determination of inorganic phosphate. Modification of isobutyl alcohol procedure, *Anal. Chem. 21*, 965−967.
28. Green, L. A., and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, *Proc. Natl. Acad. Sci. U.S.A. 73*, 2424−2428.
29. Marshall, C. (1995) Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal regulated kinase activation, *Cell 80*, 179−185.
30. Shao, Y., Akmentin, W., Toledo-Aral, J. J., Rosenbaum, J., Valdez, G., Cabot, J. B., Hilbush, B. S., and Haleoua, S. (2002) Pincher, a pinocytic chaperone for nerve growth factor/TrkA siganaling endosomes, *J. Cell Biol. 157*, 679−691.

[4] Matsuzaki et al., unpublished data.

31. Nikaido, T., Ohmoto, T., Sankawa, U., Hamanaka, T., and Totsuka, K. (1982) Inhibition of cyclic AMP phosphodiesterase by flavonoids, *Planta Med. 46*, 162−166.
32. Yao, H., York, R. D., Misra-Press, A., Carr, D. W., and Strok, P. J. (1998) The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor, *J. Biol. Chem. 273*, 8240−8247.
33. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. S. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor, *Nature 392*, 622−626.
34. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Strok, P. J. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway, *Cell 89*, 73−82.
35. Guo, F.-F., Kumahara, E., and Saffen, D. (2001) A CalDAG-GEFI/rap1/B-raf cassette couples M_1 muscarinic acetylcholine receptors to the activayion of ERK1/2, *J. Biol. Chem. 276*, 25568−25581.
36. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase, *Science 273*, 959−963.
37. Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., Deloulme, J. C., Chan, G., and Storm, D. R. (1998) Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation, *Neuron 21*, 869−883.
38. Abel, T., Nguyen, P. V., Barad, M., Deuel, T. A. S., and Kandel, E. R. (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory, *Cell 88*, 615−626.
39. Frey, U., Huang, Y.-Y., and Kandel, E. R. (1993) Effects of cAMP simulate a late stage LTP in hippocampal CA1 neurons, *Science 260*, 1661−1664.
40. Vitolo, O. V., Sant'Angelo, A., Costanzo, V., Battaglia, F., Arancio, O., and Shelanski, M. (2002) Amyloid β-peptide inhibition of the PKA/CREB pathway and long-term potentiation: Reversibility by drugs that enhance cAMP signaling, *Proc. Natl. Acad. Sci. U.S.A. 99*, 13217−13221.
41. Miyata, Y., Sato, T., Yano, M., and Ito, A. (2004) Activation of protein kinase C βII/ϵ-c-Jun NH_2-terminal kinase pathway and inhibition of mitogen-activated protein/extracellular signal-regulated kinase 1/2 phosphorylation in antitumor invasive activity induced by the polmethoxy flavonoid, nobiletin, *Mol. Cancer Ther. 3*, 839−847.

BI050643X