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Increase by FK960, a novel cognitive enhancer, in glial cell line-derived neurotrophic factor production in cultured rat astrocytes

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Abstract

We examined the effect of *N*-(4-acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate (FK960), a novel anti-dementia drug, on neurotrophic factor production in cultured rat astrocytes. FK960 (100 nM) increased mRNA and protein levels of glial cell line-derived neurotrophic factor (GDNF). FK960 did not affect mRNA levels of neurotrophic factors other than GDNF. The effect of FK960 was not affected by antagonists of dopamine and α7-nicotinic acetylcholine receptors. FK960 stimulated phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase (ERK) without any effect on phosphoryolation of p38 and c-Jun N-terminal kinase. FK960 increased the levels of c-Fos and phosphorylation of cAMP responsive element binding protein (CREB). The effect of FK960 on c-Fos was inhibited by PD98059 (10 μM), an ERK kinase inhibitor, and cycloheximide (1 μg/ml), a transcription inhibitor, and the effect of FK960 on CREB phosphorylation was blocked by PD98059. The effect of FK960 on GDNF mRNA expression was attenuated by PD98059, curcumin (10 μM), an activator protein-1 inhibitor, cycloheximide and actinomycin D (10 μg/ml). These results suggest that FK960 stimulates GDNF production in c-Fos- and CREB-dependent mechanisms in cultured astrocytes and that ERK signal is responsible for both c-Fos expression and CREB phosphorylation in the cascades.

Keywords: FK960; Neurotrophic factor; Glial cell line-derived neurotrophic factor (GDNF); Extracellular signal-regulated kinase (ERK); c-Fos; cAMP responsive element binding protein (CREB); Astrocyte

1. Introduction

N-(4-Acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate (FK960), a novel anti-dementia drug, improves memory deficits in rats [1] and monkeys [2]. In addition, FK960 facilitates neurotransmission in the sub-regions of the hippocampus [3] and increases synaptic density in the hippocampal CA3 region of aged rats [4]. These effects of FK960 were abolished by administration of cysteamine, which was shown to deplete hippocampal somatostatin [1,3]. The observation suggests that the effect of FK960 is mediated by activation of the cholinergic-somatostatinergic link in the hippocampal formation. In this line, Inoue et al. [5] reported that FK960 enhanced high K^+-evoked release of somatostatin in the hippocampal slices. On the other hand, Hodgkiss and Kelly [6] found that FK960 increased the amplitude of the excitatory postsynaptic potentials in CA1 neurons of rat hippocampus and the effect of FK960 was blocked by the α7-nicotinic acetylcholine receptor antagonists methyllycaconitine and α-bungarotoxin. This finding suggests that α7-nicotinic acetylcholine receptors are involved in the effect of FK960. Alternatively, Tada et al. [7] have recently shown that FK960 enhanced glutamate release from cultured hippocampal astrocytes and this effect was inhibited by H-89, a

Abbreviations: FK960, *N*-(4-acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; RT-PCR, reverse transcription-polymerase chain reaction; PKA, protein kinase A; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; CREB, cAMP responsive element binding protein; MEM, minimum essential medium

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protein kinase A (PKA) inhibitor. This suggests the new idea that FK960 facilitates hippocampal neurotransmission by targeting astrocytes.

Astrocytes play physiologically and pathologically important roles in neuronal activities [8]. The cells produce various neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily [9]. These factors contribute to the growth, development, and plasticity of selective neuronal populations in the nervous system [10–12]. Neurotrophic factors are also involved in memory acquisition processes and the pathogenesis of psychotic disorders [13,14]. These observations raise the possibility that neurotrophic factors are involved in the effect of FK960. However, it is not known whether the drug affects the production of neurotrophic factors in astrocytes. We report here that FK960 increases GDNF production. Furthermore, we examined the intracellular signal mechanism for the effect of FK960 in increasing mRNA expression of GDNF. Since we have studied the role of mitogen-activated protein (MAP) kinases in the effects of neuroprotective agents such as T-588 [15] and CV-2619 [16] in cultured astrocytes, we focused on the role of MAP kinase signal pathway.

2. Materials and methods

2.1. Materials

Drugs were obtained from the following sources: MMLV reverse transcriptase, random hexanucleotides and *Taq* DNA polymerase, Invitrogen (Carlsbad, CA); RNase inhibitor, Takara (Shiga, Japan); Vistra Green, Amersham (Buckinghamshire, UK); GDNF ELISA kit, Promega (Madison, WI); BCA protein assay kit, Pierce (Rockford, IL); 2′-amino-3′-methoxyflavone (PD98059), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), methyllycaconitine, α-bungarotoxin and staurosporine, Calbiochem (La Jolla, CA); anti-phospho-ERK1/2 and anti-ERK1/2 rabbit antibodies, Cell Signaling Technology (Beverly, MA); anti-phospho-p38 rabbit antibody, Biosource International (Camarillo, CA); anti-p38 and anti-CREB rabbit antibodies, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390), *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89) and curcumin, Sigma–Aldrich Co. (St. Louis, MO); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), Tocris Cookson Ltd. (Bristol, UK); dizocilpine (MK-801), Research Biochemical International (Natick, MA); anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK and anti-c-Fos rabbit antibodies, Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho CREB rabbit antibody, Upstate Biotechnology (Lake Placid, NY); enhanced chemiluminescence detection kit, NEN (Boston, MA). FK960 was a gift from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

2.2. Astrocyte culture

All experimental protocols conformed to the Guiding Principles for the Care and Use of Animals approved by the Japanese Pharmacological Society. Astrocytes were prepared from cerebrum of 1–2-day-old Wistar rats as described previously [17,18]. Cells were seeded at 1×10^4 cells/cm^2 in 75 cm^2 culture flasks and grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum. After the primary culture reached confluence (10–14 days), the culture flasks were shaken at 250 rpm overnight to remove small process-bearing cells (mainly oligodendrocyte progenitors and microglia) on the protoplasmic cell layer. The monolayer cells were trypsinized, plated onto appropriate size culture wells and grown for 14–21 days. At this stage, more than 90% of cells had glial fibrillary acidic protein. Astrocytes were cultured in serum-free MEM for 48 h before treatment with agents.

2.3. Measurement of neurotrophic factor mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured astrocytes according to the acid-phenol method followed by repeated isopropanol precipitation [19]. Briefly, astrocytes growing in 35 mm dishes were homogenized in 250 μl of 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol and 0.5% sarcosyl. The homogenate was mixed with water-saturated phenol (equal volume) and chloroform/isoamylalchol = 49:1 (1/50, v/v). After centrifugation, the upper water phase was mixed with an equal volume of isopropanol. Isopropanol precipitation was repeated twice. cDNA was synthesized from 1 μg total RNA with MMLV reverse transcriptase (200 U), random hexanucleotides (0.2 μg) and RNase inhibitor (20 U) in 10 μl of a buffer supplied by the enzyme manufacture. The reverse transcription product was added to PCR mixture containing 0.2 mM dNTP mix, 5 μM of each oligonucleotide primer and 0.5 U *Taq* DNA polymerase in 15 μl of buffer supplied by the enzyme manufacture. The following primer pairs were used. GDNF [20], 5′-ATGAAGTTATGGGATGTCGT-3′ and 5′-CAGGGTCAGATACATCCACA-3′; BDNF, 5′-ATGACCATCCTTTTCCTTACTATGGT-3′ and 5′-TCTTCCCCTTTTAATGGTCAGTGTAC-3′; NGF [21], 5′-TGGACCCAAGCTCACCTCA-3′ and 5′-GTGGATGAGCGCTTGCTCCT-3′; basic fibroblast growth factor (bFGF) [22], 5′-GCCTTCCCACCC GGCCACTTCAAGG-3′ and 5′-GCACACACTCCCTTGATGGACACAA-3′; neurotrophin-3 (NT-3) [23] 5′-GC-

TGATCCAGGCGGATATCT-3′ and 5′-GCTGATCCAGGCGGATATCT-3′; β-actin, 5′-GATGGTGGGTATGGGTCAGAAGGA-3′ and 5′-GCTCATTGCCGATAGTGATGACCT-3′. The PCR products were separated through 1.5% agarose gel and stained with Vistra Green. The amplified DNA fragment was visualized by FluroImager 595 (Amersham Bioscience, Sunnyvale, CA) and fluorescence intensity was measured with NIH Image 1.6. Pilot experiments were performed to determine the range of PCR cycle numbers where the density of amplified PCR product was proportional to the amount of the applied reverse transcription product. Expression levels of neurotrophic factor mRNAs were normalized by those of β-actin mRNA. The primer for GDNF mRNA detected two transcripts of GDNF mRNA and total of the two bands is shown as mRNA level.

2.4. Determination of GDNF by enzyme-linked immunosorbent assay (ELISA)

Astrocytes in six-well plates were cultured in serum-free MEM for 48 h. Then, cells were treated with 100 nM FK960 in 1 ml of serum-free MEM for 6 and 12 h. The contents of immunoreactive GDNF released into culture medium were determined using an ELISA kit according to the supplier's protocol.

2.5. Immunoblotting of MAP kinases and transcription factors

Serum-starved cells in 35 mm dishes were incubated with FK690 and the other agents in the serum-free MEM. After the treatment, the cells were lysed in 60 μl of ice-cold homogenate buffer (20 mM Tris–HCl (pH 7.4), 1% SDS, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 mM NaF and 2 mM Na_3VO_4) at 4 °C for 30 min. The lysate was centrifuged at 15,000 × *g* for 10 min at 4 °C, and aliquots were taken for protein determination using a BCA protein assay kit. The cell lysate (20 μg protein) was applied to SDS-PAGE and blotted to PVDF membranes. For measurement of phosphorylation of MAP kinases, the blotted membranes were incubated with an anti-phospho-ERK1/2 rabbit antibody (1:1000), an anti-phospho-p38 rabbit antibody (1:1000) or an anti-phospho-JNK rabbit antibody (1:1000) in Tris-buffered saline containing 0.05% Tween-20 and 2% skimmed milk. The membranes were subsequently incubated with a peroxidase-conjugated secondary antibody and the immunoreactive bands were detected with an enhanced chemiluminescence detection kit. After detection of the phospho-proteins, the antibodies were stripped from the membrane by incubation with 62 mM Tris–HCl (pH 6.8), 2% SDS and 100 mM 2-mercaptoethanol at 50 °C for 30 min. Then, levels of ERK, p38 and JNK on the membranes were measured by re-probing with rabbit antibodies against ERK1/2 (1:2000), p38 (1:1000) and JNK (1:1000), respectively. For measurement of transcription factor proteins, the blotted membranes were incubated with rabbit antibodies against c-Fos (1:800), phospho-CREB (1:1000) and CREB (1:1000). The immunoreactive bands were detected as described above.

2.6. Statistical analysis

Statistical analysis of the experimental data was carried out by Student's *t*-test or one-way ANOVA followed by the Dunnett's test, using a software package (Stat View 5.0) for Apple Macintosh computer. Values of $P < 0.05$ were considered to be significant, and results were expressed as means ± S.E.

3. Results

Fig. 1 shows the time course of the effect of FK960 on mRNAs for several neurotrophic factors in cultured rat astrocytes. FK960 at 100 nM increased the levels of GDNF mRNA: the effect was maximum at 6 h after addition of the drug and gradually reduced thereafter. In agreement with the previous report [20], the primer used here detected two transcripts of GDNF mRNA (Fig. 1A). FK960 did not affect the levels of BDNF, NGF, NT-3, bFGF and β-actin mRNAs during the treatment for 24 h (Fig. 1A and B). Fig. 2 shows the dose-response curve for the effect of FK960 on GDNF mRNA levels in cultured astrocytes. The significant effect of FK960 on GDNF mRNA levels was observed at the concentrations above 100 nM. The effect of FK960 on GDNF was also observed at protein level. Immunoreactive GDNF in the culture medium increased at 6 and 12 h after FK960 at 100 nM and the levels were 25–60 pg/mg protein of astrocytes (Fig. 3).

It is known that GDNF production is increased by activation of dopamine and glutamate (*N*-methyl-D-aspartic acid (NMDA) and AMPA/kainite types) receptors in cultured astrocytes and glioma [24,25]. The increases in GDNF mRNA levels by FK960 were not affected by the NMDA receptor antagonist MK-801 (1 μM), the AMPA/kainate receptor antagonist CNQX (1 μM), the dopamine D_1 receptor antagonist SCH23390 (10 nM) and dopamine D_2 receptor antagonist haloperidol (100 nM) (Table 1). In addition, methyllycaconitine (100 nM) and α-bungarotoxin (100 nM), nicotinic acetylcholine receptor antagonists, did not attenuate the effect of FK960 on GDNF production, although they blocked the effect of FK960 on synaptic transmission [6].

MAP kinases have key roles in the transcriptional regulation of various genes. FK960 increased active (phosphorylated) forms of ERK1/2 in cultured astrocytes, although it did not affect total levels of ERK1/2 (Fig. 4A). The significant effect of FK960 on phosphorylated forms of ERK1/2 was observed at 5 min after the treatment and the increased level continued up to 30 min

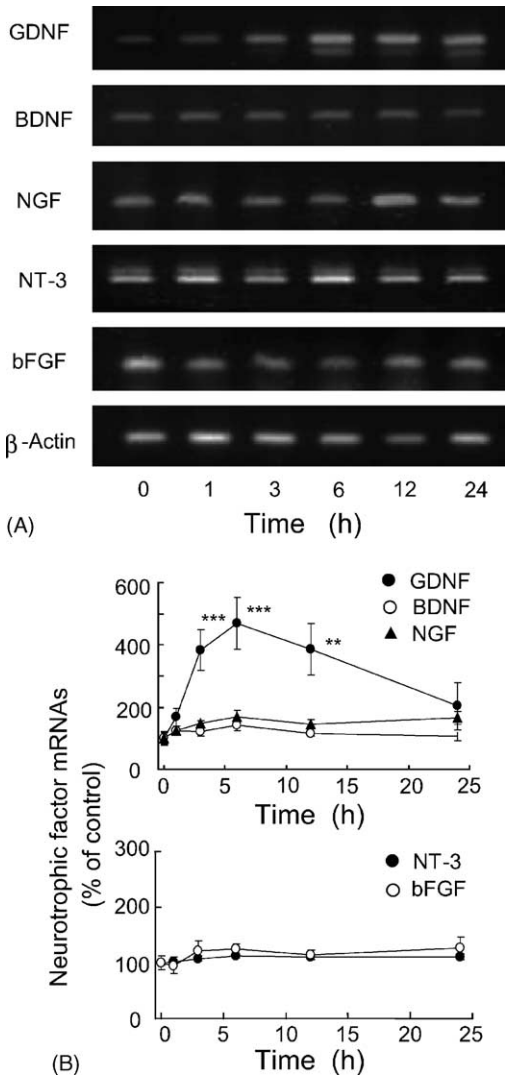

Fig. 1. Effect of FK960 on neurotrophic factor mRNA levels in cultured astrocytes. Serum-starved astrocytes were treated with 100 nM FK960 for 0.5–24 h. (A) Typical patterns of RT-PCR products for GDNF, BDNF, NGF, NT-3, bFGF and β-actin are indicated. (B) Time-courses of changes in neurotrophic factor mRNA levels. Expression levels of neurotrophic factor mRNAs were normalized by those of β-actin mRNA and shown as percentage of control. The ratios of neurotrophic factor mRNA/β-actin mRNA in non-treated astrocytes were as follows: GDNF, 0.162 ± 0.035; BDNF, 0.553 ± 0.111; NGF, 0.579 ± 0.101; NT-3, 0.860 ± 0.033; bFGF, 0.692 ± 0.088. Results are means ± S.E. mean of 8–10 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. zero-time (Dunnett's test).

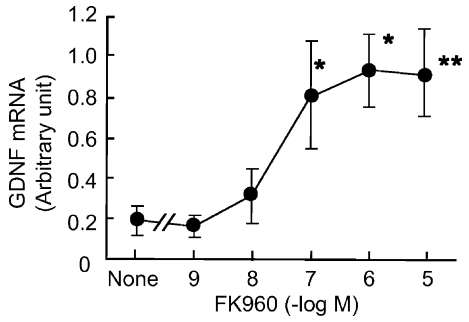

Fig. 2. Dose–response of the increases in astrocytic GDNF mRNA levels by FK960. Astrocytes were treated with the indicated concentrations of FK960 for 6 h. Expression levels of GDNF mRNA were normalized by those of β-actin mRNA and shown as ratio of GDNF mRNA to that of β-actin. Results are means ± S.E. mean of 9–10 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. none (Dunnett's test).

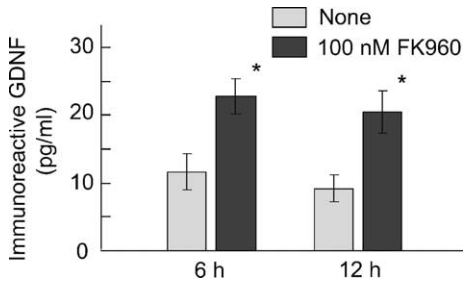

Fig. 3. Effect of FK960 on GDNF release from cultured astrocytes. Cells in six-well plates were incubated in 1 ml of serum-free MEM with (closed column) or without (open column) 100 nM FK960 for 6 and 12 h. Then, concentrations of immunoreactive GDNF in the medium were measured by an ELISA kit. Results are means ± S.E. mean of six experiments. $^{*}P < 0.05$ vs. none (Student's *t*-test).

(Fig. 4B). FK960 did not increase the phosphorylated forms of p38 and JNK in cultured astrocytes (Fig. 4A and B). The phosphorylation of ERK1/2 by FK960 was inhibited by the MAP/ERK kinase inhibitor PD98059 (10 μM) ($IC_{50} = 2$–7 μM for MAP/ERK kinase [26]) (Fig. 4C). Treatment with 100 nM FK960 caused a transient increase in the levels of c-Fos, a subunit of transcription factor AP-1, where the maximum effect was shown at 1–3 h and the c-Fos level returned to the basal level at 6 h (Fig. 5A). The FK960-induced expression of c-Fos was inhibited by cycloheximide (1 μg/ml, a protein synthesis inhibitor) and PD98059 (10 μM), but not by the PKA inhibitor H-89 (1 μM) ($IC_{50} = 0.05$ μM for PKA, 0.5 μM for protein kinase G, 31 μM for protein kinase C, 30 μM for Ca/calmodulin kinase [27]) (Fig. 5B and C). FK960 increased the levels of phosphorylated form of CREB, which is required for activation of this transcription

Table 1
Effects of some receptor antagonists on the FK960-induced increase in GDNF mRNA in cultured astrocytes

	GDNF mRNA (a.u.)
None	0.179 ± 0.033 (21)
FK960 (100 nM)	$0.732 \pm 0.079^{***}$ (21)
FK960 + MK-801 (1 μM)	$0.655 \pm 0.240^{**}$ (3)
FK960 + CNQX (1 μM)	$0.910 \pm 0.089^{***}$ (3)
FK960 + SCH23390 (10 nM)	$0.656 \pm 0.183^{**}$ (6)
FK960 + haloperidol (100 nM)	$0.607 \pm 0.064^{***}$ (6)
FK960 + methyllycaconitine (100 nM)	$0.666 \pm 0.204^{**}$ (6)
FK960 + α-bungarotoxin (100 nM)	$0.689 \pm 0.170^{**}$ (6)

Cultured astrocytes were treated with 100 nM FK960 for 6 h. The receptor antagonists were included in the medium 30 min before addition of 100 nM FK960. The expression levels of GDNF mRNA were normalized by those of β-actin mRNA. Results are means ± S.E. mean and the numbers of experiments are in the parentheses.

$^{**}P < 0.01$ vs. none (Student's *t*-test).

$^{***}P < 0.001$ vs. none (Student's *t*-test).

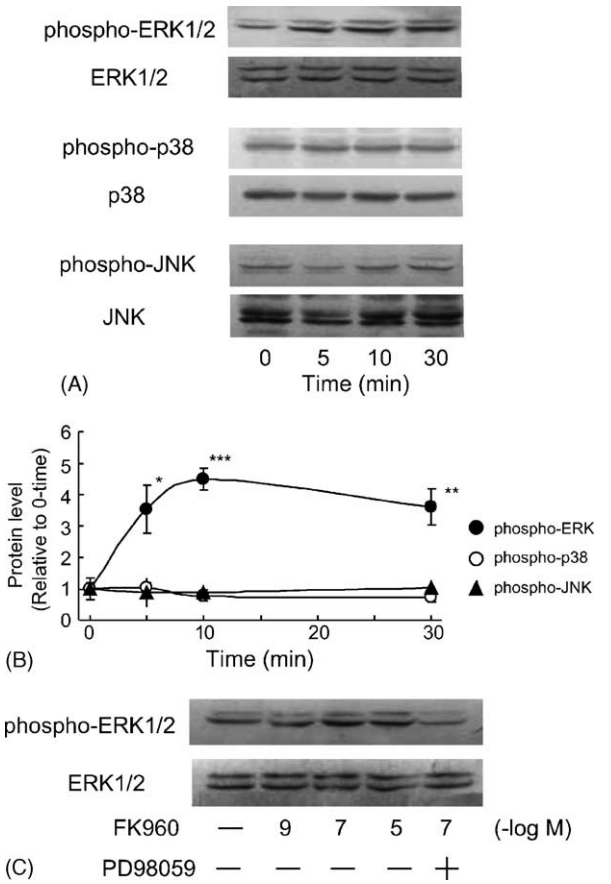

Fig. 4. Effect of FK960 on the phosphorylation of ERK1/2, p38 and JNK. (A) Astrocytes were treated with 100 nM FK960 for the time indicated. Cell lysate was applied to SDS-PAGE followed by immunoblot analysis with antibody against the phosphorylated form of ERK1/2, p38 or JNK (top blots). Subsequently, the blots were re-probed with anti-ERK1/2, p38 and JNK antibodies to confirm that equal amount of proteins were loaded in each lane (bottom blots). (B) Densitometric determination of immunoreactive bands for the phosphorylated forms of ERK1/2, p38 and JNK were carried out as described in Methods. Results are means ± S.E. mean of five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ vs. non-treated cells (Dunnett's test). (C) Effects of PD98059 on the FK960-induced phosphorylation of ERK. Before treatment with FK960, cultured astrocytes were pre-incubated with 10 μM PD98059 for 20 min. FK960 (100 nM) was added to the assay medium and cells were further treated for 10 min. The result is a representative of three experiments.

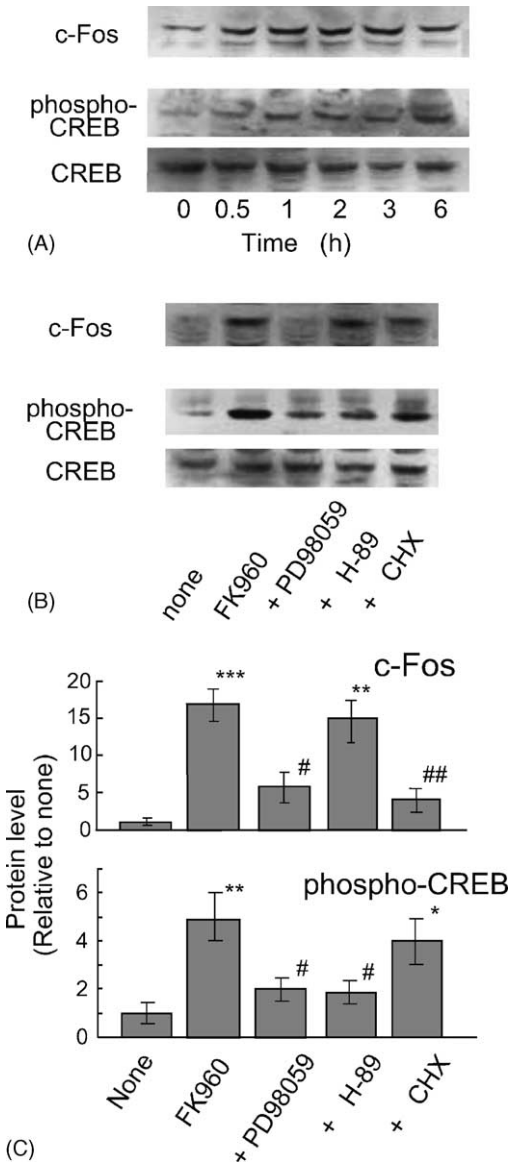

Fig. 5. Effect of FK960 on the levels of c-Fos, phosphorylated-CREB and total-CREB. (A) Astrocytes were treated with 100 nM FK960 for 0.5–6 h. Cell lysate was applied to SDS-PAGE followed by immunoblot analysis with antibodies against c-Fos, phosphorylated CREB and CREB. The result is a representative of four experiments. (B) Effects of PD98059, H-89 and cycloheximide (CHX) on the c-Fos expression and CREB phosphorylation by FK960. PD98056 (10 μM), H-89 (1 μM) and cycloheximide (1 μg/ml) were included in the assay medium 30 min before addition of 100 nM FK960. Then, astrocytes were incubated for 2 h. The result is a representative of four experiments. (C) Quantification of c-Fos and phosphorylated CREB proteins. Densities of immunoreactive bands for c-Fos and phospho-CREB were determined as described in Section 2. Results are means ± S.E.M. of 4 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. none, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. FK960 (Student's *t*-test).

factor, without affecting total levels of CREB protein (Fig. 5A). The phoshorylation of CREB by FK960 was inhibited by PD98059 and H-89 (Fig. 5B and C).

Cycloheximide (1 μg/ml) and actinomycin D, transcription inhibitors, completely prevented FK960-induced increase in astrocytic GDNF mRNA levels (Table 2). The effect of FK960 was partially inhibited by PD98059 at 10 μM: further inhibition was not observed even at 100 μM (data not shown). The p38 inhibitor SB203580 (20 μM), the protein kinase C inhibitor staurosporine (10 μM) and the intracellular Ca^{2+} chelator BAPTA/AM (10 μM) had no effects on FK960-induced increase in GDNF mRNA. The FK960-induced astrocytic GDNF expression was partially inhibited by H-89 at 1 μM: further inhibition was not observed even at 50 μM. In contrast, KT5720 (2 μM), a specific inhibitor of PKA ($K_i = 50$ nM for PKA [28]), did not affect FK960-induced increase in GDNF mRNA. Dexamethason (1 μM) and pyrrolidine dithiocarbamate (100 μM) ($IC_{50} = 10$–100 μM for NF-κB-DNA complex formation [29]) did not affect the increase in GDNF expression in cultured astrocytes.

Table 2
Effects of various signal transduction inhibitors on the FK960-induced increase in GDNF mRNA in cultured astrocytes

	GDNF mRNA (a.u.)
None	0.171 ± 0.024 (36)
FK960 (100 nM)	0.836 ± 0.048*** (36)
FK960 + actinomycin D (10 μg/ml)	0.151 ± 0.041[a] (8)
FK960 + cycloheximide (1 μg/ml)	0.111 ± 0.055[a] (8)
FK960 + PD98059 (10 μM)	0.402 ± 0.090*,[b] (8)
FK960 + SB203580 (20 μM)	0.840 ± 0.114*** (8)
FK960 + BAPTA/AM (10 μM)	0.733 ± 0.093*** (8)
FK960 + staurosporine (10 μM)	0.708 ± 0.099*** (8)
FK960 + H-89 (1 μM)	0.460 ± 0.093**,[b] (8)
FK960 + KT5720 (2 μM)	0.905 ± 0.056*** (6)
FK960 + curcumin (1 μM)	0.306 ± 0.113[a] (8)
FK960 + dexamethason (1 μM)	0.698 ± 0.130*** (8)
FK960 + pyrrolidine dithiocarbamate (100 μM)	0.789 ± 0.133*** (8)

Cultured astrocytes were treated with 100 nM FK960 for 6 h. The inhibitors, except for dexamethason, were included in the medium 30 min before addition of 100 nM FK960. Dexamethason was included in the medium 24 h before addition of FK960. The expression levels of GDNF mRNA were normalized by those of β-actin mRNA. Results are means ± S.E. mean and the numbers of experiments are in the parentheses.

* $P < 0.05$ vs. none.

** $P < 0.01$ vs. none.

*** $P < 0.001$ vs. none.

[a] $P < 0.001$ vs. 100 nM FK960 (Student's t-test).

[b] $P < 0.01$ vs. 100 nM FK960 (Student's t-test).

Curcumin (1 μM) (IC_{50} = 6.9 nM for AP-1/DNA complex formation [30]) inhibited partially the FK960-induced GDNF mRNA expression. We confirmed that the inhibitors at the concentrations used here had no effect on the basal expression levels of astrocytic GDNF mRNA (data not shown).

4. Discussion

In this study, we found that FK960 stimulated selectively the expression of GDNF mRNA in cultured rat astrocytes and this effect was accompanied by an increase in GDNF production. Previous studies showed that the pharmacological effect of FK960 might be mediated by somatostatin [1,3,5] or nicotinic acetylcholine receptors [6]. In addition, GDNF production is modulated by activation of glutamate and dopamine receptors in cultured astrocytes [24,25]. In the circumstances, we first examined whether these receptors are involved in the effect of FK960 on GDNF mRNA in cultured astrocytes, although the drug has been shown no appreciable affinities for these receptors. The present study shows that FK960-induced increase in GDNF mRNA expression is not blocked by antagonists of nicotinic acetylcholine, glutamate and dopamine receptors. These observations suggest that the effect of FK960 on GDNF mRNA is not mediated by the specific receptors in astrocytes. Although somatostatin receptors are present in astrocytes [31], it is also unlikely that the somatostatin system is involved in FK960-induced increase in GDNF mRNA. We observed that the somatostatin synthesis inhibitor cysteamine at 100 μM did not affect the FK960-induced increase in GDNF mRNA (data not shown). In addition, somatostatin and its mRNA are not detectable in cortical astrocytes [32]. Thus, the mechanism by which FK960 increases GDNF mRNA in astrocytes appears to be different from those pointed out previously.

The present study examined the intracellular signals that are responsible for FK960-induced increase in GDNF mRNA in cultured astrocytes. FK960 increased phosphorylation of ERK in cultured astrocytes. The effect of FK960 on MAP kinase family was specific for ERK: the drug did not affect phosphorylation of p38 and JNK. Furthermore, FK960 increased c-Fos protein and phosphorylation of CREB, and these effects were inhibited by the MAP/ERK kinase inhibitor PD98059. These observations suggest that FK960 increases c-Fos expression and CREB phosphorylation through activation of ERK1/2. The role of ERK signal pathway in c-fos expression and CREB phosphorylation was also observed in endothelin B receptor signaling in cultured astrocytes [33]. We also found that H-89, a PKA inhibitor, inhibited FK960-induced increase in GDNF mRNA and phosphorylation of CREB in cultured astrocytes. However, we found that KT5720, other PKA inhibitor, did not affect FK960-induced increase in CREB phosphorylation and FK960 did not affect cAMP production in cultured astrocytes (data not shown). These results suggest that H-89 inhibits CREB phosphorylation in a PKA-independent mechanism in cultured astrocytes. In contrast, the recent studies show that the inhibitory effect of H-89 on ERK is dependent on cAMP or PKA [34,35]. Then, it was unlikely that H-89 inhibited CREB phosphorylation via ERK signal, although we did not examine the effect of FK960 on ERK.

The present study shows the transcriptional regulation of FK960-induced increase in GDNF mRNA in cultured astrocytes. FK960-induced increase in GDNF mRNA was inhibited by actinomycin D, cycloheximide, PD98059, H-89 and curcumin. The inhibitions by actinomycin D and cycloheximide were complete, but those of others were partial. PD98059 inhibited both the c-Fos expression and CREB phosphorylation. Cycloheximide inhibited the c-Fos expression but not CREB phosphorylation and H-89 inhibited the CREB phosphorylation but not c-Fos expression. These observations suggest that FK960-induced increase in GDNF mRNA is mediated by activation of c-Fos and CREB and the ERK signal plays a key role in both the c-Fos expression and CREB phosphorylation. In agreement with this idea, endothelin-1 that causes c-Fos expression and CREB phosphorylation increases GDNF levels and the effect is inhibited by PD98059 [36]. There is evidence that NF-κB can regulate expression [37,38]. But, we observed that dexamethason and pyrrolidine dithiocarbamate, which inhibited the NF-κB-mediated increase in astrocytic GDNF expression [36], did not affect FK960-induced increase in GDNF mRNA. It

is unlikely that NF-κB plays a role in the effect of FK960 on GDNF expression.

GDNF was initially isolated as a potent neurotrophic factor for cultured dopaminergic neurons from the developing substantial nigra [39]. This neurotrophic factor protects neurons in forebrain ischemia of animal models and promotes survival and axonal re-generation of a wide variety of neuronal populations in nerve injury models [12,40–43]. With respect to cognitive function, exogenous GDNF enhances synaptic efficiency and transmitter release [44,45] and the study using GDNF heterogymous mutant mice shows that endogenous GDNF plays an important role in cognitive abilities [46]. Furthermore, astrocytic GDNF production has been proposed to explain neuroprotective effects of deprenyl [47], melatonin [47], apomorphine [48], Vitamin D_3 [49] and riluzol [50] and the therapeutic effect of antidepressants [51]. In this study, we observed that FK960 increased GDNF levels in cultured astrocytes. The levels (20 pg/ml medium or 25–60 pg/mg protein of astrocytes) are similar to those in the previous studies [47–50] and corresponding to those in rat brain (data not shown). Then, it is likely that GDNF may be involved in the cognitive-enhancing effect of the drug, although it is not known whether the increased levels are enough to alter synaptic transmission.

In conclusion, we have found that FK960 selectively increases GDNF mRNA in cultured astrocytes. Although the exact mechanism for the selective increase in GDNF mRNA is not known, the present study suggests that the increase in GDNF mRNA is mediated by c-Fos and CREB, which are regulated by an ERK signal. It is possible that FK960, like T-588 [15], directly or indirectly interacts with intracellular signal proteins to stimulate ERK signal. Further studies are required to clarify how FK960 affects the ERK signal in astrocytes.

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