Basic Fibroblast Growth Factor Evokes a Rapid Glutamate Release through Activation of the MAPK Pathway in Cultured Cortical Neurons

We examined the possibility that basic fibroblast growth factor (bFGF) is involved in synaptic transmissions. We found that bFGF rapidly induced the release of glutamate and an increase in the intracellular Ca\(^{2+}\) concentration through voltage-dependent Ca\(^{2+}\) channels in cultured neuronal cells. bFGF also evoked a significant influx of Na\(^{+}\). Tetanus toxin inhibited the bFGF-induced glutamate release, revealing that bFGF triggered exocytosis. The mitogen-activated protein kinase (MAPK) pathway was required for these acute effects of bFGF. We also found that pretreatment with bFGF significantly enhanced high K\(^{+}\)-elicited glutamate release also in a MAPK activation-dependent manner. Therefore, we propose that bFGF exerts promoting effects on excitatory neuronal transmission via activation of the MAPK pathway.

Neuronal transmissions require mechanisms that modulate the balanced interaction of multiple factors, which control functional maintenance and plasticity in the central nervous system. Many factors including growth factors and the excitatory amino acid glutamate control neuronal functions. In particular, brain-derived neurotrophic factor (BDNF), one of the neurotrophins) plays a fundamental role in neuronal transmission and plasticity (1–7). We also reported that rapid glutamate release was induced by BDNF through the glutamate transporter in cultured cerebellar and cortical neurons (8, 9). However, very little work has been done concerning the roles of other neurotrophic factors in the acute modulation of central nervous system and peripheral nervous system functions (10, 11).

Initially, bFGF was identified as an angiogenic mitogen, but it is now recognized as a neurotrophic factor. For example, it has been found that bFGF exerts neurotrophic activities in cortical and hippocampal neurons (12, 13). However, the expression of bFGF in the central nervous system increases during postnatal development (14), implying that bFGF also has important roles in synaptic maturation. Indeed, bFGF modulates the efficacy of hippocampal synaptic transmission. bFGF enhances the generation of long term potentiation in hippocampal neurons (15, 16). These findings suggest that bFGF is involved in glutamatergic transmission, because it is widely accepted that the release of glutamate (presynaptic), as well as the NMDA and the AMPA receptor (postsynaptic), is essential for long term potentiation (17, 18). Several reports have indicated that these ionotropic glutamate receptor activities were regulated by bFGF. bFGF regulates the intracellular Ca\(^{2+}\) concentration after activation of the NMDA receptor (19). Long term treatment with bFGF decreases the NMDA receptor-dependent increase in the Ca\(^{2+}\) concentration, and the Ca\(^{2+}\) attenuation is required for the neuroprotective effects in excitotoxic cell death (20, 21). The expression of the glutamate receptor protein is also changed by bFGF. bFGF increased the AMPA receptor subunit GluR1 protein but did not alter levels of GluR2/3, GluR4, or the NMDA subunit NR1 (22). The expression of NMDA receptor protein and calbindin D28 (Ca\(^{2+}\)-binding protein) was down-regulated by bFGF (23). These results raise the possibility that chronic bFGF treatment regulates not only neuronal survival but also postsynaptic plasticity in glutamatergic neurons. However, it is not known what kind of acute effects bFGF exerts on glutamatergic transmission and how bFGF stimulates neuronal transmission.

In the present study, we found that bFGF induced glutamate release in cultured cortical neurons. It was revealed that bFGF triggers the release through the exocytotic system in an extracellular Ca\(^{2+}\)- and MAPK activation-dependent manner. We propose that not only neurotrophins but also bFGF exerts an acute effect on the excitability of glutamatergic neurons.

EXPERIMENTAL PROCEDURES

Cell Preparation—Primary dissociated cortical cultures were prepared from the cerebral cortex of 2- or 3-day-old rats (SLC, Sizuoka, Japan) and maintained on poly-L-lysine-coated plastic dishes in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Neurons were cultured in a mixture of FBS, horse serum, and bovine chondroitin sulfate to promote neuronal differentiation (24). The medium was changed every 2–3 days. The neurons were used for experiments when they had differentiated into large neurons with dark somata and long neurites. The neurons were stimulated by bFGF (25). The bFGF was dissolved in MEM and added to the culture medium. The final concentration of bFGF was 100 ng/ml.

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‡ The abbreviations used are: bFGF, basic fibroblast growth factor; t-PDC, t-trans-pyridylidino-2,4-dicarboxylic acid; PDBA, P2-threo-β-benzoxazolylaspartate; MAPK, mitogen-activated protein kinase; MAP, mitogen-activated protein; PLC-γ, phospholipase C-γ; IP\(_3\), inositol 1,4,5-trisphosphate; PI 3-kinase, phosphatidylinositols 3-kinase; NMDA, N-methyl-D-aspartic acid; DAB, 3,3′-diaminobenzidine; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; TTX, tetrodotoxin; SFBI-AM, 1,3-benzenedicarboxylic acid, 4,4′-[(1,4,10-trioxa-7,13-diaza-cyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyi)]bisbetaraki[(acetyl oxy)methyl]ester; GABA, γ-aminobutyric acid; TeTx, teta-nustoxin; MEF, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FMI-43, N'-β-threonylcarboxamidemethyl)aminoacryloyl)benzoylarginine; 5-MeANP, α-amino-3-hydroxy-5-methylisoxazolepropionate; APV, amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

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bFGF-induced Rapid Glutamate Release

Fig. 1. bFGF rapidly induced glutamate release in cultured cortical neurons. Cultured cortical neurons were prepared from 2- to 3-day-old rats and cultured for 6–9 days. A, time course analysis of the acute effect induced by bFGF. Panel a, the bFGF-induced release was maintained for 1–2 min and then there was a return to the basal level. The neurons were treated with bFGF at 100 ng/ml by bath application. Panel b, BDNF-induced release. BDNF was applied to cultures at 100 ng/ml. Panel c, release of glutamate evoked by high K⁺ (HK⁺) assay buffer containing 50 mM KCl. B, bFGF-induced glutamate release was dose-dependent. The effect of BDNF (100 ng/ml) was also examined (gray bar). The amounts of glutamate released by bFGF (100 ng/ml) and BDNF (100 ng/ml) were similar in sister cultures. Vehicle (final 2 ng/ml BSA) had no effect. Before exposing the culture to the two growth factors (1 min), basal fractions (1 min) were collected. Data represent the mean ± S.D. (n = 6). Statistical analysis was performed with Student’s t test. **, p < 0.01 versus basal. C, no GABA was released on stimulation with bFGF (100 ng/ml). In contrast, HK⁺ evoked the release of both glutamate and GABA. Data represent the mean ± S.D. (n = 6). Statistical analysis was performed using Student’s t test. **, p < 0.01 versus basal.

Japan) as reported previously (24, 25). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 units/ml, Worthington) at 37 °C. The dissociated cells were plated at a final density of 4 or 5 × 10⁶/cm² on polyethyleneimine-coated 12- and 24-well plates (4- and 2-cm² surface area/well, respectively; Corning). The culture medium consisted of 5% precolostrum newborn calf serum, 5% heated-inactivated horse serum, 1% rat serum, and 89% of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 15 mM HEPES buffer, pH 7.4, 0.01% Na₂SeO₃, and 1.9 mg/ml NaHCO₃. After maintenance for 6–8 days, the analysis was performed. Hippocampal and cerebellar cells were prepared from 2- to 3-day-old hippocampus and 5-day-old cerebellum, respectively. The procedures for these cultures were the same as for the cortical culture. These cultures were maintained for 9–12 days.

Immunocytochemistry—Cells were double stained with anti-MAP2 (rabbit IgG; a gift from Dr. H. Murofushi, The University of Tokyo) and anti-c-Fos antibody (rabbit IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). First, cultured cells were fixed in 4% paraformaldehyde containing 0.05% Triton X-100 at room temperature (25 °C) for 20 min and then incubated overnight with anti-MAP2 (1:5000). Secondary antibodies were applied at room temperature for 1 h. To visualize the staining, aVectastain ABC kit (Vector Laboratories) together with 0.02% (w/v) 3,3’-diaminobenzidine (DAB) dissolved in 0.05 M Tris- HCl buffer, pH 7.6, containing 0.01% (w/v) H₂O₂ was used. Next, cells were incubated with the anti-c-Fos (1:1000) antibody and visualized using DAB together with 0.1% (w/v) (NH₄)₂S·H₂O.

Detection of Amino Acid Neurotransmitters—The amounts of amino acids released from the cultured neurons were measured as described previously (25). Briefly, the amounts released into the assay buffer (modified HEPES-buffered Krebs Ringer solution: KRH containing 130 mM NaCl, 5 mM KCl, 1.2 mM Na₂HPO₄, 1.8 mM CaCl₂, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4) were measured by HPLC (Shimazu, Kyoto, Japan) with a fluorescence detector (Shimazu). The high K⁺ (HK⁺) solution consisted of 85 mM NaCl, 50 mM KCl, 1.2 mM Na₂HPO₄, 1.8 mM CaCl₂, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4. The Ca²⁺-free solution was prepared by omitting the CaCl₂. Sample fractions were collected every 1 min into tubes on ice by the batch method. They were then filtered with 0.22-μm membranes to remove cell debris. Samples were treated with o-pthalaldehyde and 2-mercaptoethanol for 5 min at 37 °C before being injected into the HPLC system and analyzed using a fluorescence monitor (excitation wavelength, 340 nm; emission wavelength, 445 nm). bFGF and BDNF dissolved in phosphate-buffered saline containing bovine serum albumin (BSA, 1 mg/ml) were added to cultured neurons by bath application. PD98059 (Calbiochem) and U0126 (Promega, Madison, WI), MAPK pathway inhibitors, were applied for 40 min at 50 and 10 μM, respectively. LY294002 (Calbiochem), a PI 3-kinase inhibitor, was applied for 30 min at 30 μM. Nifedipine (Research Biochemicals International, Natick, MA) and tetrodotoxin (TTX, as Itoxan, Valence, France) were applied at 10 and 0.5 μM, respectively. Thapsigargin (1 μM) (Research Biochemicals) was added 30 min prior to the application of bFGF. Tetanus toxin (10 μg, List Biological Laboratories, Inc., Campbell, CA) was applied to the culture for 8–12 h before bFGF. t-Trans-2,4-PDC (t-PDC) (Research Biochemicals) and ni-threo-j-benzyloxyaspartate (nTBOA) (a gift from Suntory Institute for Bioorganic Research, Osaka, Japan) were applied at 10 μM. The glutamate release experiments were performed at least four times with independent cultures to confirm reproducibility. Representative data are shown in the figures.

Ca²⁺, Na⁺, and FM1-43 Imaging—Cells were cultured on polyethyl-
**RESULTS**

**bFGF-induced Glutamate Release in Cultured Cortical Neurons**—To determine the acute effect of bFGF on presynaptic function, we examined the bFGF-induced glutamate release in cultured cortical neurons. The time course of the release is shown in Fig. 1A. The release of glutamate was observed im-
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Fig. 3. bFGF induced glutamate release through an extracellular Ca\(^{2+}\) and exocytosis. A, Cd\(^{2+}\) (100 \(\mu\)M), nifedipin (10 \(\mu\)M), and TTX (0.5 \(\mu\)M) abolished the bFGF-induced release. Data represent the mean ± S.D. (n = 6). **, p < 0.01 versus basal (Student’s t test). B, bFGF induced glutamate release through an exocytotic system. bFGF still induced glutamate release in the presence of t-PDC (10 \(\mu\)M) or DL-TBOA (10 \(\mu\)M). TTX (10 \(\mu\)M) treatment for 8 h completely blocked the bFGF-induced release. Data represent the mean ± S.D. (n = 6). **, p < 0.01 versus basal (Student’s t test). C, hippocampal and cerebellar neurons responded to bFGF. Panel a, bFGF-induced Ca\(^{2+}\) increases in cultured hippocampal (HIP) and cerebellar (CBL) neurons were observed. The tracings indicate three cells, which were selected in the same well of a plate. Panel b, bFGF-induced acute glutamate release was also confirmed in cultured hippocampal (HIP) and cerebellar (CBL) neurons. Data represent the mean ± S.D. (n = 6). **, p < 0.01 versus basal (Student’s t test).

Immediately after the application of bFGF at 100 ng/ml and was maintained for 1–2 min (panel a in Fig. 1A). Neurotrophin BDNF-induced release in a sister culture is also shown (panel b in Fig. 1A). The time course analysis indicated that the bFGF-induced release was similar to the BDNF-induced one in time lapse. HK\(^{-}\) (50 mM KCl)-induced depolarization also evoked the release of glutamate (panel c in Fig. 1A). Compared with the HK\(^{-}\)-evoked release, the bFGF- and BDNF-induced releases tended to be sustained, while the former achieved a much higher level (4–10-fold the growth factor-induced increases). The dose dependence of the bFGF-induced effect was examined (Fig. 1B). The release of glutamate began at 3 ng/ml and reached a plateau at 10–100 ng/ml. The maximum release was about 250–350% of the basal level in a series of independent cultures. Vehicle solution (BSA, 2 ng/ml) did not have an effect. The maximum levels of bFGF- and BDNF-induced release were almost the same at 100 ng/ml. These results indicated that not only BDNF but also bFGF induces an acute and transient release of glutamate in cultured cortical neurons.

Next, we examined whether bFGF stimulates the release of other amino acid neurotransmitters. We found that the effect of bFGF was restricted to glutamate, which is an excitatory neurotransmitter (Fig. 1C). GABA, an inhibitory neurotransmitter, was not induced. Releases of glycine and taurine were not induced by bFGF either (data not shown). HK\(^{-}\), however, evoked the release of not only glutamate but also GABA. These results indicated that bFGF specifically influenced an excitatory amino acid transmitter in cultured cortical neurons.

**bFGF Induced an Increase in the Intracellular Ca\(^{2+}\) Concentration.**—To elucidate the mechanism of the bFGF-induced glutamate release, we focused on the intracellular Ca\(^{2+}\) accumulation induced by bFGF, because the mobilization of intracellular Ca\(^{2+}\) is believed to be an important step in neurotransmitter release. bFGF induced acute increases in the intracellular Ca\(^{2+}\) concentration in cortical neurons (Fig. 2). A marked increase was elicited immediately and followed by continuous oscillations (panel a in Fig. 2B). Some 71–85% of cells in cultures responded to bFGF. The Ca\(^{2+}\) increase was observed in the cell body and neurites (Fig. 2A). A similar response was observed on application of BDNF (panel b in Fig. 2B). Approximately 73–82% of cultured cells were BDNF-responsive. Previously, we reported that BDNF induced the release of glutamate in an intracellular Ca\(^{2+}\)-dependent manner and that the increase in Ca\(^{2+}\) was mediated by the IP\(_3\) receptor (which is known to exist on intracellular Ca\(^{2+}\) stores) rather than by an influx of extracellular Ca\(^{2+}\) (8, 9). Therefore, we next examined the influence of thapsigargin (which induces depletion of Ca\(^{2+}\) from intracellular stores at 10 \(\mu\)M) on the bFGF-stimulated accumulation of Ca\(^{2+}\). Interestingly, although the BDNF-induced Ca\(^{2+}\) increase was inhibited by thapsigargin, the bFGF-induced increase was not affected (panels c and d in Fig. 2B). Under extracellular Ca\(^{2+}\)-free conditions, no Ca\(^{2+}\) increase was induced by bFGF, but it was still observed in response to BDNF (panels e and f in Fig. 2B). Cd\(^{2+}\) (voltage-dependent Ca\(^{2+}\) channel blocker, 100 \(\mu\)M) and TTX (Na\(^{+}\) channel blocker, 0.5 \(\mu\)M) also blocked the bFGF-induced Ca\(^{2+}\) increase (Fig. 2C), indicating that the increase occurred through voltage-dependent Ca\(^{2+}\) channels. On the other hand, Cd\(^{2+}\) and TTX did not inhibit the BDNF-stimulated Ca\(^{2+}\) increase (data not shown). Previously, we showed that the intracellular Ca\(^{2+}\) concentration was either steady or displayed spontaneous synchronized oscillations (9). We had identified the growth factor-triggered or spontaneous synchronized oscillations as a synaptic transmission mediated by glutamate. Here we confirmed that the bFGF-induced Ca\(^{2+}\) increases (the first Ca\(^{2+}\) transient) were not affected by glutamate receptor antagonists, APV (10 \(\mu\)M) or CNQX (10 \(\mu\)M), but the subsequent oscillatory Ca\(^{2+}\) activities (synchronized oscillations after the first Ca\(^{2+}\) transient) were abolished. Furthermore, the bFGF-induced increase in the number of c-Fos (one of the immediate early genes)-positive neurons was examined by immunohistochemistry with anti-c-Fos antibody. Both bFGF and BDNF potentiated the number of neurons showing expression of c-Fos (control BSA, 7.93 ± 3.13%; bFGF, 70.5 ± 5.03%; BDNF, 51.55 ± 5.09%, percentages (c-fos/MAP2) of double positive cells, treatments with these factors were performed...
for 20 min). The induction of c-Fos expression by both these factors was not changed by APV or CNQX, indicating that the induction is triggered directly by bFGF and not through secondary effects such as stimulation by the released glutamate. Therefore, we performed Ca\(^{2+}\)/H\(_{11001}\) imaging using resting cortical cultures, because the first rapid Ca\(^{2+}\)/H\(_{11001}\) increase induced by growth factors was easy to clarify.

**bFGF Induced Glutamate Release via an Exocytosis Mechanism**—To test whether the bFGF-induced glutamate release is controlled by an exocytotic system, the Ca\(^{2+}\)/H\(_{11001}\) dependence of the release and the involvement of exocytosis were examined (Fig. 3). Under extracellular Ca\(^{2+}\)/H\(_{11001}\)-free conditions, bFGF was not able to induce the release of glutamate (Fig. 3A). Cd\(^{2+}\) also abolished the bFGF-induced release. In the presence of nifedipine, an L-type Ca\(^{2+}\) channel blocker, and TTX, which are expected to block Ca\(^{2+}\) influx through the voltage-dependent Ca\(^{2+}\) channels, the bFGF-induced release was abolished, suggesting that the bFGF-induced glutamate release was dependent on Ca\(^{2+}\) influx through the voltage-dependent Ca\(^{2+}\) channels.

To further identify the mechanism behind the bFGF-induced release, we tested the effect of tetanustoxin (TeTx, which inhibits synaptic vesicle-dependent release). Pretreatment with TeTx for 8–12 h before the application of bFGF completely inhibited the release of glutamate. Furthermore, the influence of glutamate transporter inhibitors was examined. In the presence of t-PDC (10\(\mu\)M) and DL-TBOA (10\(\mu\)M), the bFGF-induced release was not affected (Fig. 3B), suggesting that it occurred through exocytosis. Furthermore, we examined the acute effects of bFGF on the release of glutamate and accumulation of intracellular Ca\(^{2+}\) in cultured hippocampal and cerebellar neurons. These cultured neurons also responded to bFGF (Fig. 3C), suggesting that bFGF exerted acute effects on central nervous system glutamatergic neurons. By contrast, cultured astroglial cells (95–99% pure judging from immunocytochemistry) exhibited no response to bFGF (glutamate release: basal, 24.5 \(\pm\) 3.88; bFGF-induced, 26.6 \(\pm\) 3.52 (\(\times 10^{-10}\) mol/well), \(n = 6\)). All these results suggested that the bFGF induced the release of excitatory transmitters through an extracellular Ca\(^{2+}\)-depend-
ent pathway in central nervous system neurons.

To further characterize the bFGF-induced release and the involvement of the exocytotic assay with the style dye FM1-43 (Fig. 4, A and B). After depolarization-induced FM dye loading, we tested whether bFGF triggers a reduction in the fluorescent intensity of FM. As expected, HK and bFGF reduced the intensity (panels a and b in Fig. 4B). However, the destaining effect of BDNF tended to be weaker than that of bFGF (panels c and d in Fig. 4B). Vehicle (solution containing BSA) had no effect. Although HK also attenuated the intensity of fluorescence, the extent of the destaining by HK was less than expected. However, because the number of FM fluorescent buttons, which responded to HK, seemed to be much larger than that which responded to bFGF, the HK-evoked glutamate release was thought to be more extensive. The reduction in FM dye by bFGF meant that bFGF likely evoked the depolarization. Thus, we performed an Na imaging assay using a Na indicator SBFI-AM in cultured cortical neurons. Acute application of bFGF induced a significant influx of Na, suggesting that bFGF acutely evoked cell depolarization (panels a and c in Fig. 4C). BDNF induced an accumulation of Na as well (panel c in Fig. 4C), although the extent of the influx was less than that caused by bFGF. Furthermore, we confirmed that HK also induced an influx of Na, which was 2–3-fold greater than the bFGF-induced one.

MAPK Signal Transduction Was Required for the bFGF-Induced Glutamate Release—What kind of intracellular signal pathways are involved in the bFGF-induced glutamate release? First, the effect of genistein, a broad tyrosine kinase inhibitor, on the bFGF-induced release was tested. bFGF did not induce glutamate release after treatment with genistein (1 μM) in cultured cortical neurons (data not shown). Next, we examined the effect of an inhibitor of the MAPK pathway on the bFGF-induced release. MEK (an upstream regulator of MAPK) was required for the survival-promoting effects of bFGF on cultured cortical and hippocampal neurons (27). U0126 and PD98059, inhibitors of MEK, completely blocked the bFGF-induced glutamate release (Fig. 5A). On the other hand, BDNF still induced the release in the presence of these MEK inhibitors as reported previously (8). Furthermore, to confirm that bFGF and BDNF stimulated the activation of MAPK in the cultured cortical neurons, we performed Western blotting using anti-phospho-MAPK antibody. bFGF and BDNF induced phosphorylation of MAPK, although the level of phosphorylation was somewhat lower with bFGF. U0126 and PD98059 markedly attenuated the bFGF-stimulated phosphorylation of MAPK, although the inhibitory effect of U0126 was stronger than that of PD98059. The BDNF-induced phosphorylation of MAPK was also completely blocked by U0126. These results indicated that bFGF and BDNF similarly activate MAPK in cultured cortical neurons. It was therefore revealed that activation of the MAPK pathway is involved in the bFGF-induced, but not BDNF-induced, release of glutamate.

bFGF Potentiated HK-evoked Glutamate Release through the Activation of MAP Kinase Pathway—Here, we found that bFGF activates MAPK and that the activation is essential for the bFGF-induced glutamate release. As shown in Fig. 6A, interestingly, the phosphorylation level of MAPK gradually increased for at least 20 min after the application of bFGF. The phosphorylation of MAPK began to be observed at 3 min after bFGF exposure. The intensity of phosphorylation then increased, reaching a plateau at 20–40 min. Because the bFGF-induced glutamate release occurred within 1–2 min, the weak but significant MAPK phosphorylation at the early phase (within 3 min) may lead to the glutamate release. On the other hand, there is the possibility that MAPK activation at a later phase (20–40 min after bFGF stimulation) is also involved in the modulation of synaptic transmission. Thus, in addition to the acute effects of bFGF, we examined the effect of pretreatment with bFGF on the HK-evoked release. Time course analysis showed that treatment with bFGF for 20–60 min enhanced the HK-evoked release (Fig. 6, B and C), and vehicle (BSA solution) had no effect (Fig. 6B), suggesting that pretreatment with bFGF potentiated the activity-dependent release. The effects of inhibitors of the MAPK pathway were tested next. Both U0126 and PD98059 (data not shown) completely abolished the bFGF-enhanced release. There are several reports that PI 3-kinase is also activated by bFGF (28, 29). However, a PI 3-kinase inhibitor (LY294002, 30 μM) had no effect on the HK-evoked release with or without bFGF treatment (Fig. 6C), excluding the involvement of PI 3-kinase-mediated intracellular signaling systems. These results indicated that the later phase phosphorylation of MAPK is required for the bFGF-potentiated glutamate release.

**DISCUSSION**

Growth factors play important roles in the regulation of synaptic transmission in central nervous system neurons. Reports have indicated that BDNF and other neurotrophins trigger and modulate synaptic transmission in the acute phase. However, as concerns the effects of bFGF, very little was known. Here, we report an acute effect of bFGF on neurotransmitter release and the underlying intracellular signaling. We
found that bFGF induced a rapid and transient glutamate release in cultured cortical neurons. Sequential experiments revealed that exocytosis, which requires an influx of Ca^{2+} and the activation of the MAPK pathway, is involved in the bFGF-induced release. Furthermore, we found that pretreatment with bFGF significantly enhanced the high K^{+}-elicited glutamate release in a MAPK activation-dependent manner. These results suggest that bFGF acutely influences excitability in central nervous system neurons through activation of the MAPK pathway.

The present study indicated that bFGF induced the release of glutamate, but not GABA, in cultured cortical neurons. In contrast, GABA as well as glutamate were released by HK^{+}-induced depolarization, suggesting that bFGF specifically acts on glutamatergic neurons to trigger glutamate release. In cultured hippocampal neurons, bFGF modulated GABA-mediated neuronal transmissions (30). However, in our system, there was no change in the amount of GABA released by bFGF, bFGF and BDNF triggered continuous synchronized Ca^{2+} oscillations in cultured cortical neurons (Fig. 2B). We previously reported that synchronized spontaneous Ca^{2+} oscillations were potentiated by acute application of BDNF and that BDNF-induced glutamate release was essential for these potentiated oscillations (9). We had confirmed that cortical cells displaying Ca^{2+} oscillations were glutaminase (a marker for glutamatergic neurons)-positive by immunocytochemistry, suggesting that glutamate, but not GABA-mediated, transmissions were enhanced by these growth factors. Thus, we concluded that bFGF plays a dominant role in stimulating glutamatergic neurons in cortical cultures.

The increase in the intracellular Ca^{2+} concentration seems to be stimulated through different mechanisms among growth factors. Phospholipase C (PLC), which produces IP_{3}, is activated by growth factors, and IP_{3} triggers release of Ca^{2+} from intracellular storage sites (31). Neurotrophins are known to increase the intracellular Ca^{2+} concentration through the release of Ca^{2+} from intracellular stores (32–34). In addition, *Drosophila* TRP proteins and some mammalian homologs (TRPC1–7 proteins) are thought to mediate capacitative Ca^{2+} influx (35, 36) in a membrane potential-independent manner. Interestingly, Li et al. (37) reported that TRPC3 mediates Ca^{2+} and Na^{+} entry downstream of a pathway, including TrkB (BDNF-specific receptor), PLC-γ, and IP_{3} in pontine neurons. We previously showed that BDNF increases the Ca^{2+} concentration in a PLC-γ/IP_{3}-dependent manner and that this increase is necessary for the BDNF-induced glutamate release in cultured cortical and cerebellar neurons (8, 9). On the other hand, pharmacological experiments revealed that the Ca^{2+} influx induced by bFGF was via voltage-dependent Ca^{2+} channels and that bFGF-induced glutamate release was dependent on the Ca^{2+} influx. In contrast, BDNF still induced both the release of glutamate and an increase in Ca^{2+} under conditions in which the influx of extracellular Ca^{2+} was blocked, implying that BDNF and bFGF stimulate Ca^{2+} increases through different mechanisms. These results raise the possibility that bFGF- and BDNF-induced glutamate releases are mediated through different mechanisms. To test this possibility, we examined the contribution of exocytosis to the bFGF-induced release, because a reverse transport system (through a glutamate transporter) was involved in the BDNF-induced release in developing cortical neurons (9). Pretreatment with TTX completely inhibited the bFGF-induced release (Fig. 3), while the transporter inhibitors had no effect. Furthermore, we confirmed that bFGF
reduced the FM1-43 intensity, while BDNF had a weaker effect than bFGF (Fig. 4, A and B). Furthermore, Na+ imaging experiments suggested that bFGF evoked significant cell depolarization in comparison with BDNF. All these results imply that exocytosis was the dominant system in the bFGF-induced glutamate release. The question then arises whether the BDNF-induced effect in our system is mediated through conventional synapses or whether there is spillover of glutamate via synapses or non-exocytotic release, as suggested for some transmitters (38–40). Katsumori et al. (41) has reported that depolarization-evoked release was occurred via reverse transport in hippocampal slices in neurones rather than via exocytosis as in adults. Therefore, in addition to exocytosis, a reverse transport system may be involved in the BDNF-induced glutamate release at this cortical stage. At a more mature stage, BDNF may exert an acute effect via exocytosis.

The glutamate release was induced by bFGF in a dose-dependent manner (Fig. 1), suggesting that bFGF receptor-stimulated intracellular signaling is required. What kind of intracellular signaling is required for the bFGF-induced glutamate release? It is possible that a MAPK pathway is involved. This pathway includes Ras (Raf kinase), MEK (MAP kinase), S6 kinase (p90 ribosomal S6 kinase), and MAPK. For the survival-enhancing effect of bFGF on cultured cortical and hippocampal neurons, MEK was required (27). Recently, Yang et al. (11) indicated that glial cell line-derived neurotrophic factor acutely modulated the excitability in midbrain dopaminergic neurons through MAPK-dependent A-type K⁺ channel inhibition. Therefore, we examined the contribution of the MAPK pathway to the bFGF-induced glutamate release. MEK inhibitors completely inhibited the release (Fig. 5). On the other hand, the BDNF-induced release was not blocked. Both bFGF and BDNF stimulated activation of MAPK in the cultured cortical neurons. These results indicate that the MAPK pathway is involved in the bFGF-induced, but not BDNF-induced, glutamate release even though bFGF and BDNF similarly activate MAPK in cultured cortical neurons (Fig. 6).

There is a question of whether the MAPK pathway is upstream or downstream of the Ca²⁺ influx because the bFGF-dependent glutamate release was mediated by the Ca²⁺ increase. Pende et al. (42) reported that bFGF rapidly induces an extracellular Ca²⁺-independent phosphorylation of MAPK. On the other hand, Ca²⁺ also induces activation of the MAPK pathway (43). We tested the bFGF-induced Ca²⁺ increase in the presence of MEK inhibitors, PD98059 and U0126 partially blocked the increase (ratio values (bFGF-stimulated/basal): none, 262 ± 109%; PD98059, 137 ± 45.2%; U0126, 135 ± 49.9%; n = 12, n indicates the cell numbers selected). Furthermore, we observed the bFGF-stimulated phosphorylation of MAPK under extracellular Ca²⁺-free conditions (data not shown). These results indicate that the MAPK pathway is located upstream of bFGF-stimulated Ca²⁺ influx. Glial cell line-derived neurotrophic factor-modulated excitation of dopaminergic neurons is primarily mediated by MAPK-dependent A-type K⁺ channel inhibition, and this inhibition seemed to result in an increase in Ca²⁺ current (11). In the present study, K⁺ channel inhibition may be functioning as well as in the dopaminergic neurons. We cannot exclude the possibility that MAPK worked downstream of the bFGF-induced Ca²⁺ influx because MEK inhibitors significantly but did not completely inhibit the Ca²⁺ increase. There is adequate evidence that the increase in intracellular Ca²⁺ evoked by depolarization induces the activation of MAPK. For example, glutamate and potassium are effective in activating MAPK in hippocampal neurons (44–47). Therefore, both Ca²⁺-dependent and -independent MAPK activation may be required for the bFGF-induced glutamate release, although further investigation is needed to clarify the mechanisms by which Ca²⁺ and MAPK regulate each other.

Studies have suggested that BDNF potentiates activity-dependent synaptic transmission. For example, BDNF produces a long-lasting (2–3 h) enhancement of field excitatory postsynaptic potential in the hippocampus that requires local protein synthesis (48). BDNF enhances glutamate release, and novel protein synthesis is required for the enhancement by BDNF in cortical neurons (49). We also reported that BDNF enhanced depolarization-evoked glutamate release in a PLC-γ-dependent manner in cultured cortical neurons (50). However, the underlying signaling, which is essential for the activity-dependent synaptic modulation by bFGF, is not known. The time course experiment showed that the rapid bFGF-induced glutamate release finished within 1–2 min of the bFGF stimulation. However, the activation of MAPK was sustained for 20–40 min. Interestingly, the HK⁺-evoked glutamate release was significantly potentiated by bFGF pretreatment, and MAPK was essential for the potentiation. These studies indicate that different signal transductions are important in the late-phase modulation of synaptic efficacy by each growth factor.

Recent studies have suggested that bFGF is a multifunctional growth factor in neuronal cells (51). bFGF and FGF1 (high affinity receptor for bFGF; Ref. 52) are present in the developing and adult brain (14). Astrocytes also synthesized and released bFGF (53). Here we showed a novel function of bFGF in central nervous system neurons at an acute phase. Thus, in developing and adult brain, bFGF, which is derived from neurons or astrocytes, may modulate glutamatergic transmission.

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bFGF-induced Rapid Glutamate Release

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Basic Fibroblast Growth Factor Evokes a Rapid Glutamate Release through Activation of the MAPK Pathway in Cultured Cortical Neurons
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