Differential Activation of Brain-derived Neurotrophic Factor Gene Promoters I and III by Ca\(^{2+}\) Signals Evoked via L-type Voltage-dependent and N-Methyl-d-aspartate Receptor Ca\(^{2+}\) Channels*

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Although the brain-derived neurotrophic factor (BDNF) gene is activated by the intracellular Ca\(^{2+}\) signals evoked via Ca\(^{2+}\) influx into neurons, little is known about how the activation of alternative BDNF gene promoters is controlled by the Ca\(^{2+}\) signals evoked via N-methyl-d-aspartate receptors (NMDA-R) and L-type voltage-dependent Ca\(^{2+}\) channels (L-VDCC). There is a critical range in the membrane depolarization caused by high K\(^+\) concentrations (25–50 mM KCl) for effective BDNF mRNA expression and transcriptional activation of BDNF gene promoters I and III (BDNF-PI and -PIII, respectively) in rat cortical culture. The increase in BDNF mRNA expression induced at high K\(^+\) was repressed not only by nicardipine, an antagonist for L-VDCC, but also by DL-amino-5-phosphonooxalate, an antagonist for NMDA-R, which was supported by the effects of antagonists on the Ca\(^{2+}\) influx. Although the promoter activations at 25 and 50 mM KCl were different, BDNF-PIII was activated by either the Ca\(^{2+}\) influx through NMDA-R or L-VDCC, whereas BDNF-PI was predominantly by the Ca\(^{2+}\) influx through L-VDCC. Direct stimulation of NMDA-R supported the activation of BDNF-PIII but not that of BDNF-PI. Thus, the alternative BDNF gene promoters responded differently to the intracellular Ca\(^{2+}\) signals evoked via NMDA-R and L-VDCC.

A variety of genes whose products are involved in expressing neuronal functions including synaptic plasticity can be activated by the Ca\(^{2+}\) signals evoked via Ca\(^{2+}\) influx into neurons, which is associated with an electrical activity of neurons (1). The main sites for extracellular Ca\(^{2+}\) influx leading to an activation of gene expression are the N-methyl-d-aspartate receptors (NMDA-R), a subtype of ion channels of glutamate receptors, and L-type voltage-dependent Ca\(^{2+}\) channels (L-VDCC) (2). The Ca\(^{2+}\) influx through both sites evokes intracellular Ca\(^{2+}\) signals through several signaling routes mainly involving calcium/calmodulin-dependent protein kinases (3–5) and extracellular signal-related protein kinase/mitogen-activated protein kinase (6, 7). The Ca\(^{2+}\) signals are received by the Ca\(^{2+}\) signal-responsive DNA regulatory elements by a mediation of specific DNA-binding proteins, such as the cyclic AMP/calcium response element-binding protein (CREB) and serum response element-binding protein (SREBP) and serum-responsive factor, respectively (6, 7). Irrespective of the context of Ca\(^{2+}\) signaling in neurons, however, the physiological role of the Ca\(^{2+}\) signaling evoked via NMDA-R versus L-VDCC in neurons, and the intracellular mechanisms for differentially conveying the Ca\(^{2+}\) signals for gene expression are still controversial.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and plays a key role in the survival, differentiation, and synaptic plasticity of neurons (8). The rat BDNF gene consists of four short exons, exons I, II, III, and IV, and a 3’ exon, exon V, encoding the mature BDNF protein, giving rise to eight species of BDNF transcripts each of which contains exon I, II, III, or IV and a long or short exon V (9). Four promoters were mapped upstream of exons I, II, III, and IV, respectively, which are differentially activated by the kainic acid-induced seizure in distinct regions of the rat brain (9). It is becoming evident that the alternative promoters are used as a versatile mechanism to create diversity and flexibility in the regulation of gene expression (10). Therefore, it is important to know the transcriptional regulation of BDNF gene promoters I, II, III, and IV for a better understanding of the cellular mechanisms controlled by BDNF.

Although the Ca\(^{2+}\) signal-mediated activation of the BDNF gene has well been established (2, 11, 12), it remains unclear how the alternative promoters of the BDNF gene are regulated by the Ca\(^{2+}\) signals evoked via L-VDCC and NMDA-R. Using DNA transfection in primary rat cortical neuronal cell culture, the DNA elements responsible for the Ca\(^{2+}\) signal-mediated activation of BDNF promoter III have been identified (4, 5), and furthermore, we have characterized the BDNF promoter I as a Ca\(^{2+}\) signal-responsive promoter (13, 14). In the present study, therefore, we examined whether the BDNF gene promoters I and III responded differentially or similarly to the Ca\(^{2+}\) signals evoked via NMDA-R or L-VDCC. During this methyl-phenyl]-3-pyridinecarboxylic acid methyl ester; DMEM; Dulbecco’s modified Eagle’s medium.
study, we also found that the mode of BDNF gene activation changed as the KCl concentration in the medium was raised and that this change was mediated by changes in modes of Ca\(^{2+}\) influx into neurons dependent upon the level of membrane depolarization.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Nicardipine, NMDA, L-\text{amin}-5\text{-phosphonovalerate (APV)}, and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[\text{2-(trifluoromethyl)}-\text{phenyl}]-3-pyridinedicarboxylic acid methyl ester (Bay-K8644) were purified from Sigma.

**Cell Culture**—Primary cultures of cortical neurons were prepared from the cerebral cortices of 18-day-old rat (Cry:SD) embryos as described (13). Briefly, small pieces of cerebral cortex were treated with 0.125% trypsin (DIFCO) and 1 mM EDTA for 15 min at 37 °C and thoroughly dissociated in DMEM. After centrifugation, the cell pellet was treated with 0.004% DNase I (Sigma) and 0.05% trypsin inhibitor (Sigma), and the suspended cells were seeded at 5 × 10^6 cells in a 60-mm culture dish (Iwaki). The cells were grown for 48 h in DMEM medium (10% fetal calf serum), and then the medium was replaced with serum-free DMEM containing glucose (4.5 mg/ml), transferrin (5 μg/ml), insulin (5 μg/ml), sodium selenite (5 μg/ml), bovine serum albumin (1 mg/ml), and kanamycin sulfate (100 μg/ml) (TIS medium). Cytosine arabinoside (Sigma) was also added to prevent the growth of glial cells. 2 h before DNA transfection, the medium was replaced with fresh TIS medium from which cytosine arabinoside had been removed.

**DNA Transfection and Luciferase Assay**—DNA transfection of cortical neurons was carried out on 3 days in culture. Plasmid DNAs containing BDNF promoters I and III, respectively, were prepared to generate pGL3-BDNF-pI and pGL3-BDNF-pIII by inserting the amplified DNA fragments covering the region (−528 to +138) of BDNF promoter I and the region (−629 to +281) of BDNF promoter III into the HindIII site of the pGL3-Basic firefly luciferase reporter vector (Promega), respectively. The primers used for amplification of BDNF promoter I were described before (13), and those for BDNF promoter III are as follows: forward primer, 5'-ATGCTGAGAAGGCTGACCTTCTCATTGCTCTTGTGTTAGGTTCTCAACGTCCTG-3'; and reverse primer, 5'-CCCAAGCTTTCCCAAGGTTCTACGCTTATCT-3'. As an internal control vector, we used pRL-EFluc, which was constructed by inserting the elongation factor 1α (EF1α) gene promoter into the HindIII-EcoRI site of the pRL-null Renilla luciferase control reporter vector (Promega). The procedure used for DNA transfection was the calcium phosphate precipitation method (15). The calcium phosphate/DNA precipitates were prepared by mixing one volume of calcium phosphate/DNA precipitates for 30 min. We added 40 or 80 μl of plasmid DNA (10 μg) to 250 μl CaCl_2 solution with an equal volume of 2× HBS (50 mM HEPES-NaOH, pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4). After the calcium phosphate/DNA precipitate mixture had been added to each dish and incubated for 30 min, the cells were incubated in a 10% CO_2 atmosphere after addition of fresh TIS medium. Following incubation in 10% CO_2 at 37 °C for 24 h, the transfected cortical cells were stimulated with high K^+ (25 or 50 mM KCl), 100 μM NMDA, or vehicle for a further 6–12 h before harvest, and cellular extracts were prepared for luciferase assay. There was no loss of cortical cells in culture after DNA transfection, because of the short treatment of cells with calcium phosphate precipitates for 30 min. We added 40 or 80 μl of 2× TIS solution to the culturing medium (4 ml/dish), to adjust the final KCl concentration to 25.3 or 50.3 mM, including the 5.3 mM KCl originally contained in the DMEM medium. The conditions for luciferase assay were described before (13). 20 μl of cell extract was used for firefly and Renilla luciferase assays using the Dual-LuciferaseTM Reporter Assay System (Promega) and the ratio of luminescence signals from the reaction mediated by firefly luciferase to those from the reaction mediated by Renilla luciferase, both measured with a Luminometer TD-20/20 (Promega), was calculated. Fold increase of transcription was shown as a multiple of the control. The procedure for the luciferase assays was provided by the supplier (Promega). The primary culture of rat cortical neurons contains a small population of glial cells, which can also be transfected with plasmid DNA. However, we could not detect the induction of BDNF promoter in primary culture of glial cells under high K^+ (13), indicating that the induction of BDNF gene promoters is caused in neuronal cells.

**Preparation of Total Cellular RNA and Northern Blotting**—Total cellular RNA was extracted by the acid guanidine phenol-chloroform method as described (15). Aliquots of total cellular RNA (10 μg) were suspended and subjected to an electrophoresis on denaturing formaldehyde gels and then transferred onto nylon membrane filters (Hybond N; Amersham Pharmacia Biotech). The conditions for hybridization were described before (16). To compare the kinetics of BDNF and β-actin mRNA expression, we performed the hybridization using the same hybridization membrane filters after reprobing. For reprobing, the radiolabeled probes were removed from the filters by shaking them with 2× SSPE solution three times. BDNF and β-actin mRNAs were detected by an Imaging scanner (BAS 2000; Fuji) and calculated as a multiple of the control (fold increase). cDNA probes for hybridization were derived from mouse for β-actin (nucleotide positions 81–1208) and from rat for BDNF (nucleotide positions 1–1892).

**Ca^{2+} Uptake Experiment**—The Ca^{2+} influx into the cortical neurons was measured by the procedure of Lazarewicz et al. (17). 10 min before stimulating the cells, 5 μM nicardipine or 200 μM APV was added to the cultivation medium. Incubation after stimulating the cells with high K^+ in the presence of 1 μCi of ^{45}CaCl_2 (Amersham Pharmacia Biotech; 0.37–1.5 GBq/mg of calcium) was carried out for 5 min, and the reaction was terminated by rapid aspiration of the medium and three washes with phosphate-buffered saline containing 2 mM EGTA. Cells were solubilized in 0.5 M NaOH for radioactivity determination. The radioactivity per dish was measured, and the Ca^{2+} uptake in each dish was normalized to the control, in which the cells had not been stimulated, and calculated as a multiple of the control (fold increase). The amounts of protein recovered from each dish were almost the same (approximately 2 mg/dish; data not shown).

**Statistical Analysis—**All values represent the means ± S.D. of the number of separate experiments performed in duplicate, as indicated in the corresponding figures. Comparisons between groups were made using Student’s t test followed by F-test, with p < 0.05 as the minimum significant level.

**RESULTS**

**Optimum Range of Membrane Depolarization for BDNF Gene Promoter Activation**—Because it is well established that the BDNF gene can be activated by membrane depolarization (11, 12), we investigated the effect of KCl concentrations in the medium on the gene activation. As shown in Fig. 1A, Northern blotting analysis revealed that the BDNF mRNA expression was up-regulated at 25 and 50 mM KCl but markedly reduced above 75 mM KCl. In contrast to this, c-fos induction was detected not only at 25 and 50 mM but also above 75 mM KCl (data not shown). The Ca^{2+} uptake by cultured cortical cells increased from 10 mM, reached a plateau level at 50–75 mM KCl, and maintained its elevated level until 100 mM KCl (Fig. 1C). To investigate whether the changes in BDNF gene activation involve a selective or co-ordinated activation of respective BDNF gene promoters, we transfected pGL3-BDNF-PI and pGL3-BDNF-PIII, which carry the BDNF promoter I (BDNF-pI) and promoter III (BDNF-pIII), respectively, to the cultured rat cortical cells using calcium phosphate precipitation. As shown in Fig. 1B, the luciferase activities derived from the firefly luciferase reporter gene were obvious at 25 and 50 mM KCl with both pBDNF-PI and pBDNF-PIII, corresponding to the changes in BDNF mRNA expression detected by Northern blotting (Fig. 1A). The transcriptional activity of both BDNF-PI and -PIII was greater at 25 mM than at 50 mM KCl. The c-fos mRNA expression detected by Northern blotting (Fig. 1A) was not shown. For reprobing, the radiolabeled probes were removed from the filters by shaking them with 2× SSPE solution three times. BDNF and β-actin mRNAs were detected by an Imaging scanner (BAS 2000; Fuji) and calculated as a multiple of the control (fold increase). The amounts of protein recovered from each dish were almost the same (approximately 2 mg/dish; data not shown).

**Measurement of NMDA-R Activation and BDNF Gene Expression by Membrane Depolarization**—To determine whether the BDNF gene activation induced by membrane depolarization is due to the Ca^{2+} influx through L-VDCC alone or not, we investigated the BDNF mRNA expression in the cortical neurons stimulated with 25 or 50 mM KCl in the presence of APV, a specific NMDA-R antagonist, or nicardipine, a specific L-VDCC...
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Fig. 1. Effect of KCl concentrations in the medium on BDNF mRNA expression, BDNF promoter activity and 45Ca2+ uptake. A, following the incubation of cortical cells with TIS medium for 24 h, the KCl solution (2 mM) or vehicle was added to adjust the final KCl concentration to 5, 10, 25, 50, 75, 100, or 125 mM. The total cellular RNA was prepared for Northern blotting 6 h after starting the incubation. Arrows, BDNF mRNA; #, β-actin mRNA. B, after the cells had been incubated with TIS medium for 2 h, they were transfected with 8 μg/60-mm dish of pGL3-BDNFPi or pGL3-BDNFPIII plus pRL-EF1α by the DNA-calcium phosphate precipitation method, incubated for another 40 h, and then collected to prepare cellular extracts for luciferase assay 12 h after stimulation. C, after the cells had been incubated in TIS medium for 24 h, 1 μCi of 45Ca2+ was added 10 min prior to the addition of KCl solution (2 mM) or vehicle at the indicated concentrations. The cells were incubated for another 5 min, and the radioactivity incorporated by the cells was measured. The bars in each experiment (A–C) represent the means ± S.D. from three separate experiments.

antagonist. As shown in Fig. 2A, both the increases in BDNF mRNA expression induced by 25 and 50 mM KCl in culture were effectively inhibited by APV (200 μM), the extent of the inhibition being greater at 25 mM than at 50 mM KCl. Administration of nicardipine (5 μM) partially inhibited the increase in BDNF mRNA expression at 25 or 50 mM KCl, the inhibitory level of which was smaller than that by APV. Consistent with these observations, the Ca2+ uptake by cortical cells at 25 and at 50 mM KCl was inhibited by APV, to an extent greater than that by nicardipine (Fig. 2B). These results indicate that the membrane depolarization at 25 and at 50 mM KCl evoked Ca2+ influxes not only through L-VDCC but also through NMDA-R, both of which are responsible for the increases in the BDNF mRNA expression. Consistent with the observation that the 45Ca2+ uptake was higher at 50 mM than at 25 mM KCl (Fig. 2B), the increase in the intracellular Ca2+ concentration was approximately 2-fold higher at 50 mM than at 25 mM KCl (Fig. 2C). However, the induction of the BDNF gene expression was greater at 25 mM than at 50 mM KCl (Fig. 2A).

Different Responses of BDNF-Pi and -PIII to the Ca2+ Channel Antagonists—To investigate the effect of the Ca2+ influx through NMDA-R and L-VDCC on BDNF-Pi and -PIII, we measured the activities of both promoters by DNA transfection in culture (Fig. 3). Upon elevation of the KCl concentration to 25 mM, the BDNF-Pi was markedly activated, but this activation was completely abolished by APV as well as by nicardipine (Fig. 3A). Upon elevation of the KCl concentration to 50 mM, however, the activation of BDNF-Pi, the level of which was lower than that at 25 mM KCl, was abolished by nicardipine to almost the control level but not inhibited by APV (Fig. 3A). On the other hand, the activation of BDNF-PIII upon elevation of the KCl concentration to 25 mM was reduced by APV to almost the same level as the nonstimulated control and by nicardipine to about half the level of induction (Fig. 3B). At 50 mM KCl, about a 40% reduction of BDNF-PIII activation was obtained with APV and nicardipine (Fig. 3B). Thus, different responses of promoter activities were observed with BDNF-Pi and -PIII between 25 and 50 mM KCl. The APV-sensitive activities of BDNF-Pi markedly changed between 25 and 50 mM KCl (Fig. 3A), the same tendency being observed with BDNF-PIII, but less markedly (Fig. 3B). In addition, the nicardipine-sensitive activities of BDNF-Pi were completely abolished by nicardipine at 25 and 50 mM KCl, whereas those of BDNF-PII remained when nicardipine was added at 25 or 50 mM KCl. The induction pattern of BDNF mRNA expression detected by Northern blotting (Fig. 2A) resembled that of BDNF-PIII rather than BDNF-Pi (compare Fig. 3B with Fig. 2A). The antagonistic effect of APV on BDNF-Pi activation was saturated at 200 μM (data not shown), and an addition of MK801, another specific antagonist for NMDA-R, also produced an inhibitory effect on both the BDNF mRNA expression and the promoter activations, which was the same as that of APV (data not shown). The inhibitory effect of nicardipine on BDNF-Pi activation was saturated at 2 μM (data not shown), and other L-VDCC-specific antagonists, nifedipine and nimodipine, revealed the same effect as nicardipine (data not shown).

Changes in the Susceptibility of BDNF-Pi to APV Caused by L-VDCC Agonist—Because the different responses of promoters I and III at 25 and 50 mM KCl could be due to the fact that L-VDCC is unable to open below 25 mM KCl because of insufficient membrane depolarization, we measured the BDNF-Pi activity when Bay-K8644, a specific L-VDCC agonist, was added simultaneously upon elevation of the KCl concentration to 25 mM (Fig. 4). Although only a small induction of BDNF-Pi activity was detected with Bay-K8644 alone, the simultaneous addition of Bay-K8644 with 25 mM KCl markedly induced the activation of BDNF-Pi. In contrast to the activation at 25 mM KCl alone (Fig. 3A), the activation of BDNF-Pi induced by
Bay-K8644 together with 25 mM KCl was not inhibited by APV (Fig. 4). On the other hand, the administration of nicardipine reduced the BDNF-PI activation to almost the same level as the control (Fig. 4). Thus, the simultaneous addition of Bay-K8644 at 25 mM KCl changed the susceptibility of BDNF-PI to APV. The small BDNF-PI activation induced by Bay-K8644 alone was inhibited by both APV and nicardipine, as was the BDNF-PI activation at 25 mM KCl (Fig. 3A).

**Activation of BDNF-PIII via Ca²⁺ Influx through NMDA-R—Nicardipine was fully active on BDNF-PII but only partially active on BDNF-PIII, irrespective of the K⁺ concentration used (Fig. 3). These observations raised the possibility that BDNF-PIII can respond to the Ca²⁺ influx evoked via NMDA-R but BDNF-PII cannot. To test this possibility, we stimulated the cortical neurons with NMDA (100 μM) and measured the promoter activities of BDNF-PI and -PIII. To exclude a possible Ca²⁺ influx through L-VDCC that might be caused by a specific activation of NMDA-R, we treated the cortical neurons with NMDA in the presence of nicardipine. As shown in Fig. 5A, the BDNF-PI activation induced by NMDA was largely reduced by nicardipine, to about the level obtained by APV, indicating that the Ca²⁺ influx through L-VDCC caused by NMDA-stimulation mostly accounted for the BDNF-PII activation. On the other hand, the BDNF-PIII activation induced by NMDA was not inhibited by nicardipine but completely inhibited by APV. The ⁴⁵Ca²⁺ uptake was induced by NMDA stimulation and partially inhibited by nicardipine as well as by APV (Fig. 5B), indicating that the activation of NMDA-R induces the Ca²⁺ influx through not only NMDA-R but also L-VDCC.

Finally, we examined the time course of BDNF-PI and -PIII...
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activation after incubating the cells with 50 mM KCl plus APV (L-VDCC-driven Ca\(^{2+}\) influx) or NMDA plus nicardipine (NMDA-R-driven Ca\(^{2+}\) influx). As shown in Fig. 6A, the BDNF-PI activity was stimulated by the L-VDCC-driven Ca\(^{2+}\) influx but not by the NMDA-R-driven one until 24 h after incubation. In contrast, the BDNF-PIII activity was stimulated by both the L-VDCC- and NMDA-R-driven Ca\(^{2+}\) influxes although the L-VDCC-driven Ca\(^{2+}\) influx was approximately 2-fold more effective than the NMDA-R-driven one (Fig. 6B).

DISCUSSION

Among the many possible roles of alternative promoters, such as varying the efficiency of transcription initiation, varying the turnover or translation efficiency of mRNA isoforms with different leader exons, changing the tissue specificity, reacting differentially to some extracellular signals, developmentally regulating gene expression, and generating protein isoforms differing at the amino terminus, it has been suggested that the alternative promoters of the BDNF gene are involved in the control of tissue- and brain region-specific expression of the gene (9). Elucidation of the other usages of alternative promoters is important to reveal the mechanisms controlling the neuronal functions mediated by BDNF. Because it has well been established that the expression of the BDNF gene can be controlled by the intracellular Ca\(^{2+}\) signals (2, 11, 12, 18), we focused on the effect of the Ca\(^{2+}\) signals evoked via L-VDCC and NMDA-R on the alternative BDNF gene promoters. Therefore, we measured the promoter activities of BDNF-PI and BDNF-PIII by transfecting the plasmid DNAs containing the BDNF gene promoter I or III and luciferase reporter gene to primary rat cortical neurons in culture and then stimulating the cells with high K\(^{+}\) (25 or 50 mM KCl) or NMDA (100 \(\mu\)M) in the presence of their respective antagonists, nicardipine and APV.

In this study, we demonstrated that there were effective K\(^{+}\) concentrations (25–50 mM KCl) for the activation of the BDNF gene (Fig. 1, A and B), although the extent of Ca\(^{2+}\) influx was still large above 75 mM KCl (Fig. 1C), and that between 25 and 50 mM KCl, different modes of BDNF gene activation were evoked via the Ca\(^{2+}\) influx through L-VDCC and NMDA-R, which was demonstrated by the different sensitivities of BDNF gene activation to APV and nicardipine (Figs. 2 and 3). The increase in BDNF mRNA expression at 25 mM KCl was inhibited completely by APV but only partially by nicardipine, whereas that at 50 mM KCl was inhibited partially by both (Fig. 2A). A difference in sensitivity to the antagonists between 25 and 50 mM KCl was also observed for the transcriptional activation of the BDNF gene promoters (Fig. 3). The BDNF-PI activation at 25 mM KCl was completely repressed not only by nicardipine but also by APV, whereas that at 50 mM KCl was repressed by nicardipine but not by APV (Fig. 3A). The BDNF-PIII activation was repressed by nicardipine and by APV but was more resistant to APV at 50 mM than at 25 mM KCl (Fig. 3B). These observations suggested that the Ca\(^{2+}\) influx through L-VDCC at 25 mM KCl was induced dependent upon an activation of NMDA-R but independently of NMDA-R activation at 50 mM KCl; that is, the L-VDCC at 25 mM KCl is unable to open by itself and needs the help of NMDA-R, which leads to the Ca\(^{2+}\) influx through L-VDCC, whereas that at 50 mM KCl is able to open without the mediation of NMDA-R. In support of this notion, the Ca\(^{2+}\) influx was almost abolished by APV at 25 mM KCl but not at 50 mM KCl (Fig. 2B). The fact that the selective activation of L-VDCC by Bay-K8644 at 25 mM KCl increased the nicardipine-sensitive activation of BDNF-PI and abolished the APV-sensitive one (Fig. 4) also supported the above notion; that is, Bay-K8644 directly helped to open L-VDCC at 25 mM KCl, leading to the Ca\(^{2+}\) influx through L-VDCC without the mediation of NMDA-R.

The L-VDCC activation mediated by NMDA-R at 25 mM KCl was thought to be due to a glutamate release induced by membrane depolarization and a subsequent activation of NMDA-R (19), which might lead to an increase in the membrane potential that rises as the KCl concentration in medium is elevated (20). To test this notion, we added botulinus toxin to the culturing medium of 25 mM KCl to repress a possible glutamate release from neurons. However, botulinus toxin did not inhibit the increase in BDNF mRNA expression at 25 mM KCl (data not shown). The release of glutamate is also caused by a reversal of glutamate transporter, which can be induced by AMPA/kainate receptor activation (21). Thus, it is still unclear whether or not the glutamate release is involved in the NMDA-R activation induced by membrane depolarization at 25 mM KCl.

That the Ca\(^{2+}\) influxes through not only L-VDCC but also NMDA-R and vice versa when the cortical neurons in culture are stimulated by high K\(^{+}\) (50 mM KCl) or glutamate, respectively, could additively influence the Ca\(^{2+}\)-responsive gene expression was already pointed out by Hardingham et al. (22). In the present study, we confirmed this notion by demonstrating that the Ca\(^{2+}\) influx through both Ca\(^{2+}\) channels is caused by either stimulation of membrane depolarization or NMDA-R (Fig. 2, A and B). Considering these observations, it seems essential to include the counterpart channel antagonist to L-VDCC or NMDA-R when stimulating the cortical neurons in culture with high K\(^{+}\) or NMDA to study the Ca\(^{2+}\) signalings specifically evoked via L-VDCC versus NMDA-R.

Because the BDNF-PI activation was markedly repressed by nicardipine at both 25 and 50 mM KCl and resistant to APV at 50 mM KCl (Fig. 3A), the BDNF-PI seems to respond to the Ca\(^{2+}\) signals evoked via L-VDCC but not to those evoked via NMDA-R. On the other hand, the BDNF promoter III activation at 50 mM KCl was sensitive to both APV and nicardipine (Fig. 3B), indicating that BDNF-PIII can respond to the Ca\(^{2+}\)
signals evoked via L-VDCC and NMDA-R. The BDNF-PIII activation at 25 mM KCl in the presence of nicardipine (Fig. 3B) also seemed to be due to the Ca\(^{2+}\) influx through NMDA-R induced by membrane depolarization at 25 mM KCl (Fig. 2B). In support of these observations, the stimulation of NMDA-R in the presence of nicardipine activated BDNF-PIII but not BDNF-PI (Fig. 5A). During the time course of activation, in addition, BDNF-PI remained activated by the Ca\(^{2+}\) influx through L-VDCC but not through NMDA-R for at least 24 h after stimulation, whereas BDNF-PIII remained activated by the Ca\(^{2+}\) influx through not only L-VDCC but also NMDA-R (Fig. 6). Thus, we clearly demonstrated that BDNF-PI predominantly responded to the Ca\(^{2+}\) signals evoked via L-VDCC, whereas BDNF-PIII responded to the Ca\(^{2+}\) influxes through L-VDCC and NMDA-R to almost the same degree.

It has been reported that BDNF-PIII is activated by Ca\(^{2+}\) signals evoked via L-VDCC through intracellular signaling routes involving calcium/calmodulin-dependent protein kinase IV and CREB (4, 5). In the present study, we demonstrated that BDNF-PIII is also activated by Ca\(^{2+}\) signals evoked via NMDA-R and BDNF-PI predominantly by those evoked via L-VDCC. Quite recently, Hardingham et al. (22) reported that the Ca\(^{2+}\) influx through NMDA-R and L-VDCC induces distinct kinetics of CREB phosphorylation at serine 133; that is, although the Ca\(^{2+}\) influx through NMDA-R and L-VDCC is initially equally potent at inducing CREB phosphorylation, CREB remained phosphorylated at serine 133 even 1 h after stimulation upon L-VDCC activation, whereas NMDA-R activation caused only a transient increase in CREB phosphorylation. We also observed that the CREB phosphorylation was sustained for longer than 1 h upon L-VDCC activation, which was caused by membrane depolarization at 50 mM KCl in the presence of APV, but such a sustained and strong CREB phosphorylation was not observed upon NMDA-R activation, which was caused by NMDA stimulation in the presence of nicardipine (data not shown), indicating that the Ca\(^{2+}\) signals evoked via L-VDCC and NMDA-R are transferred through different signaling pathways in terms of CREB phosphorylation. The c-fos promoter receives both the Ca\(^{2+}\) signals transduced by NMDA-R and L-VDCC through cAMP/calcium response element and serum response element which are located approximately −60 and −310 nucleotides on the c-fos promoter, re-
spectively (6, 7). Therefore, it seems worth investigating whether the same Ca\(^{2+}\) signaling pathways are involved in the BDNF-PIII activation. In addition, it has recently been demonstrated that the phosphorylation of not only CREB at Ser-133 but also the CREB-binding protein, which facilitates the unwinding of DNA and interacts with the basal transcriptional machinery, is required for regulating the Ca\(^{2+}\) signal-mediated gene expression (22, 23). Therefore, it is of interest to investigate which transcriptional factor(s) and whether the CREB-binding protein is involved in the activation of BDNF-PI and BDNF-PIII controlled by Ca\(^{2+}\) signals.

The fact that the induction levels of BDNF-PI and -PIII tended to be reduced from 25 mM to 50 mM KCl, although the intracellular Ca\(^{2+}\) concentrations were higher at 50 mM than at 25 mM KCl (Figs. 1–3), indicates that the higher the intracellular Ca\(^{2+}\) concentration, the stronger the repressive activities exerted on BDNF gene transcription, which might also account for the nonresponsiveness of BDNF-PI and -PIII above 75 mM KCl (Fig. 1B). Thus, it seems likely that certain repressive activities are exerted on the BDNF gene transcription when the intracellular Ca\(^{2+}\) concentration is raised. On the other hand, the Ca\(^{2+}\) signals evoked via L-VDCC induce the BDNF gene activation more effectively than those evoked via NMDA-R because the L-VDCC activation induced the BDNF promoter activation higher than did the NMDA-activation (Fig. 6B), even though the Ca\(^{2+}\) uptake was lower upon the L-VDCC than NMDA-R activation (Figs. 2B and 5B). This might be due to a difference in the localization of these channels in neurons or to different intracellular signaling machineries associated with these channels (24, 25). Thus, it seems worth inquiring how the BDNF gene activation are regulated by the concentration or the localization of intracellular Ca\(^{2+}\), which could be controlled by L-VDCC and NMDA-R activation.

The observation that the pattern of change in BDNF mRNA expression resembled that in BDNF-PII activities (Figs. 2A and 3B) indicates that the transcription controlled by BDNF-PII and not by BDNF-PI mainly accounted for the BDNF mRNA expression in cultured cortical cells. In support of this observation, the basal promoter activity of BDNF-PIII was approximately 10–20-fold higher than that of BDNF-PI or -PII (data not shown). Using a reverse transcription-polymerase chain reaction method, furthermore, we found that the expression of exon I, II, and III but not exon IV transcripts were induced in rat cortical neurons under high K\(^{+}\) and the exon III transcripts were expressed more abundantly than the exon I or II ones under either high or low K\(^{+}\) (data not shown). Thus, the alternative BDNF gene promoters may confer not only a differential responsiveness to the Ca\(^{2+}\) signals evoked via L-VDCC and NMDA-R but also a differential efficacy of transcription initiation on the BDNF mRNA expression. The activation of BDNF promoter II showed the same sensitivities to APV and nicardipine as that of BDNF-PI (data not shown). However, it is still unknown whether the BDNF promoters I, II, and III are coordinately activated by Ca\(^{2+}\) signals. To assess this point, it is necessary to investigate whether the alternative BDNF gene promoters could be activated in the same or different neurons. The differential usages of BDNF gene promoters in neurons should be involved in controlling the multiple BDNF functions in mammalian nervous systems.

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