Neuron, Vol. 7, 625–635, October, 1991, Copyright © 1991 by Cell Press

L-type Voltage-Sensitive Calcium Channels Mediate Synaptic Activation of Immediate Early Genes

Timothy H. Murphy,* Paul F. Worley,*† and Jay M. Baraban*‡
*Department of Neuroscience
†Department of Neurology
‡Department of Psychiatry and Behavioral Sciences
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Summary

Although L-type voltage-sensitive calcium channels (VSCCs) have been well characterized electrophysiologically, their role in synaptic physiology has remained unclear. To assess their involvement in synaptic regulation of gene expression, we have examined the effects of selective VSCC antagonists on basal, synaptically mediated activation of several transcription factor genes in cultured cortical neurons. Basal expression of c-*fos*, *jun*-B, *zif268*, and *fos*-B is rapidly suppressed by exposure to L-type VSCC antagonists and increased by (−)BayK-8644, a VSCC agonist. Although VSCC antagonists block kainate-induced rises in intracellular calcium and gene expression, these agents have little effect on spontaneous electrical activity or synaptically induced calcium transients in these neurons. These findings suggest that even though L-type VSCCs contribute a relatively minor component of synaptic calcium transients, they appear to play a key role in coupling synaptic excitation to activation of transcriptional events thought to contribute to neuronal plasticity.

Introduction

L-type voltage-sensitive calcium channels (VSCCs) have been extensively characterized biochemically (for review see Campbell et al., 1988) and electrophysiologically (for review see Tsien et al., 1988). They possess several distinctive features, including a high threshold for activation, sensitivity to dihydropyridine drugs, and slow inactivation. Although these channels are expressed in brain neurons, their function remains unclear, as blockade of these channels has little, if any, effect on synaptic transmission (Kuan and Scholfield, 1986; Yu et al., 1988). Recently, VSCCs have been localized to the base of proximal dendrites and neuronal cell bodies (Westenbroek et al., 1990; Ahlijanian et al., 1990), prompting the proposal that they play a critical role in linking dendritic electrical activity to regulatory processes in the soma.

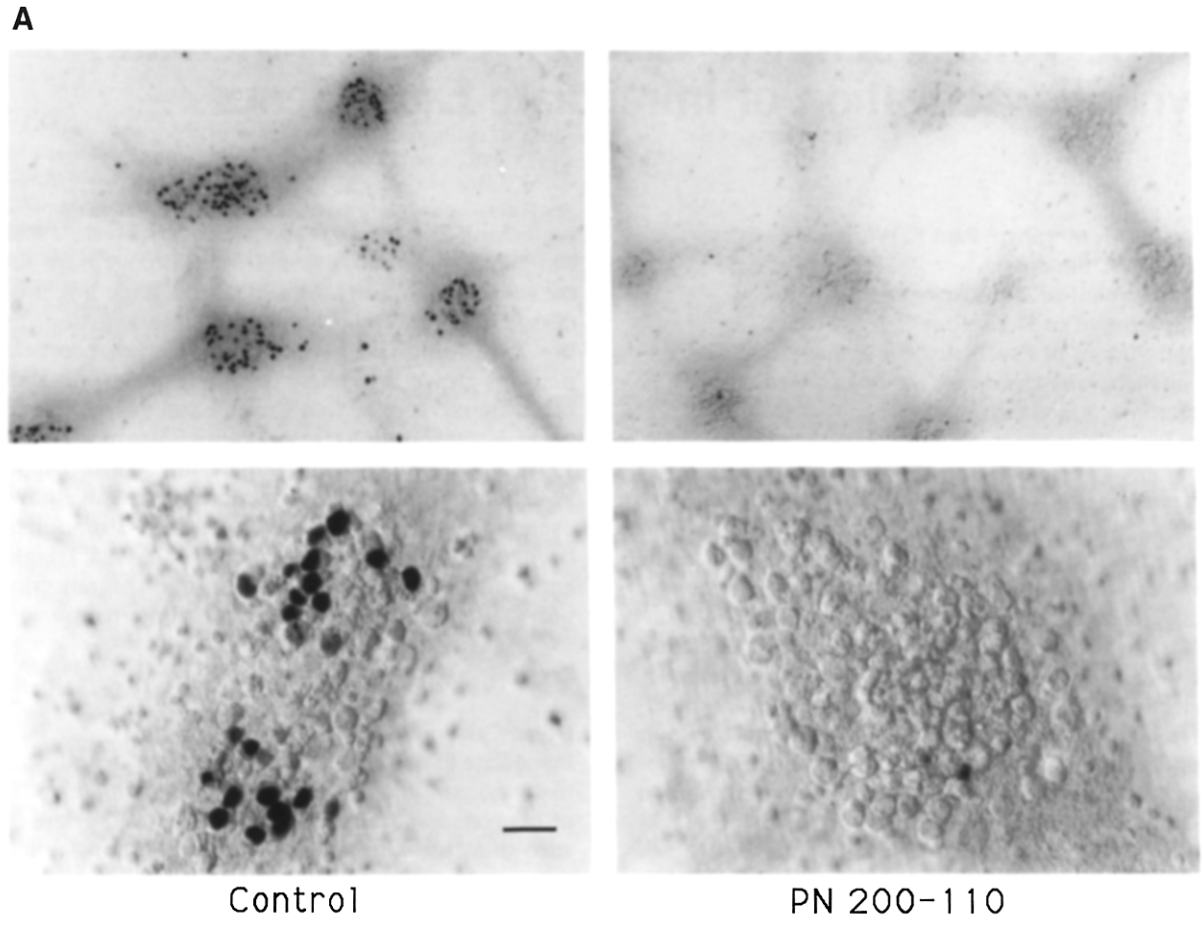
Synaptic stimulation has been recently shown to activate rapidly several transcription factor genes, such as c-*fos* and *zif268* in brain (Cole et al., 1989; Wisden et al., 1990; for review see Lau and Nathans, 1990; Sheng and Greenberg, 1990), thought to be involved in orchestrating changes in gene expression underlying neuronal plasticity (Goelet et al., 1986; Morgan and Curran, 1989). These findings have focused attention on how synaptic signals are processed and how they regulate gene expression. In previous studies, we have demonstrated that cortical neurons grown in primary culture display spontaneous synaptic activity that drives basal expression of several transcription factor genes (Murphy et al., 1991). Accordingly, to investigate the role of L-type VSCCs in this signal transduction process, we have examined whether selective agents that modulate L-type VSCCs (for review see Tsien et al., 1988; Triggle and Janis, 1987) affect synaptic activation of transcription factor genes in cultured cortical neurons.

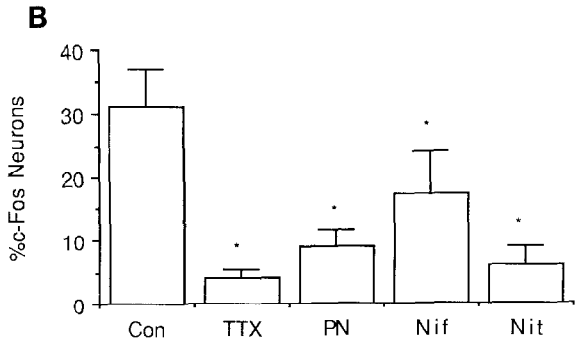
Results

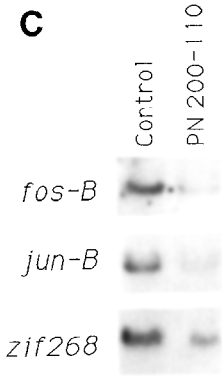
L-type Calcium Channel Antagonists Block Immediate Early Gene Expression

In previous studies, we found that approximately 30% of neurons in cultures maintained for 21–24 days in vitro show prominent nuclear c-Fos immunostaining that is induced by spontaneous synaptic activity present in these mature cultures (Murphy et al., 1991). To assess the role of L-type VSCCs in mediating synaptic activation of immediate early genes, we examined the effect of selective L-type VSCC antagonists on basal levels of c-Fos immunostaining in primary cortical cultures. In prior studies examining synaptic regulation of c-Fos, we found that exposure to tetrodotoxin (TTX) for 4–6 hr markedly reduced c-Fos immunostaining (Murphy et al., 1991). Treatment of cultures with the VSCC antagonist PN200-110 (0.5 μM) produced a similar effect; after 6 hr of continuous treatment, basal c-Fos immunostaining is reduced by greater than 70% (Figure 1A), while at 2 hr no effect on c-Fos immunostaining is detected. Nifedipine (0.5 μM) and nitrendipine (1 μM), other VSCC antagonists, also reduce c-Fos immunoreactivity (Figure 1B). Half-maximal effects of nitrendipine and PN200-110 were observed at approximately 100 nM. Furthermore, the effect of these agents does not appear to be restricted to c-Fos, as basal mRNA levels of several other immediate early gene transcription factors, such as *zif268* (Christy et al., 1988), *jun*-B (Ryder et al., 1988), and *fos*-B (Zerial et al., 1989), are also reduced by PN200-110 (Figure 1C).

To ensure that the effect of these agents is not merely due to their ability to block synaptic activity, we monitored the effect of PN200-110 on spontaneous synaptic activity in whole-cell recordings. In contrast to TTX, PN200-110 did not reduce spontaneous synaptic potentials or currents in 5 of 5 neurons examined after at least 8 min of continuous exposure (Figure 1D). Furthermore, records from neurons treated for 5–7 hr with PN200-110 (500 nM; 5 cells) or nitrendipine (1 μM; 7 cells) indicated that the spontaneous cur-







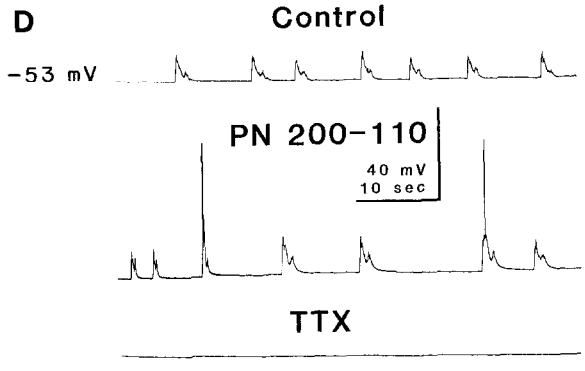


Figure 1. 1,4-Dihydropyridine Voltage-Sensitive Calcium Antagonists Block Basal Immediate Early Gene Expression

(A) Hoffman photomicrographs of c-Fos immunostaining in groups of control and PN200-110-treated (500 nM; 6 hr) neurons. Calibration, 30 and 120 μm.

(B) The effect of dihydropyridines and TTX on basal c-Fos immunoreactivity. Shown are the means ± SEM of at least 3 separate duplicate experiments. All compounds were added for 6 hr. PN200-110 at 500 nM, nifedipine at 500 nM, nitrendipine at 1 μM, and TTX at 1 μM. *$P < 0.05$ in a paired t test comparing control and the indicated treatments.

(C) Northern blot hybridization of mRNA from control and PN200-110-treated (4 hr; 500 nM) cultures probed with the indicated ^{32}P-labeled cDNAs. Regions with molecular weights of the indicated RNA species are shown. This experiment was repeated with similar results.

(D) PN200-110 does not reduce spontaneous synaptic potentials. Shown is the whole-cell current-clamp record from a 25 day in vitro cortical neuron with a −53 mV resting membrane potential before PN200-110, 8 min after continuous PN200-110 (1 μM), and 3 min after TTX (1 μM).

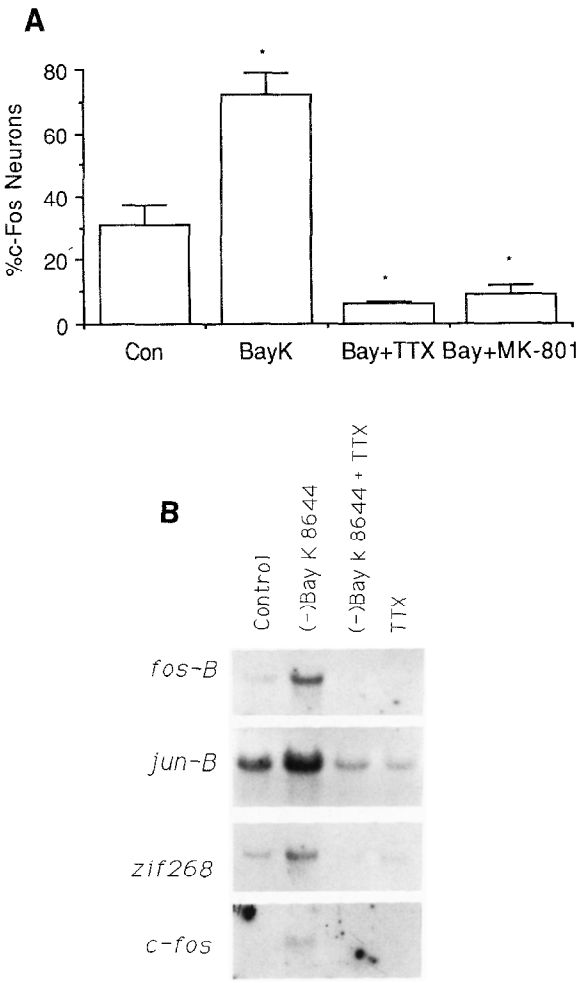


Figure 2. 1,4-Dihydropyridine VSCC Agonist Increases Immediate Early Gene Expression

(A) (−)BayK-8644 increases c-Fos immunoreactivity. Shown are the means ± SEM of the percentage of c-Fos-immunoreactive neurons after the following treatments: basal (no drug additions), (−)BayK-8644 (2 hr; 1 μM), (−)BayK-8644 (2 hr) in the presence of TTX (1 μM), or MK-801 (10 μM) (pretreated 4 hr). *$P < 0.05$ in a paired t test.

(B) Northern blot of mRNA from control cultures, from cultures treated with (−)BayK-8644 for 1 hr, or from cultures pretreated with TTX for 3 hr and then treated with or without (−)BayK-8644 and TTX for an additional 1 hr (last two lanes). Blots were probed with the indicated ^{32}P-labeled cDNAs as described in Figure 1 and Experimental Procedures. The blots had lower basal mRNA levels than those shown in Figure 1.

rents, potentials, and ability to fire action potentials were not obviously compromised when compared with 11 vehicle control neurons. This blockade of immediate early gene activation appears to be specific, since PN200-110 did not affect induction of c-Fos immunoreactivity by phorbol 12,13-diacetate (8 μM), or treatment with forskolin (10 μM) and 3-isobutylmethylxanthine (500 μM). In contrast to dihydropyridines, ω-conotoxin (500 nM), an N-type VSCC blocker (McCleskey et al., 1987), did not affect basal c-Fos immunoreactivity (data not shown).

Table 1. Differential Activation of c-Fos by Kainate and NMDA

	% c-Fos-Immunoreactive Neurons
Control	33 ± 3
TTX	4 ± 1[a]
TTX + kainate	24 ± 3[a]
TTX + (−)BayK-8644	6 ± 1
TTX + kainate + (−)BayK-8644	62 ± 8[a]
TTX + NMDA	17 ± 6
TTX + NMDA + (−)BayK-8644	19 ± 7
TTX + PDA	67 ± 4
TTX + PDA + NMDA	33 ± 4[a]

Shown are the means ± SEM of at least 3 separate duplicate experiments. Cultures were treated with TTX (1 μM) for 4 hr and 2 additional hours in the presence of TTX and either 15 μM kainate, kainate and (−)BayK-8644 (1 μM), (−)BayK-8644, NMDA (10 μM), NMDA and (−)BayK-8644, PDA (10 μM; phorbol 12,13-diacetate), or NMDA and PDA. Paired t tests were used to compare the percentage of immunoreactive neurons in the following cultures: control versus TTX; TTX versus TTX and kainate; TTX versus TTX and (−)BayK-8644; TTX versus TTX and NMDA; TTX and kainate versus TTX, kainate, and (−)BayK-8644; TTX and PDA versus TTX, PDA, and NMDA.

[a] The second member of the pair for comparisons with $P < 0.05$.

L-type Calcium Channel Agonists Enhance Immediate Early Gene Expression

The ability of L-type VSCC antagonists to suppress basal immediate early gene expression suggests that spontaneous synaptic activity is sufficient to activate L-type VSCCs. Accordingly, we determined whether potentiation of these currents by a calcium channel agonist, (−)BayK-8644, would increase c-Fos expression. (−)BayK-8644 at 1 μM markedly increases c-Fos basal immunoreactivity (Figure 2A) and c-*fos*, *jun*-B, *zif268*, and *fos*-B mRNA levels (Figure 2B). Studies of the action of (−)BayK-8644 on calcium channels demonstrate that this agent acts to potentiate depolarization-induced calcium currents (Nowycky et al., 1985). Consistent with this property of (−)BayK-8644, we found that its ability to increase transcription factor mRNA or c-Fos immunoreactivity was blocked by TTX (Figure 2), which suppresses synaptically induced depolarization. N-methyl-D-aspartate (NMDA) receptor antagonists also blocked (−)BayK-8644-induced c-Fos expression, suggesting that activation of this receptor is required to evoke L-type VSCC currents.

Induction of c-Fos Expression by Kainate

As these studies suggest that synaptic activation of L-type VSCCs mediate induction of immediate early gene expression, we assessed whether neurotransmitter activation of these channels would exert a similar effect. Since cortical neurons in primary culture display prominent depolarizing responses to kainate that could lead to L-type VSCC activation and rises in intracellular calcium, we investigated the effect of this receptor agonist on c-Fos immunostaining. In initial studies, we confirmed that, even in the presence of MK-801 (Wong et al., 1986) to block possible effects

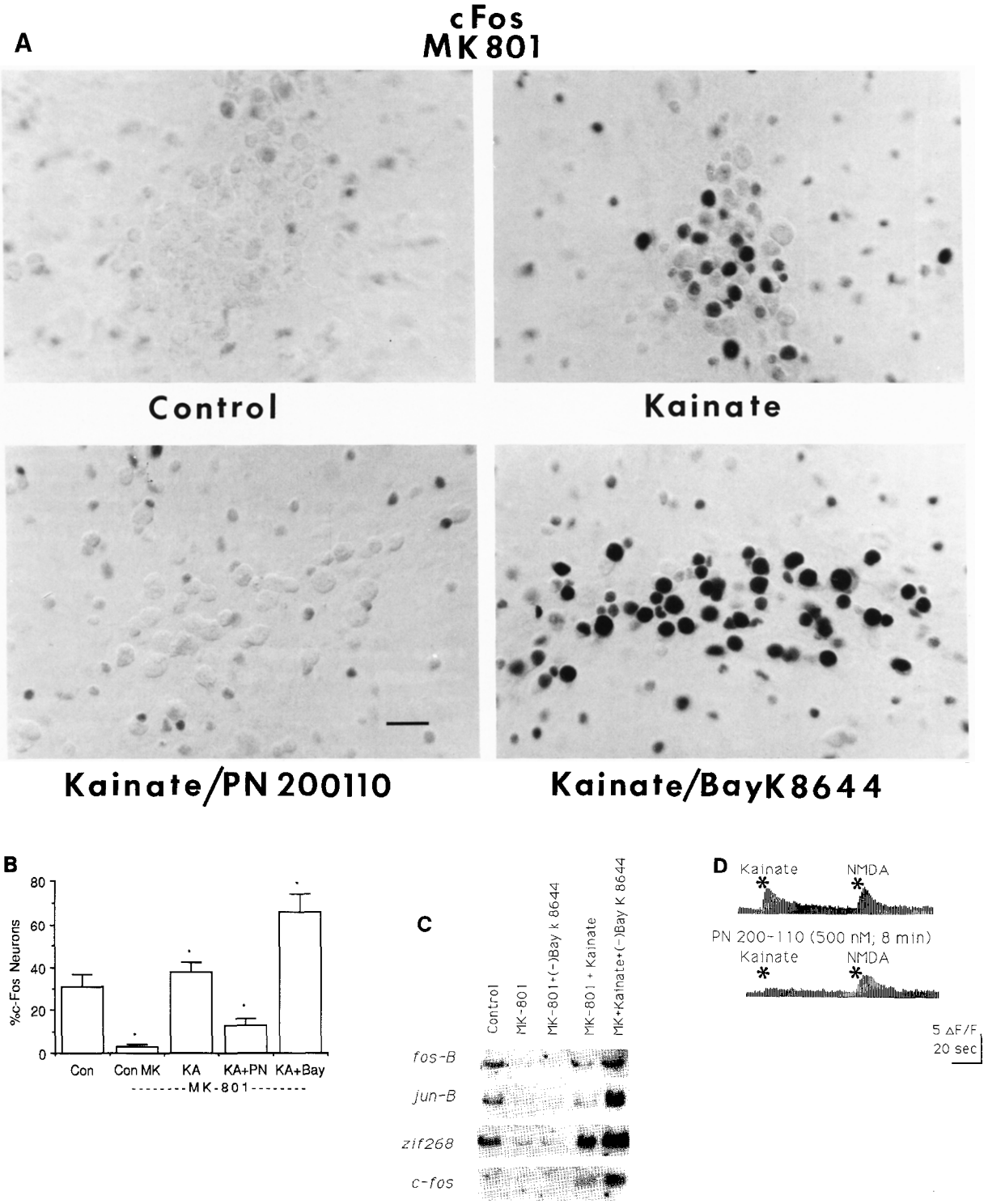


Figure 3. Kainate Restores Basal Immediate Early Gene Expression

(A) Shown are Hoffman photomicrographs of c-Fos immunostaining of MK-801-treated cultures (10 μM; 4 hr MK-801 pretreatment followed by 2 hr in the presence of kainate [15 μM], kainate and PN200-110 [0.5 μM], or kainate and (−)BayK-8644 [1 μM]). Bar, 30 μm.

(B) Percentage of neurons with c-Fos immunoreactivity following kainate stimulation. Shown are the means ± SEM of at least three separate duplicate experiments. *$P < 0.05$ in paired t tests comparing control with MK-801 treated, MK-801 with kainate, kainate with kainate plus PN200-110, and kainate with kainate plus (−)BayK-8644. Concentration and time of drug addition are the same as in (A).

(C) Northern blot of RNA from cultures pretreated with 3 μM MK-801 for 3 hr and treated in the presence of MK-801 and the indicated compounds (concentrations in [A]) for an additional 1.25 hr. Replicate blots were probed with the indicated ^{32}P-labeled cDNAs. Shown are regions corresponding to weights of the respective RNAs probed. (−)BayK-8644 potentiation of kainate was observed by Northern blot analysis in 3 separate experiments.

(D) Selective blockade of kainate-induced increases in intracellular calcium by PN200-110. Kainate (50 μM) and NMDA (50 μM) were applied locally by pressure ejection, and calcium was measured as described in Figure 5. PN200-110 (500 nM) was added to the bathing medium.

of kainate on NMDA receptors, local application of kainate (by pressure ejection) produced a large sustained, but reversible, depolarization (4 of 4 cells) (see Figure 4A). After pretreatment with MK-801 to reduce basal c-Fos levels (Murphy et al., 1991), addition of kainate (15 μM) for 2 hr produced reliable increases in c-Fos immunostaining (Figures 3A and 3B). Kainate also increased c-Fos expression in TTX-pretreated cultures (Table 1). Lower concentrations of kainate (5 μM) failed to induce c-Fos expression reliably. Although 15 μM was nontoxic (as assessed by propidium iodide exclusion) for at least 3 hr, concentrations in excess of 15 μM were avoided because of possible acute toxicity. The ability of 15 μM kainate to stimulate c-Fos staining was blocked by a 5 min pretreatment with the antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honore et al., 1988) (50 μM; 96% ± 8% reduction; $P < 0.05$, paired t test), whereas 10 μM CNQX had no significant effect (data not shown).

Dihydropyridines Modulate the Kainate Response

Following the initial characterization of kainate-induced activation of immediate early genes, we assessed whether this response also displayed sensitivity to L-type VSCC antagonists. Following pretreatment of cultures with MK-801 to reduce basal expression of c-Fos, we found that PN200-110 blocked kainate-stimulated c-Fos (Figures 3A and 3B). Conversely, the VSCC agonist (−)BayK-8644 potentiated kainate induction of c-Fos immunostaining and c-*fos*, *jun*-B, and *fos*-B mRNAs (Figure 3C). This effect of (−)BayK-8644 is dependent on its coadministration with kainate, since, as described above, in the presence of MK-801 or TTX, but the absence of kainate, (−)BayK-8644 was without significant effect. These findings are consistent with previous reports that (−)BayK-8644 enhances calcium currents activated by depolarization (for review see Tsien et al., 1988).

To confirm that kainate increases intracellular calcium via L-type VSCCs, we used the fluorescent calcium indicator fluo-3 (Minta et al., 1989) to monitor intracellular calcium directly. Consistent with reports on other primary neuronal cultures (Murphy and Miller, 1989; Mayer et al., 1987; Yuste and Katz, 1991; Courtney et al., 1990), we found that application of kainate by pressure ejection resulted in rapid increases in intracellular calcium that were reduced 64% ± 11% ($n = 5$ experiments; $P < 0.05$) by the L-type VSCC antagonist PN200-110 (500 nM) (Figure 3D). NMDA-induced increases in intracellular calcium were not affected by PN200-110 (4% ± 6% reduction; $n = 9$ experiments) (Figure 3D), confirming the specificity of PN200-110 for L-type VSCCs in this system. In contrast to the effect of PN200-110, (−)BayK-8644 (1 μM) more than doubled calcium-induced fluo-3 fluorescence in response to kainate in 3 of 4 separate experiments.

NMDA Agonist Effects

Local application of NMDA by pressure ejection in the presence of TTX resulted in sustained 40–60 mV depolarizations from the resting membrane potential (data not shown) and increases in intracellular calcium (Figure 3D). Although NMDA is a potent depolarizing agent, application of concentrations between 1 μM and 30 μM for 2 hr in the presence of TTX produced only low levels of c-Fos-immunoreactive cells (Table 1). Furthermore, exposure to NMDA (10–30 μM) for 5 min followed by the antagonist 2-amino-5-phosphonovalerate (APV) at 300 μM failed to increase c-Fos-immunoreactive cells by more than 10% when measured 2 hr later. In contrast to its action on kainate-stimulated and basal synaptic expression of transcription factor genes, (−)BayK-8644 (1 μM) did not potentiate NMDA (10 μM) (Table 1). Application of 10 μM NMDA for 2 hr did not result in significant toxicity when assessed by propidium iodide exclusion. However, NMDA treatment reduced phorbol ester induction of c-Fos (Table 1), suggesting that it may be difficult to dissociate NMDA toxicity from induction of immediate early genes.

Kainate Receptor Blockade Reduces Spontaneous Synaptic Activity and Basal Gene Expression

To assess whether quisqualate/kainate receptor activation may also mediate synaptically induced c-Fos expression, we examined the effect of a kainate receptor antagonist on basal synaptic activity and c-Fos expression. Application of CNQX by pressure ejection reversibly reduced spontaneous synaptic currents by >50% in 4 of 5 neurons examined (Figure 4B), confirming that, as expected, quisqualate/kainate receptors mediate, in part, basal synaptic activity. Treatment of cultures with 50 μM CNQX significantly reduced basal c-Fos immunoreactivity (52% ± 12% reduction; $P < 0.05$). CNQX (10 μM) was unable to affect basal c-Fos staining significantly. CNQX treatment was performed in the presence of 1 mM glycine (which did not affect basal c-Fos) to prevent any blockade of NMDA receptor activity due to CNQX's relatively low affinity at the NMDA receptor–glycine modulatory site (Pellegrini-Giampietro et al., 1989). In the presence of 1 mM glycine, CNQX (50 μM) was unable to block the toxicity due to 15 μM NMDA exposure (continuous 24 hr), but reliably blocked 15 μM kainate toxicity (24 hr exposure), suggesting receptor specificity under the conditions used. The 2 hr of 15 μM kainate exposure used for immunostaining experiments was nontoxic.

Effect of L-type Channel Antagonists on Synaptic Alterations in Intracellular Calcium

Although we found that L-type VSCC antagonists blocked kainate-induced calcium transients, we wanted to examine the effects of these agents on synaptically induced calcium transients. Using the calcium-sensitive probe fluo-3 (Minta et al., 1989), we observed spontaneous calcium transients that presumably reflected prominent spontaneous synaptic activity exhibited by these cultures. In some instances, we could easily record these transients using

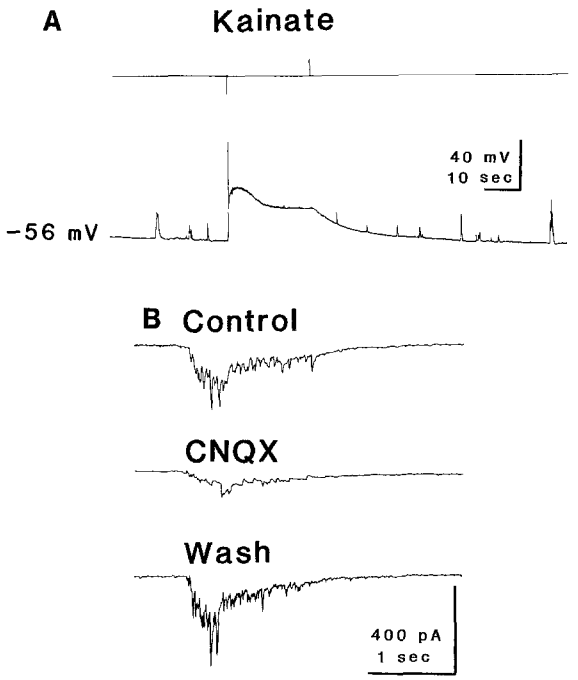


Figure 4. Spontaneous Activity Mediated by Kainate Receptors

(A) Kainate produces large, sustained depolarizations. Shown is a neuron under whole-cell current clamp with a −56 mV resting membrane potential during local application of 30 μM kainate for the time in the top trace. This recording was made in the presence of 10 μM MK-801.

(B) The effect of locally applied CNQX (100 μM) on spontaneous synaptic currents in a voltage-clamped (−60 mV) neuron. Top trace, control; middle trace, 30 s after CNQX; bottom trace, 3 min later. Glycine (1 mM) was present throughout the recording.

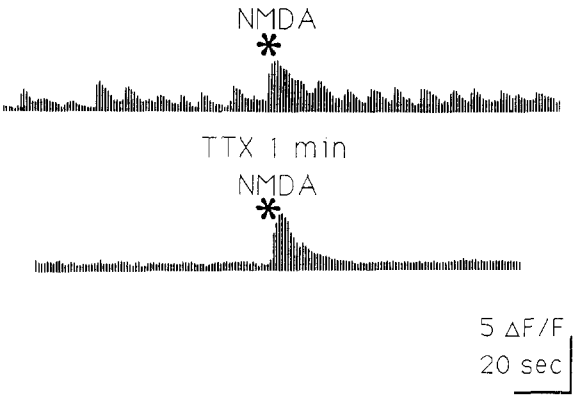


Figure 5. Spontaneous Synaptic and Agonist-Stimulated Intracellular Calcium Transients

Fluctuations in basal calcium-induced fluo-3 fluorescence were measured by a photodiode (30 ms illumination every 1.1 s) positioned over 2 or 3 clustered neuronal cell bodies. NMDA (40 μM) was used as a positive control. TTX (1 μM) was added to block synaptic activity as indicated. This record depicts one of the best examples of basal calcium oscillations observed. Addition of TTX (1 μM) completely blocks the oscillations, but fails to reduce the calcium response induced by 40 μM NMDA.

a photodiode positioned over clustered neurons and found that they were blocked by TTX (Figure 5). However, to elicit synaptically mediated alterations in intracellular calcium reliably, we employed electrical stimulation of neighboring groups of neurons with a fine bipolar stimulating electrode. This procedure resulted in rapid, TTX-sensitive increases in intracellular calcium in target neurons measured by the photodiode (Figure 6). Current-clamp records indicated that electrical stimulation was of sufficient magnitude to produce action potentials (Figure 6A). To investigate the relative contribution of NMDA receptors, kainate receptors, and L-type VSCCs to synaptic calcium transients, specific antagonists were applied during electrical stimulation. Application of CNQX (250 μM; pressure ejection in the presence of 1 mM glycine; n = 7) produced a reversible 68% ± 6% decrease in the electrically evoked calcium response ($P < 0.05$). APV (400 μM; pressure ejection; n = 4) produced a 49% ± 9% reduction in the calcium transient ($P < 0.05$). In contrast, bath application of PN200-110 (0.5 μM) or nitrendipine (0.5–1 μM) for up to 8 min did not significantly alter the electrically evoked postsynaptic calcium response (96% ± 9% of control; n = 9). Since blockade of L-type VSCCs is use dependent (Tsien et al., 1988), in 2 experiments, nitrendipine (1 μM) was applied in the presence of kainate to ensure opening of L-type VSCCs and access to the blocker. Despite this maneuver, nitrendipine reduced the electrically evoked calcium transient by 20% or less.

Discussion

Although it is now generally accepted that expression of immediate early genes in neurons can be rapidly regulated by synaptic activity, little is known about the mechanisms leading from synaptic neurotransmitter receptor activation to altered gene expression. To investigate this process, we have utilized primary cortical cultures, as these display basal levels of transcription factor gene expression that are dependent on spontaneous synaptic activity. In previous studies, we have demonstrated that synaptic activity and basal immediate early gene expression develop in parallel as these cultures mature over the course of 1–3 weeks in vitro (Murphy et al., 1991). In addition, blockade of synaptic activity with TTX suppresses basal expression of several immediate early genes within a few hours. Accordingly, to investigate the role of L-type VSCCs in coupling synaptic activity to transcriptional regulation, we have examined the effect of selective L-type VSCC agents on immediate early gene expression in these cultures. Our findings that L-type VSCC antagonists markedly reduce basal expression of several immediate early genes imply that (as suggested by their selective localization in the vicinity of neuronal somata) these channels play a critical role in coupling dendritic synaptic activity to gene expression in the soma.

Several lines of evidence indicate that the ability of

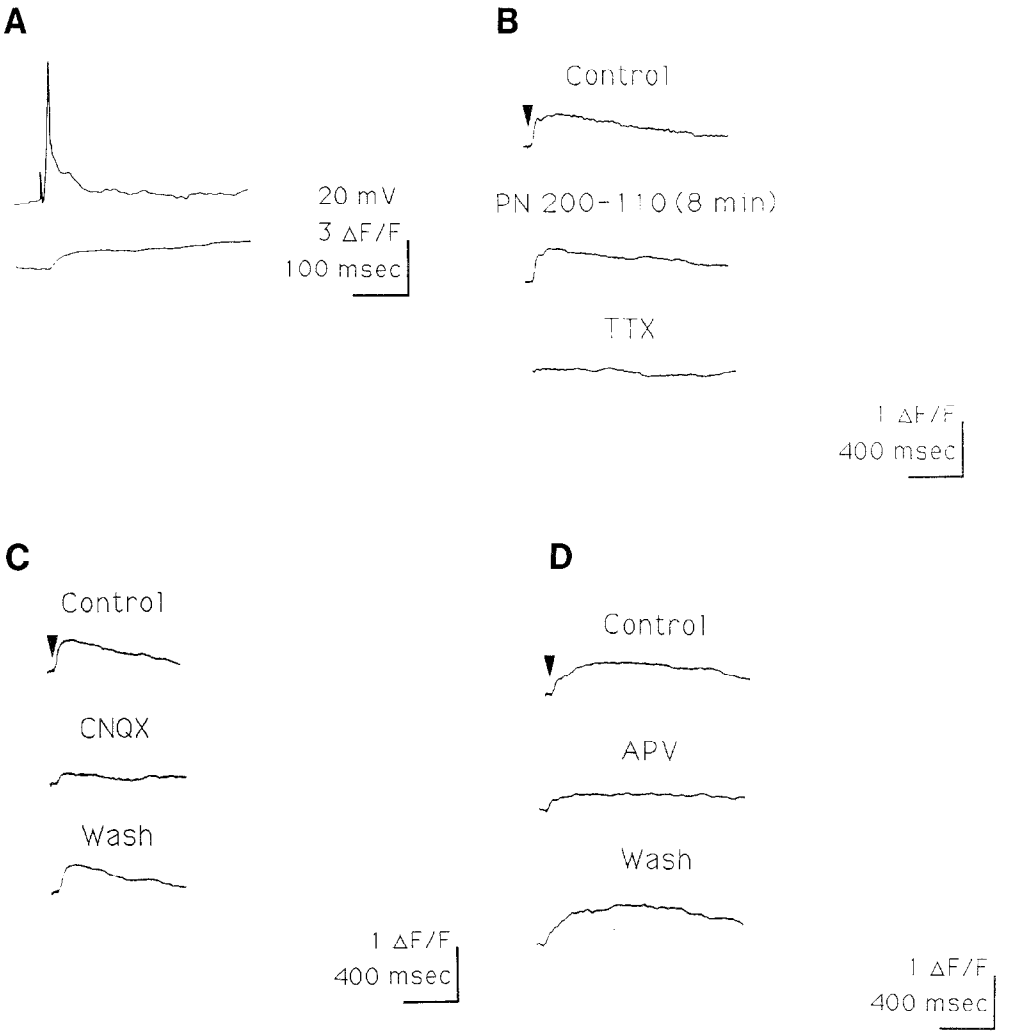


Figure 6. Electrically Evoked Synaptic Calcium Transients

Shown is the effect of electrical stimulation of a neighboring group of neurons (~1000 μm away; single shock 90 V per 100–200 μs) on calcium-induced fluo-3 fluorescence in target neurons.

(A) Coincidence of action potential (−53 mV holding potential) and fluo-3 fluorescence during electrical stimulation. Membrane potential and fluorescence are from different experiments.

(B) PN200-110 (0.5 μM; 8 min exposure) fails to reduce synaptic calcium-induced fluo-3 fluorescence. TTX (1 μM) was added for 3 min.

(C) Local application of the kainate receptor antagonist CNQX (250 μM; pressure ejected; see Experimental Procedures) reduces synaptic calcium-induced fluo-3 fluorescence. This experiment was performed in the presence of 1 mM glycine.

(D) Local application of the NMDA receptor antagonist APV (400 μM; pressure ejected; see Experimental Procedures) reduces synaptic calcium-induced fluo-3 fluorescence.

dihydropyridine L-type VSCC antagonists to reduce basal expression of immediate early genes results from the specific blockade of L-type VSCCs. Similar effects were found for three antagonist compounds: PN200-110, nifedipine, and nitrendipine. These compounds were effective at concentrations (0.1–1 μM) comparable to those needed to block calcium fluxes in other neuronal preparations (Carboni and Wojcik, 1988; Kingsbury and Balazs, 1987; Courtney et al., 1990; Regan et al., 1991). It is unlikely that these agents act by blocking synaptic transmission, since in these cultures, as described for other preparations (Kuan and Scholfield, 1986; Kamiya et al., 1988; Yu et al., 1988), synaptic transmission was not affected by dihydropyridine antagonists. Furthermore, blockade of immediate early gene expression by these agents appears to be specific, since induction of c-Fos by phorbol ester or forskolin is unaffected by these agents.

Further evidence that these agents act by blocking L-type VSCCs is provided by studies with kainate. Kainate-induced depolarization leads to increases in intracellular calcium that are, in part, mediated by L-type VSCCs (Murphy and Miller, 1989; Courtney et al., 1990). In cortical cultures pretreated with MK-801 or TTX to reduce basal expression of immediate early genes, kainate was able to induce immediate early

gene expression. We found that PN200-110 completely blocked the expression of immediate early genes by kainate. Furthermore, kainate-induced increases in intracellular calcium were greatly reduced by 500 nM PN200-110, confirming that kainate produces an L-type VSCC current in this culture system that is blocked by PN200-110. The effect of PN200-110 on kainate-induced intracellular calcium is selective, as NMDA-induced increases in intracellular calcium are not affected. Thus, concentrations of PN200-110 that block basal immediate early gene expression that is dependent upon synaptic transmission reduce kainate-induced c-Fos expression as well as rises in intracellular calcium mediated by L-type VSCCs.

VSCC agonists act by enhancing L-type calcium currents that are triggered by depolarization (Nowycky et al., 1985). Consistent with this property, we found that (−)BayK-8644, an L-type VSCC agonist, potently enhanced basal immediate early gene expression. The effect of (−)BayK-8644 was completely blocked by addition of TTX, consistent with (−)BayK-8644 enhancing an existing calcium current resulting from spontaneous activity. NMDA antagonists, which reduce a component of synaptic activity in these cultures (Murphy et al., 1991), also blocked the ability of (−)BayK-8644 to enhance basal c-Fos, suggesting that under these conditions NMDA receptor activity is required for synaptic activation of L-type VSCCs.

Consistent with the proposed role of kainate receptors in synaptic transmission in cortical slices (Jones and Baughman, 1988), CNQX, a kainate receptor antagonist (Honore et al., 1988), reduced both spontaneous synaptic activity and basal c-Fos expression. The effect of CNQX suggested that activation of kainate receptors may in part mediate synaptic expression of immediate early genes. Consistent with this idea, kainate was able to induce c-Fos expression in TTX-treated cultures, suggesting that it can mimic the endogenous transmitter. Synaptic activation of the kainate receptor would provide a mechanism for VSCC activation. Kainate induction of c-Fos was blocked by VSCC antagonists and augmented by VSCC agonists. Thus, kainate-induced immediate early gene expression is regulated by dihydropyridines in a manner similar to that of endogenous transmitter. However, NMDA receptor antagonists block basal synaptically induced immediate early gene expression but fail to block kainate-induced gene expression. Conceivably, NMDA receptor antagonists might indirectly reduce transmitter release by reducing network synaptic activity, thereby decreasing kainate receptor activation (Murphy et al., 1991). Alternatively, synaptic activation of both kainate and NMDA receptors is needed to elicit sufficient depolarization to activate L-type VSCCs, whereas the exogenous agonist kainate, which produces a persistent, nondesensitizing activation of the quisqualate/kainate glutamate receptor, alone is sufficient.

Previous studies in cerebellar granule cells (Szekely et al., 1989) indicate that NMDA receptor activation can directly induce c-Fos in neurons. Although, application of NMDA produced large increases in intracellular calcium in cortical neurons, we did not observe statistically significant induction of c-Fos by NMDA in TTX-pretreated cultures. NMDA was applied at concentrations that did not produce toxicity as measured by vital stain. However, we cannot rule out that toxic effects of NMDA may compromise the expression of immediate early genes. Consistent with this, induction of c-Fos in cortical neurons by phorbol ester was reduced by NMDA treatment (Table 1). The observed induction of immediate early genes by NMDA in cerebellar granule cells may reflect differences in sensitivity to NMDA toxicity. Alternatively, NMDA receptor activation alone may be insufficient to induce immediate early gene expression in a large percentage of cortical neurons, even though it appears to be necessary, as NMDA receptor blockers suppress basal, synaptically induced immediate early gene expression.

In vitro studies of c-*fos* activation have demonstrated that multiple second messenger pathways can act in parallel to increase its transcription. For example, growth factors and phorbol esters are thought to act via serum response elements present in its 5′ upstream sequence, while cyclic AMP and calcium act through a cyclic AMP response element (Sheng et al., 1990). Interestingly, in PC12 cells activation of c-*fos* by KCl depolarization or veratridine is also blocked by L-type VSCC antagonists (Morgan and Curran, 1986). The results of our studies in cortical neurons suggest that synaptic activation of immediate early genes may be mediated via this cyclic AMP and calcium responsive element.

Zif268 mRNA and protein are expressed at relatively high levels in neurons of normal adult cortex. This basal or constitutive expression appears to be driven by ongoing physiological afferent inputs (Worley et al., 1991). Accordingly, it is possible that L-type VSCCs are involved in coupling natural synaptic activity and transcription factor expression in vivo. Studies performed in cultured hippocampal neurons and adrenal chromaffin cells suggest that activation of L-type VSCCs by KCl depolarization leads to the delayed expression of brain-derived neurotrophic factor (Zafra et al., 1990) and proenkephalin genes (Kley et al., 1987), suggesting that these genes may be regulated by immediate early gene products. Consistent with our results on glutamate agonist activation of immediate early gene expression in cortical neurons, kainate but not NMDA induces brain-derived neurotrophic factor mRNA (Zafra et al., 1990). Furthermore, recent studies in cultured spinal cord neurons (Agoston et al., 1991) suggest that spontaneous synaptic activity is required for the expression of enkephalin and that in the absence of synaptic activity, L-type VSCC agonists can restore the enkephalin phenotype.

Interestingly, the bulk of the electrically evoked synaptic calcium transients in cultured cortical neurons are sensitive to NMDA and kainate receptor antagonists and only marginally affected by VSCC antago-

nists. These data are consistent with studies of cortical slices in which APV blocks greater than 90% of the evoked postsynaptic calcium transient (Yuste and Katz, 1991). Empirical and theoretical studies have suggested that calcium fluxes during synaptic transmission may be highly localized (Regehr and Tank, 1990; Simon and Llinas, 1985). Furthermore, selective activation of calcium-sensitive messengers in close proximity to sites of calcium entry could occur if these factors bound calcium with low affinity. Interestingly, the K_m of calmodulin for calcium is 2.4 μM (Teo and Wang, 1973), a steady-state concentration not reached in neuronal somata during synaptic transmission in hippocampal slices (Regehr et al., 1989). In support of calmodulin as a mediator of synaptic immediate early gene expression, Morgan and Curran (1986) showed that a calmodulin antagonist reduced induction of c-Fos by KCl depolarization. Accordingly, we propose that kinases or other cellular messengers responsible for triggering expression of immediate early genes may be localized in the vicinity of L-type VSCCs and may therefore be selectively regulated by calcium fluxes through these channels. In summary, although L-type VSCCs contribute only a small component of the calcium current elicited by synaptically induced depolarization, they appear to play a pivotal role in coupling strong synaptic stimulation to regulation of gene expression in the nucleus.

Experimental Procedures

Cell Culture and Media

Cell cultures were prepared from Sprague–Dawley rat cerebral cortex taken at day 17 gestation, using a papain (EC 3.4.22.2) dissociation method (Murphy and Baraban, 1990). Cultures were allowed to mature for at least 3 weeks for all experiments. The dissociated cells were resuspended at a density of 1.2×10^6 cells per ml in minimal essential medium supplemented with 5.5 g/l glucose, 2 mM glutamine, 10% fetal calf serum, 5% heat-inactivated horse serum, 50 U/ml penicillin, 0.05 mg/ml streptomycin, plated onto polylysine-coated (10 μg/ml) 35-mm culture dishes in 1.5–2 ml of medium or 12-well dishes (1 ml of medium), and placed in a 37°C CO_2-buffered incubator. The cultures were fed by addition of minimal essential medium with 5.5 g/l glucose, 5% heat-inactivated horse serum, and 2 mM glutamine, after about 4–6, 12–14, 16–17, and 19–21 days in culture, by removal and replacement of approximately 60% of the medium. Cultures were not fed for at least 24 hr before fixation for immunostaining or extraction of RNA to avoid any possible effects of refeeding on expression of transcription factor proteins. During experimental treatments prior to immunostaining or RNA measurements, cultures were maintained in minimal essential medium with 5% heat-inactivated horse serum in a 37°C 5% CO_2 incubator. Compounds of interest were added directly to the cultures in 50×–200× stock solutions. All compounds were dissolved in water, with the exception of CNQX (Cambridge Research Biochemicals), nitrendipine (Miles), nifedipine (Sigma), and PN200-110 (Sandoz), which were dissolved in dimethyl sulfoxide, diluted to 10% with water, and then added to cultures at 100×. Vehicle controls were included within each experiment. Addition of water (0.5%–2% of final volume) or dimethyl sulfoxide (0.1%) had no detectable effect on basal or stimulated c-Fos immunostaining. (−)BayK-8644 was obtained from Bayer Pharmaceuticals.

Cell viability was assessed using the non-membrane-permeating fluorescent compound propidium iodide (4 μg/ml) as described by Krishan (1975).

Electrophysiology

For electrophysiological measurements, cells were switched to a Hank's balanced salt solution (by triple exchange) that contained the following: 137 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4(7H_2O)$, 10 mM Na^+–HEPES, 1 mM $NaHCO_3$, 0.01 mM glycine, and 5 mM glucose (pH 7.4 and 340 mosmol). All recordings were made using the whole-cell variant of the patch-clamp technique at room temperature (Hamill et al., 1981), using 4–8 MΩ glass micropipettes (World Precision Instruments, 1B/20 F-4) and an Axopatch 1C amplifier as described (Murphy and Baraban, 1990). The pipette solution used for current-clamp and voltage-clamp experiments contained 140 mM potassium methyl sulphate, 2 mM $CaCl_2$, 11 mM potassium ethylene glycol-bis-N,N,N,N'-tetraacetic acid, and 10 mM K^+–HEPES. Recordings were made in a static bath (1–2 ml) within a 35-mm tissue culture dish. Agonists and antagonists were applied either by pressure ejection (15–20 psi for 0.5–5 s) from 2–6 μm tip diameter glass pipettes (compounds diluted into bathing medium) positioned 250–500 μm from the cell of interest (previous experiments indicate dilution of up to 5-fold; Murphy et al., 1988), or by addition directly into the bathing medium at 100-fold concentration dissolved in bathing medium. Direct addition of bathing medium or water alone (20 μl to a 2 ml bath) failed to affect ongoing electrical activity or cell stability.

Cell Calcium

Changes in cell calcium were estimated using the fluorescent probe fluo-3 (Minta et al., 1989). Fluo-3 undergoes a 40-fold increase in fluorescence upon binding calcium (K_d 400 nM). Fluo-3 acetoxymethylester was dissolved in dimethyl sulfoxide at 5 μg/μl and further diluted into Hank's balanced salt solution at 10 μg/ml in the presence of 0.25% pluronic. Cortical neurons were incubated with this solution for 1 hr at room temperature. Cells were then washed two times with Hanks's balanced salt solution and observed under epifluorescence (490 nm excitation) at room temperature. Fluorescence was quantitated using an EG and G Ortech photodiode (UV040BQ), amplified by a Axopatch 1C amplifier, and displayed by a strip chart recorder. A cardboard mask was placed between the cells and the photodiode to reduce the area illuminating the photodiode to ~1000 μm^2 (approximately three neuronal cell bodies). A neutral density filter was added to reduce photobleaching, and the fluorescence field diaphragm was lowered, allowing fluorescence illumination of only the cells imaged by the photodiode. To prevent further photobleaching, a shutter was added to the fluorescence lamp. The shutter was typically open for 30 ms, every 0.5–1.1 s. Synaptic calcium fluxes were stable for at least 10 min of continuous illumination at the above shutter rate. However, during experiments involving prolonged incubation with drugs, care was taken to reduce fluorescence illumination to only times of data collection. Fluorescence background was determined in areas of the culture lacking neurons and subtracted from all records shown. Results are expressed in units of $\Delta F/F$, where F equals baseline fluorescence in the presence of neurons minus nonneuronal background culture fluorescence. F was typically 3%–5% of the total fluorescence background.

Electrically Evoked Synaptic Calcium Transients

Fluo-3-loaded cultures were stimulated with a bipolar tungsten electrode placed over (but not in contact with) a group of neurons ~1000 μm away from the cells under study. Fluo-3 fluorescence was measured by a photodiode as described above, with the exception that records were taken during continuous fluorescence illumination for 1–1.6 s, with stimulation occurring 100 ms after shutter opening. Stimulation parameters were typically 50–200 μs in duration and 70–90 V.

Immunostaining

Affinity-purified polyclonal antiserum to c-Fos (Oncogene Science) was used for immunostaining. This antiserum was prepared against residues 4–17 of human c-Fos (not contained in Fra proteins). Preincubation of c-Fos serum (0.1 μg/ml) with the c-Fos

peptide at 0.1 μg/ml completely abolished immunostaining. However, incubation of c-Fos serum (0.1 μg/ml) with the corresponding Fos-B peptide at 1 μg/ml did not result in any detectable reduction in basal c-Fos immunostaining.

For immunostaining, cells were fixed for 1 hr in 4% formalin, 0.1 M sodium phosphate buffer (pH 7.4), washed with 0.05 M Tris-Cl (pH 7.4), 15 g/l NaCl (used as a buffer for all subsequent steps), permeabilized with 0.2% Triton X-100, incubated with 3% normal goat serum for 1 hr, and then incubated with primary antiserum in 1% normal goat serum at 4°C overnight. Primary antiserum was used at 0.1 μg of affinity-purified protein per ml. After removal of primary antiserum, cultures were processed using the avidin-biotin-peroxidase method of antibody detection (Vector Labs), with the addition of 0.8 mg/ml $NiCl_2$ in the color development step.

Quantitation of Immunostaining

After immunostaining, cells were preserved in glycerol and examined under Hoffman modulation/bright-field optics. To estimate the percentage of cells exhibiting nuclear immunostaining, 3–5 fields at 50× magnification within duplicate wells were picked at random, and the number of presumed neurons was counted. In these cultures, neurons tend to form distinct clumps of rounded, process-bearing cells, which are readily differentiated using Hoffman optics from flat underlying confluent nonneuronal cells. These cells can be considered neurons since they display the following characteristic features: staining with neuron-specific enolase antiserum (Marangos et al., 1979), spontaneous electrical activity, fast inward and outward voltage-sensitive currents (measured under voltage clamp), and sensitivity to NMDA toxicity (nonneuronal cells in cortical cultures are resistant [Choi et al., 1987]). After estimating the number of neurons per 50× field within an experiment, the number of stained neurons was counted in at least 3 fields in each of 2 duplicate wells (200–400 neurons). As bright-field optics are preferable for visualization of immunostaining and Hoffman optics for identification of neuronal morphology, counting of stained neurons was performed by alternating between these optical modes. To check that observer bias did not affect immunostaining results, cell counts were performed without knowledge of the treatments received by the cultures.

Northern Blot Hybridization

Extraction and preparation of RNA were performed as described (Cole et al., 1990). Briefly, 20 μg of RNA (approximately 2–3 wells of a 12-well plate) was used for each lane. The amount of RNA was estimated by measuring absorbance at 260 nm. Ethidium bromide fluorescence of 18S and 28S ribosomal RNAs indicated that equivalent amounts of RNA were present in each lane of the blots used for cDNA hybridization. Blots were prehybridized at 52°C for 3 hr and hybridized overnight at 52°C as described (Cole et al., 1990). The blots were washed twice for 30 min in 2× SSC, 0.1% SDS at room temperature, followed by four 15 min washes in 0.15× SSC, 0.1% SDS at 58°C. cDNA probes were prepared by nick translation of inserts prepared from Bluescript plasmids containing coding regions of c-*fos*, *fos*-B, *jun*-B, and *zif268*.

Statistics

All results are the means ± SEM of at least 3 separate experiments. Data were analyzed by paired t tests to examine potential group differences.

Acknowledgments

We thank Darla Rodgers for excellent secretarial assistance, Dr. Paul Manis of the Department of Neuroscience for indispensable advice in setting up the fluo-3 photodiode system, and Drs. B. Christy, K. Ryder, Y. Nekabeppu, D. Nathans, and J. Milbrandt for providing plasmids. This work was supported by a National Research Service Award Fellowship (T. H. M.), the Joseph P. Kennedy, Jr. Foundation (J. M. B.), the Lucille P. Markey Charitable Trust (J. M. B.), the Esther and Joseph A. Klingenstein Fund (P. F. W.), and United States Public Health Service grants DA-00266 (J. M. B.) and EY-08900 (P. F. W.). J. M. B. is a Lucille P. Markey Scholar.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received May 24, 1991; revised July 15, 1991.

References

Agoston, D. V., Eiden, L. E., and Brenneman, D. E. (1991). Calcium-dependent regulation of the enkephalin phenotype by neuronal activity during early ontogeny. J. Neurosci. Res. *28*, 140–148.

Ahlijanian, M. K., Westenbroek, R. E., and Catterall, W. A. (1990). Subunit structure and localization of dihydropyridine-sensitive calcium channels in mammalian brain, spinal cord, and retina. Neuron *4*, 819–832.

Campbell, K. P., Leung, A. T., and Sharp, A. H. (1988). The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. Trends Neurosci. *11*, 425–430.

Carboni, E., and Wojcik, W. J. (1988). Dihydropryridine binding sites regulate calcium influx through specific voltage-sensitive calcium channels in cerebellar granule cells. J. Neurochem. *50*, 1279–1286.

Choi, D. W., Maulucci-Gedde, M. A., and Kriegstein, A. R. (1987). Glutamate neurotoxicity in cortical cell culture. J. Neurosci. *7*, 357–368.

Christy, B. A., Lau, L. F., and Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc-finger" sequences. Proc. Natl. Acad. Sci. USA *85*, 7857–7861.

Cole, A. J., Saffen, D. W., Baraban, J. M., and Worley, P. F. (1989). Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature *340*, 474–476.

Cole, A. J., Abu-Shakra, S., Saffen, D. W., Baraban, J. M., and Worley, P. F. (1990). Rapid rise in transcription factor mRNAs in rat brain after electroshock-induced seizures. J. Neurochem. *55*, 1920–1927.

Courtney, M. J., Lambert, J. J., and Nicholls, D. G. (1990). The interactions between plasma membrane depolarization and glutamate receptor activation in the regulation of cytoplasmic free calcium in cultured cerebellar granule cells. J. Neurosci. *10*, 3873–3879.

Goelet, P., Castellucci, V. F., Schacher, S., and Kandel, E. R. (1986). The long and short of long-term memory—a molecular framework. Nature *322*, 419–422.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. *391*, 85–100.

Honore, T., Davies, S. N., Drejer, J., Fletcher, E. J., Jacobsen, P., Lodge, D., and Nielsen, F. E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. Science *241*, 701–703.

Jones, K. A., and Baughman, R. W. (1988). NMDA- and non-NMDA-receptor components of excitatory synaptic potentials recorded from cells in layer V of rat visual cortex. J. Neurosci. *8*, 3522–3534.

Kamiya, H., Sawada, S., and Yamamoto, C. (1988). Synthetic omega-conotoxin blocks synaptic transmission in the hippocampus in vitro. Neurosci. Lett. *91*, 84–88.

Kingsbury, A., and Balazs, R. (1987). Effect of calcium agonists and antagonists on cerebellar granule cells. Eur. J. Pharmacol. *140*, 275–283.

Kley, N., Loeffler, J.-P., Pittius, C. W., and Hollt, V. (1987). Involvement of ion channels in the induction of proenkephalin A gene

expression by nicotine and cAMP in bovine chromaffin cells. J. Biol. Chem. *9*, 4083–4089.

Krishan, A. (1975). Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. *66*, 188–193.

Kuan, Y. F., and Scholfield, C. N. (1986). Ca-channel blockers and the electrophysiology of synaptic transmission of the guinea-pig olfactory cortex. Eur. J. Pharmacol. *130*, 273–278.

Lau, L. F., and Nathans, D. (1990). Genes induced by serum growth factors. In Hormonal Control of Gene Transcription, Molecular Aspects of Cell Regulation, Vol. 6, G. Foulkes and P. Cohen, eds. (Amsterdam: Elsevier Biomedical), pp. 165–202.

Marangos, P. J., Schmechel, D., Parma, A. M., Clark, R. L., and Goodwin, F. K. (1979). Measurement of neuron-specific (NSE) and non-neuronal (NNE) isoenzymes of enolase in rat, monkey and human nervous tissue. J. Neurochem. *33*, 319–329.

Mayer, M. L., Mac Dermott, A. B., Westbrook, G. L., Smith, S. J., and Barker, J. L. (1987). Agonist and voltage gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured with arsenazo III. J. Neurosci. *7*, 3230–3244.

McCleskey, E. W., Fox, A. P., Feldman, D. H., Cruz, L. J., Olivera, B. M., Tsien, R. W., and Yoshikami, D. (1987). Omega-conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc. Natl. Acad. Sci. USA *84*, 4327–4331.

Minta, A., Kao, J., and Tsien, R. Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. *264*, 8171–8178.

Morgan, J. I., and Curran, T. (1986). Role of ion flux in the control of c-*fos* expression. Nature *322*, 552–555.

Morgan, J. I., and Curran, T. (1989). Stimulus-transcription coupling in neurons: role of cellular immediate early genes. Trends Neurosci. *12*, 459–462.

Murphy, T. H., and Baraban, J. M. (1990). Glutamate toxicity in immature cortical neurons precedes development of glutamate receptor currents. Dev. Brain Res. *57*, 146–150.

Murphy, S. N., and Miller, R. J. (1989). Regulation of Ca^{++} influx into striatal neurons by kainic acid. J. Pharmacol. Exp. Ther. *249*, 184–193.

Murphy, T. H., Schnaar, R. L., Coyle, J. T., and Sastre, A. (1988). Glutamate cytotoxicity in a neuronal cell line is blocked by membrane depolarization. Brain Res. *460*, 155–160.

Murphy, T. H., Worley, P. F., Nakabeppu, Y., Christy, B., Gastel, J., and Baraban, J. M. (1991). Synaptic regulation of immediate early gene expression in primary cultures of cortical neurons. J. Neurochem., in press.

Nowycky, M. C., Fox, A. P., and Tsien, R. W. (1985). Three types of neuronal calcium channels with different calcium agonist sensitivity. Nature *316*, 440–443.

Pellegrini-Giampietro, D. E., Galli, A., Alesiani, M., Cherici, G., and Moroni, F. (1989). Quinoxalines interact with the glycine recognition site of NMDA receptors: studies in guinea-pig myenteric plexus and in rat cortical membranes. Br. J. Pharmacol. *98*, 1281–1286.

Regan, L. J., Sah, D. W. Y., and Bean, B. P. (1991). Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. Neuron *6*, 269–280.

Regehr, W. G., and Tank, D. W. (1990). Postsynaptic NMDA receptor mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites. Nature *345*, 807–810.

Regehr, W. G., Connor, J. A., and Tank, D. W. (1989). Optical imaging of calcium accumulation in hippocampal pyramidal cells during synaptic activation. Nature *341*, 533–536.

Ryder, K., Lau, L. F., and Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-*jun*. Proc. Natl. Acad. Sci. USA *85*, 1487–1491.

Sheng, M., and Greenberg, M. E. (1990). The regulation and function of c-*fos* and other immediate early genes in the nervous system. Neuron *4*, 477–485.

Sheng, M., McFadden, G., and Greenberg, M. E. (1990). Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571–582.

Simon, S. M., and Llinas, R. R. (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. Biophys. J. *48*, 485–498.

Smith, S. J., and Augustine, G. J. (1988). Calcium ions active zones and transmitter release. Trends Neurosci. *11*, 458–464.

Szekely, A. M., Barbaccia, M. L., Alho, H., and Costa, E. (1989). In primary cultures of cerebellar granule cells the activation of N-methyl-D-aspartate-sensitive glutamate receptors induces c-*fos* expression. Mol. Pharmacol. *35*, 401–408.

Teo, T. S., and Wang, J. H. (1973). Mechanism of activation of a cyclic adenosine 3′-5′-monophosphate phosphodiesterase from bovine heart by calcium ions. J. Biol. Chem. *248*, 5950–5955.

Triggle, D. J., and Janis, R. A. (1987). Calcium channel ligands. Annu. Rev. Pharmacol. Toxicol. *27*, 347–369.

Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R., and Fox, A. P. (1988). Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci. *11*, 431–438.

Westenbroek, R. E., Ahlijanian, M. K., and Catterall, W. A. (1990). Clustering of L-type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons. Nature *347*, 281–284.

Wisden, W., Errington, M. L., Williams, S., Dunnett, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V. P., and Hunt, S. P. (1990). Differential expression of immediate early genes in the hippocampus and spinal cord. Neuron *4*, 603–614.

Wong, E. H. F., Kemp, J. A., Priestly, T., Knight, A. R., Woodruff, G. N., and Iversen, L. L. (1986). The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. Proc. Natl. Acad. Sci. USA *83*, 7104–7108.

Worley, P. F., Christy, B. A., Nakabeppu, Y., Bhat, R. V., Cole, A. J., and Baraban, J. M. (1991). Constitutive expression of zif268 in neocortex is regulated by synaptic activity. Proc. Natl. Acad. Sci. USA *88*, 5106–5110.

Yu, C., Jia, M., Litzinger, M., and Nelson, P. G. (1988). Calcium agonist (BayK-8644) augments voltage-sensitive calcium currents but not synaptic transmission in cultured mouse spinal cord neurons. Exp. Brain Res. *71*, 467–474.

Yuste, R., and Katz, L. C. (1991). Control of postsynaptic Ca^{2+} influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron *6*, 333–344.

Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H., and Lindholm, D. (1990). Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J. *9*, 3545–3550.

Zerial, M., Toschi, L., Ryseck, R.-P., Schuermann, M., Miller, R., and Bravo, R. (1989). The product of a novel growth factor activated gene, FosB interacts with JUN proteins enhancing their DNA binding activity. EMBO J. *8*, 805–813.