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In Primary Cultures of Cerebellar Granule Cells the Activation of *N*-Methyl-D-aspartate-Sensitive Glutamate Receptors Induces c-fos mRNA Expression

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SUMMARY

L-Glutamate, the natural agonist of quisqualate- and *N*-methyl-D-aspartate (NMDA)-sensitive excitatory amino acid receptors, elicits a rapid, transient, dose-dependent increase of the steady state level of c-fos mRNA followed by an accumulation of c-fos protein immunostaining in cell nuclei. This induction is prevented by 2-amino-5-phosphonovalerate, an isosteric glutamate receptor antagonist, and by Mg^{2+} ion and phencyclidine, two noncompetitive allosteric antagonists of NMDA-sensitive glutamate receptors. Kainate and quisqualate (up to 150 μM) failed to alter the basal expression of c-fos mRNA. Furthermore, glycine, a positive allosteric modulator of NMDA-sensitive glutamate receptors, potentiated the glutamate response in a strychnine-insensitive manner. Activation of other transmitter receptors present in these cells (γ-aminobutyric acid$_A$, γ-aminobutyric acid^B, and muscarinic) failed to increase c-fos mRNA expression. Our results provide evidence that activation of NMDA-sensitive glutamate receptors plays an exclusive role in the induction of c-fos mRNA expression and translation in primary cultures of granule cells. It can be inferred that, by this mechanism, glutamate can initiate a transcriptional program that may result in changes in the simultaneous expression of a set of target genes involved in neuron-specific responses.

In neuron-to-neuron signaling, the activation of transmitter receptors by the primary transmitter generates precise messages for the postsynaptic neurons, leading to functional modifications of regulatory processes located in plasma membranes, cytosol, and nucleus. During long-term plastic changes of neuronal function, a coordinated change in the expression of a set of genes may be required (1). In the past years a number of phylogenetically conserved genes have been identified as cellular homologs of retroviral transforming genes, termed proto-oncogenes. They are thought to control physiological processes of development, growth, and differentiation (2). In nonneuronal cells, the *c-fos* proto-oncogene is of particular interest; it can be rapidly and transiently induced at the level of transcription by a variety of external stimuli (growth factors, mitogens, differentiation-specific agents). In differentiated neuronal cells, synaptic signaling appears to be prominent in causing this induction (3) and, thereby, increasing the nuclear content of the phosphoprotein encoded by the c-fos mRNA, suggesting that c-fos protein may regulate gene expression (4). In fact, c-fos protein appears to be involved in the transcriptional regulation of some genes (5, 6), inasmuch as it interacts with other nuclear protein(s) like the transcription factor AP-1 encoded by another proto-oncogene, termed *c-jun* (7, 8). It can be inferred that *c-fos* may be of value in providing a coordinated regulation of the expression of cell-specific target genes encoding for proteins participating in neuronal plasticity responses. However, so far only an indirect relationship between *in vivo* neuronal activation and increase of *c-fos* expression has been shown in mammalian brain (9–12).

The activation of excitatory amino acid receptors sensitive to NMDA appears to play a pivotal role in the development of transsynaptically induced neuronal plasticity in both *in vivo* and *in vitro* models used to study learning, memory consolidation, or seizure activity (13–15). This information prompted us to study whether stimulation of specific excitatory amino acid receptors induces the expression of *c-fos* proto-oncogene in primary cultures of cerebellar granule cells. These cultures include a homogeneous neuron population expressing various excitatory amino acid receptor subtypes operating with metabolotropic and ionotropic effector systems similar to those described previously in the mammalian central nervous system (for review see Refs. 16 and 17). The stimulation of the NMDA-sensitive glutamate receptors increases PI hydrolysis (18, 19) and opens predominantly high conductance cationic channels allowing Ca^{2+} influx (20, 21). These events contribute to a transient elevation of free cytosolic Ca^{2+} content; when the

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; APV, 2-amino-5-phosphonovalerate; PKC, protein kinase C; PCP, phencyclidine; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PI, phosphatidylinositol; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DOG, 1-α-dioctanylglycerol; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

receptor stimulation is protracted and the increase of free Ca^{2+} is prolonged, the activation and translocation of PKC enzymes occurs (22). These NMDA-evoked responses are characteristically blocked by Mg^{2+} in a voltage-dependent manner (23) and are reduced by the isosteric glutamate receptor antagonist APV (16) and by the noncompetitive allosteric receptor inhibitor PCP (24). Moreover, the NMDA-sensitive glutamate receptor domain includes an allosteric modulatory center where glycine appears to act as a positive allosteric modulator (21, 25). Kainate, a synthetic chemical analog of glutamate, acts on a recognition site linked preferentially to low-conductance cationic channels, resulting predominantly in an influx of Na^+ and K^+ (20). Quisqualate appears to act on still another recognition site, which is probably linked via a G_o protein (26, 27) to a phospholipase C functioning as a generator of metabolotropic signals that may also activate low-conductance cationic channels. Kainate- and quisqualate-sensitive excitatory amino acid receptors are resistent to Mg^{2+}, APV, and PCP inhibition (for review see Ref. 28).

The present report demonstrates that the activation of NMDA-sensitive glutamate receptors induces the expression of *c-fos* proto-oncogene mRNA, followed by an increase of the nuclear content of c-fos protein immunoreactivity. In contrast, the stimulation of kainate- and quisqualate-sensitive excitatory amino acid receptors fails to activate *c-fos* proto-oncogene transcription.

Materials and Methods

Cell culture. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rats (Zivic Miller, Allison Park, PA) as previously described (29). The cells were grown in basal Eagle's medium (GIBCO, Grand Island, NY), supplemented with 10% fetal calf serum (GIBCO), 25 mM potassium chloride, 2 mM glutamine, and 50 μg/ml gentamycin. The experiments were performed on the 8th or 9th day of the cell culture. As previously reported (30), over 90% of the cells are glutamatergic granule cells with minimal contamination (less than 3%) of glial and endothelial elements. The presence of inhibitory GABAergic neurons (Purkinje, stellate, basket, and Golgi cells) amounts to about 5% of the total cell population. The granule cell monolayers grown on 150-mm culture dishes (Nunc) were washed once and preincubated for 15 min in Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) at 37° in a humidified atmosphere (5% CO_2/95% O_2). $MgCl_2$ (1 mM) was added to the buffer where indicated. After the different agonists were added, the incubation was continued for 40 min. The antagonist drugs were added to the culture 5 min before the agonist, except nitrendipine, which was preincubated for 15 min.

Poly (A)$^+$ RNA preparation and Northern blot. All solutions contained 0.1% diethylpyrocarbonate to inhibit RNA degradation by ribonucleases during the extraction procedure. At the end of the incubation, the cell monolayers were washed with ice-cold PBS and were lysed in a 5 M solution of guanidium isothiocyanate containing 100 mM Tris·HCl and 1 mM EDTA at pH 7.4. The cell lysate was passed five times through a 21-gauge needle to shear genomic DNA, overlaid on a 5.7 M $CsCl_2$ cushion, and ultracentrifuged for 16 hr to pellet RNA. After ethanol precipitation, total cellular RNA was resuspended in Tris/EDTA buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA) and applied to an oligo(dT) cellulose affinity column to select poly(A)$^+$ RNA. The poly(A)$^+$ RNA was size-fractionated on a denaturing 2.2 M formaldehyde/1% agarose gel by electrophoresis for 6–8 hr at 60 V. After electrophoresis, the RNA was transferred overnight to a nitrocellulose filter (Schleicher and Schuell, Keene, NH) by capillary blotting in 20× SSC according to standard procedures (1× SSC buffer contains 0.015 M trisodium citrate and 0.15 M NaCl). The filters then were rinsed in 2× SSC and baked at 80° for 2 hr. Baked filters were prehybridized in a solution containing 50% formamide, 8× Denhart's solution (1× contains 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin), 0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and 0.2% SDS, for 12 hr at 42°. The hybridization with the ^{32}P-labeled c-fos probe ($1–2 \times 10^{-6}$ cpm/ml) was carried out at 42° for 24–36 hr in the solution described for prehybridization except containing 5× Denhart's solution. Hybridization with the β-actin cDNA probe was carried out at 42° overnight. Final washings were in 0.1× SSC with 0.1% SDS at 55° for c-fos and 0.1× SSC with 0.1% SDS at 42° for filters probed with β-actin. The filters were exposed to KODAK X-OMAT film with a DuPont Cronex Lightning Plus intensifying screen at −70°. The amount of c-fos mRNA was quantified by densitometric analysis, where the intensity of the fos signal was corrected for variation in the amount of poly(A)$^+$ RNA loaded on the gel by rehybridizing the same blot with β-actin.

Probe preparation. The mouse *c-fos* genomic clone (31) was digested with the *Sau*I restriction enzyme (Boehringer-Mannheim, Indianapolis, IN) to obtain a 1.2-kb fragment encoding exon 4. The fragment was gel purified and labeled by nick translation, using [^{32}P]dATP and [^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA), to a specific activity of 5×10^8 cpm/μg of DNA. The rat β-actin gene (32) was a gift of Dr. I. Mochetti (FIDIA-Georgetown Institute for the Neurosciences).

Immunohistochemistry. The cells were fixed for 30 min at room temperature in 3% paraformaldehyde solution (pH 7.4 with PBS), washed twice for 10 min with PBS containing 10 mM glycine, and, thereafter, permeabilized with PBS/glycine plus 0.5% Nonidet P-40 for 5 min. After washing, the cells were incubated with affinity-purified polyclonal rabbit c-fos antibody (33) in 1:100 dilution for 24 hr at 4°. The antibody, which precipitates p^{55} c-fos and p^{55} v-fos proteins, was raised against a synthetic 27-amino acid fragment (127–152) conserved in c-fos and v-fos protein. The bound antibody was detected by biotin-conjugated secondary antiserum (1:100), subsequently incubated with avidin-peroxidase complex (ABC Kit; Vector Laboratories Inc., Burlingame, CA) and reacted with 0.2 mg/ml 3,3′-diaminobenzidine and hydrogen peroxide for 15 min at room temperature.

Intravital staining of the culture. After treatment with excitatory amino acid receptor agonists, the monolayers were stained for 3 min at 22° with fluroescein diacetate (15 μg/ml) and propidium iodide (4.6 μg/ml) mixture according to Jones and Senft (34). The stained cells were examined immediately with a standard epi-illumination fluroescence microscope. Fluorescein diacetate crosses the cell membranes and is hydrolyzed by intracellular esterases to produce green-yellow fluorescence. Neuronal injury curtails fluorescein diacetate staining and facilitates propidium iodide penetration and interaction with DNA to yield a bright red fluorescent complex.

Results

In a preliminary note (3), we reported that incubation of granule cell monolayers with L-glutamate increases the steady state content of c-fos poly(A)$^+$ RNA. Fig. 1A shows that glutamate (10 μM) in the absence of extracellular Mg^{2+} elicited a significant 8-fold increase of c-fos mRNA content. The Northern blot demonstrated that this increase is due to a single mRNA species of 2.2-kb size. In contrast, the same treatment failed to alter the expression of mRNAs corresponding to the structural protein β-actin. Because neither β-actin expression nor others (ras^{Ha} or $G_{i\alpha 2}$, data not shown) change during the 4 hr of post-glutamate treatment tested, we infer that the increase of c-fos mRNA was not due to a general enhancement of mRNA expression and this allowed us to use β-actin hybridization to monitor and simultaneously correct for variations in the amount of poly(A)$^+$ RNA loaded on the gels. The c-fos mRNA induction appeared 15–20 min after glutamate exposure, reach-

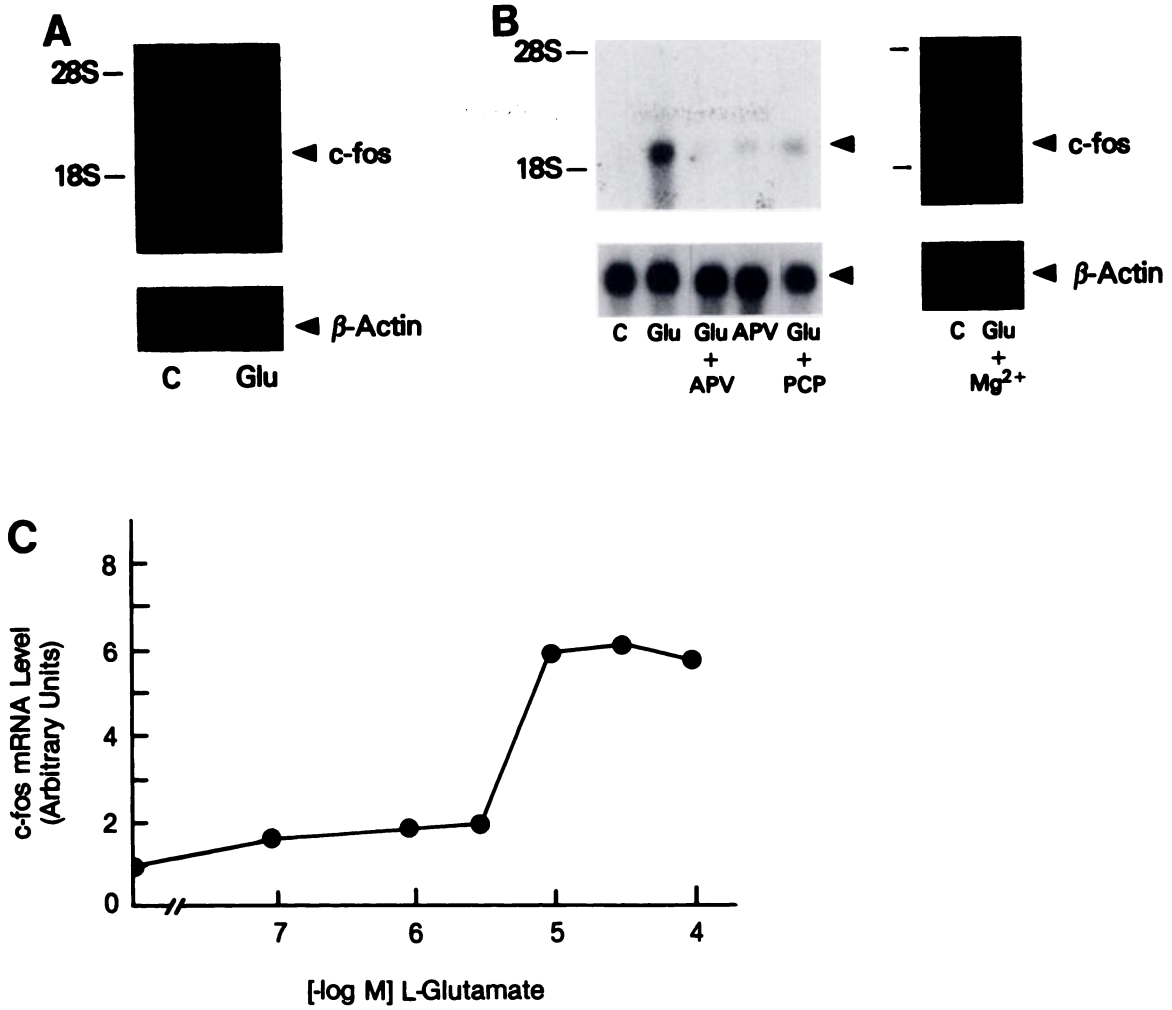

Fig. 1. L-Glutamate induces *c-fos* expression in primary cultures of cerebellar granule cells. A, Northern blot analysis of poly(A)$^+$ mRNA prepared from control (*C*) and glutamate (*Glu*)-treated (10 μM, 45 min) granule cell monolayers. The position of 28S and 18S ribosomal RNA, as well as of the 2.2-kb c-fos and 1.8-kb β-actin messages, is indicated. B, APV (100 μM), Mg^{2+} (1 mM), and PCP (700 nM) antagonize the increase in c-fos mRNA induced by glutamate (10 μM, 45 min). C, Dose-response of c-fos expression induced by L-glutamate (40 min). The experiments were carried out in Mg^{2+}-free Locke's solution; the c-fos mRNA content was analyzed, as described in Materials and Methods. The c-fos mRNA level is expressed in arbitrary units, where 1 unit is defined as the peak densitometric area of c-fos mRNA hybridization divided by the corresponding peak densitometric area of β-actin mRNA hybridization.

ing maximal levels at 40–50 min. Fig. 1C shows that the dose-response relationship of glutamate response is rather steep, with a threshold between 1 and 5 μM and a maximum at 10 μM. Using intravital staining with fluorescein diacetate/propidium iodide (34), we documented that 90–95% of the neurons were viable up to 4 hr after the treatment with glutamate, up to 50 μM.

Because granule cells contain various excitatory amino acid receptor subtypes, we investigated whether the induction of *c-fos* expression was the consequence of the preferential activation of a specific subclass of excitatory amino acid receptors. As Fig. 1B demonstrates, the stimulatory action of glutamate on c-fos mRNA content (corrected for β-actin values) is virtually blocked by APV (100 μM), an isosteric antagonist of NMDA-sensitive glutamate receptors. In the presence of 1 mM Mg^{2+} or 700 nM PCP, two highly selective, noncompetitive, voltage-dependent blockers of NMDA receptors, glutamate failed to significantly induce c-fos mRNA expression. These compounds alone, as well as the PCP solvent dimethylsulfoxide (final concentration, 0.01%) failed to modify the basal c-fos mRNA signal.

By adding PCP at various times after glutamate exposure, we could study the minimum time period of glutamate receptor stimulation necessary to increase c-fos mRNA. Fig. 2 shows that application of 10 μM glutamate followed 5 min later by 700 nM PCP was sufficient to elicit an increase of c-fos mRNA content, which peaked after 30 min (2.8-fold) and declined to the basal level by 120 min. When PCP was added together with glutamate, a change in c-fos mRNA content was virtually absent. The data of Fig. 2 also show that, when PCP was added 10 or 30 min after glutamate, the increase of c-fos mRNA became progressively stronger, suggesting that the extent of c-fos mRNA induction depends on the duration of glutamate receptor activation. These results suggest that a short-lived activation of transmembrane signaling elicited by the NMDA-sensitive glutamate receptor is sufficient to initiate a cascade of intracellular events that, in turn, brings about an increase in c-fos mRNA expression.

The selective involvement of the NMDA-sensitive glutamate receptors in triggering c-fos mRNA induction was further characterized by using structural analogs of glutamate. Fig. 3A

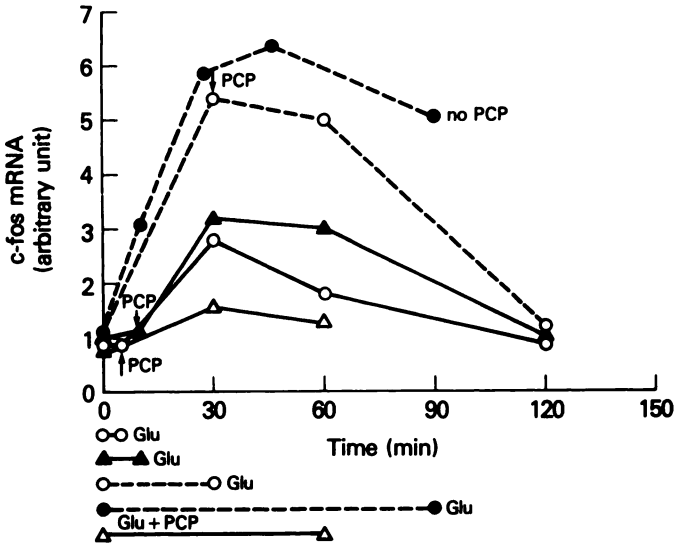

Fig. 2. Time-dependent increase of c-fos mRNA following brief stimulation of granule cells with glutamate (10 μM, added at time 0). No PCP (●–●) or 700 nM PCP was added at time 0 together with glutamate (*Glu*). (△—△), 5 min after glutamate (○—○), 10 min after glutamate (▲–▲), or 30 min after glutamate (○–○). The incubation was continued for various time periods as indicated, then the cells were harvested and the c-fos mRNA content was analyzed, as described under Materials and Methods.

shows that in Mg^{2+}-free medium, NMDA mimicked the action of glutamate. At 50 μM concentrations, it significantly (by 6–7-fold) increased c-fos mRNA content and this c-fos induction by NMDA was almost entirely (90%) inhibited by PCP (700 nM). We also studied the effect of quisqualate and kainate in granule cells. The experiments were performed in medium containing 1 mM Mg^{2+} in order to inhibit the possible effect of the endogenous glutamate that may have been released by the depolarization associated with the activation of kainate- and quisqualate-sensitive receptors. Fig 3B indicates that 50 μM quisqualate and kainate are unable to increase *c-fos* expression. Application of higher concentrations of these two amino acids (100–150 μM) also failed to produce a significant and consistent increase of c-fos mRNA (data not shown). Although quisqualate failed to elicit neurotoxicity even at 150 μM, intravital staining of the neurons (34) following the 40-min incubation with 100 μM kainate showed clear signs of neuronal degeneration and death.

Whereas PCP, a noncompetitive antagonist of NMDA-sensitive glutamate receptors, prevented the action of glutamate, pretreatment of the cells with nitrendipine (1 μM), a selective blocker of voltage-sensitive Ca^{2+} channels, failed to change the glutamate-evoked c-fos mRNA increase (Fig. 3B). Furthermore, massive granule cell depolarization elicited by 50 mM potassium or 5 μM veratridine also increased c-fos mRNA content, but this increase was inhibited by PCP and APV, suggesting that the endogenous glutamate released by depolarization mediated this response (data not shown).

Moreover, Fig. 4 demonstrates that glycine, a specific putative allosteric modulator of NMDA-sensitive glutamate receptors, in dose that by itself failed to increase c-fos mRNA content, potentiates the c-fos mRNA induction evoked by a threshold dose (5 μM) of glutamate. This glycine effect was strychnine insensitive (50 μM glycine, 25 μM strychnine); strychnine alone also failed to modify the basal level of c-fos mRNA.

In cultured cerebellar granule cells, the activation of muscarinic, $GABA_A$, or $GABA_B$ receptors failed to modify the c-fos mRNA content. Fig. 5 demonstrates that carbachol (5 μM), a highly potent activator of PI turnover through the stimulation of muscarinic M_2 receptors (24), failed to increase c-fos mRNA content. Similarly, pharmacologically effective doses of GABA (10 μM) or of the selective $GABA_B$ receptor agonist (−)-baclofen (25 μM) were found to be unable to modify c-fos mRNA content.

In primary cultures of cerebellar granule cells, the stimulation of NMDA-sensitive glutamate receptors triggers a signifi-

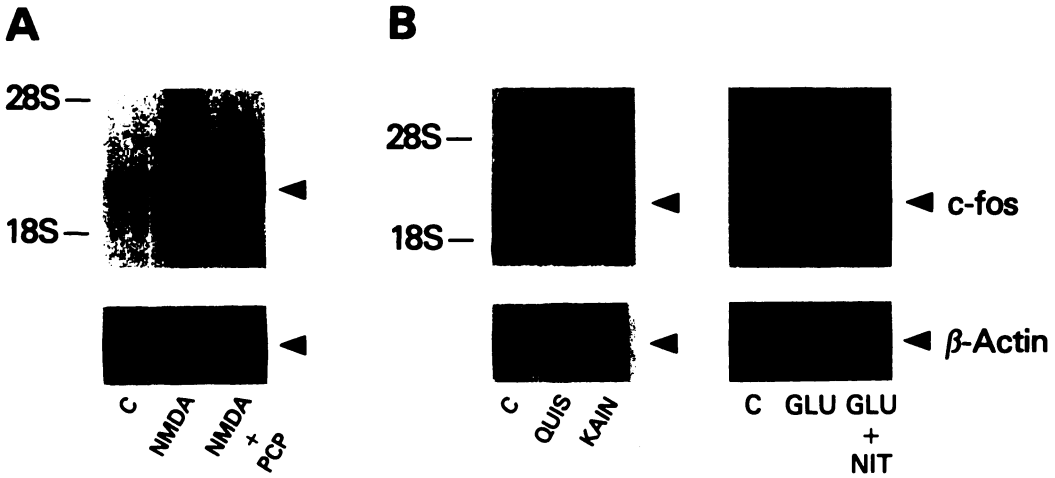

Fig. 3. Receptor selectivity of the L-glutamate-induced expression of the *c-fos* proto-oncogene. A, NMDA mimicks the action of glutamate on the c-fos mRNA level. The granule cells were washed and preincubated in Mg^{2+}-free Locke's solution, then further incubated with 50 μM NMDA for 40 min in the absence and presence of 700 nM PCP. B, Quisqualate (*QUIS*) and kainate (*KAIN*) fail to induce c-fos mRNA expression; both drugs at 50 μM final concentration were added after the preincubation period, using Locke's solution containing 1 mM Mg^{2+}, and incubated for 40 min (*left*). Nitrendipine does not reduce glutamate-induced c-fos mRNA expression; after 15-min preincubation with 1 μM nitrendipine, (NIT) 10 μM glutamate (*GLU*) was added and the cells were further incubated for 30 min in the absence of Mg^{2+} (*right*). The *arrowheads* indicate the position of the c-fos and β-actin mRNA. *C*, control.

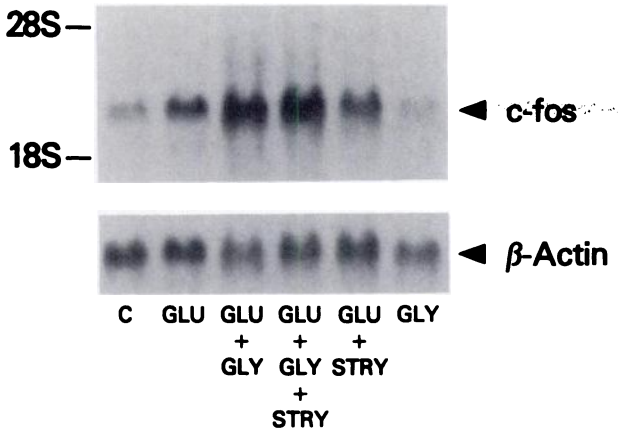

Fig. 4. Glycine potentiates the effect of glutamate on *c-fos* expression; the cells were incubated with glutamate (*GLU*) (5 μM) in combination with glycine (*GLY*) (50 μM) and strychnine (*STRY*) (25 μM) in Mg^{2+}-free Locke's solution for 40 min. *C*, control.

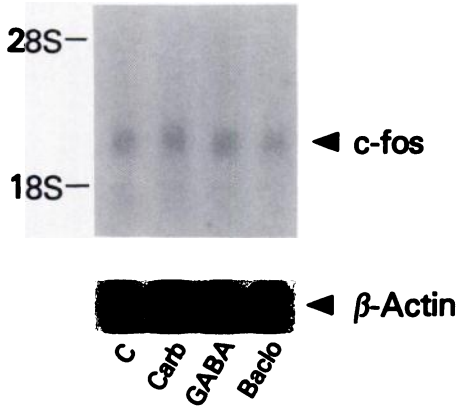

Fig. 5. Cholinergic and GABAergic agonists fail to modify the expression of the *c-fos* proto-oncogene in primary cultures of cerebellar granule cells. The monolayers were incubated with 5 μM carbachol (*Carb*), 10 μM GABA, and 25 μM (−)-baclofen (*Baclo*) for 40 min in Mg^{2+}-containing Locke's buffer. *C*, control.

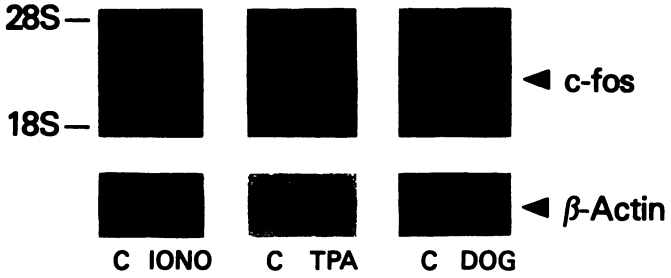

Fig. 6. The Ca^{2+} ionophore ionomycin and PKC activators TPA and DOG induce c-fos mRNA expression. The cells were incubated with ionomycin (*IONO*) (2 μM) in the presence of 1 mM Mg^{2+} and 1 μM PCP, with TPA (100 nM) and with DOG (60 μM) for 30 min. The *arrowheads* indicate the position of the c-fos and β-actin mRNA. *C*, control.

cant rise in intracellular Ca^{2+} (21) and a rapid increase in PI turnover (19), resulting in the activation and membrane translocation of PKC (22). To evaluate which putative intracellular signal is involved in *c-fos* activation, we used different pharmacological tools and found that increasing the cell Ca^{2+} content by the ionophore ionomycin (2 μM) can increase c-fos mRNA expression (Fig. 6). The drug is tested in the presence of 1 mM Mg^{2+} and 1 μM PCP in order to exclude the indirect effects of the endogenous glutamate that might be released by ionomycin. On the other hand, stimulation of PKC by an analog of the endogenous diacylglycerol, DOG, and by the tumor promoter phorbol ester TPA also induced the expression of c-fos mRNA. DOG at the concentration of 60 μM failed to evoke channel openings in outside-out patches excised from granule cells.[1] In contrast, addition of lipophilic analogs of cAMP or cGMP (dibutyril cAMP and dibutyril cGMP up to 1 mM) did not increase the level of c-fos mRNA (data not shown).

Immunocytochemical analysis performed with an affinity-purified c-fos antibody directed against the c-fos mRNA translation product showed that the induction of c-fos mRNA by glutamate was followed by an accumulation of immunoreactivity in the granule cells. This immunoreactivity appears to be located in cell nuclei; however, because of the scarcity of cytosol in granule cells, it is difficult to be absolutely sure by light microscopy that it was an exclusive nuclear localization. Whereas control cultures displayed a very weak sporadic staining of granule cell nuclei (Fig. 7A), the treatment of the cerebellar neuronal cultures with glutamate (10μM) for 20 min increased the nuclear c-fos protein immunostaining (Fig. 7B). It became more intense after 40 min (Fig. 7C) and was still present after 90 min. The increase of nuclear immunoreactivity evoked by glutamate (10 μM, 40 min) was greatly reduced by the simultaneous addition of glutamate and PCP (700 nM) (Fig. 7D). The specificity of the immunochemical reaction for the c-fos protein was shown by the lack of immunostaining following preabsorption of the antibody for the c-fos mRNA translation product with the synthetic peptide that was used as antigen (Fig. 7E).

Discussion

In primary cultures of cerebellar neurons, the excitatory amino acid glutamate induces the expression of *c-fos* proto-oncogene mRNA and the accumulation of c-fos protein immunoreactivity in the nuclei. The glutamate-evoked increase of c-fos mRNA content is prevented by specific NMDA receptor antagonists such as APV, PCP, and Mg^{2+}. These results indicate that the activation of the NMDA-sensitive glutamate receptors is preferentially involved in triggering this nuclear event. Quisqualate receptor activation may not be operative, because of the lack of glutamate action in the presence of the three specific inhibitors of NMDA-sensitive glutamate receptors. This conclusion is further supported by direct experiments showing that only NMDA mimicked the action of glutamate, whereas quisqualate and kainate, in maximal tolerated doses, were unable to increase c-fos expression. Activation of muscarinic or GABAergic receptors also failed to increase c-fos mRNA expression. The NMDA receptor specificity in inducing c-fos mRNA is further strengthened by the potentiation of the response to threshold doses of glutamate with doses of glycine that by themselves were inactive. Inasmuch as the effect of glycine was not blocked by strychnine, glycine is not acting via the activation of the inhibitory glycine receptor linked to the Cl^- channel that is strychnine sensitive. This finding suggests that glycine potentiation is mediated by the specific activation of the allosteric modulatory center of the NMDA-sensitive glutamate receptor and, therefore, is further evidence in support

[1] M. Bertolino, personal communication.

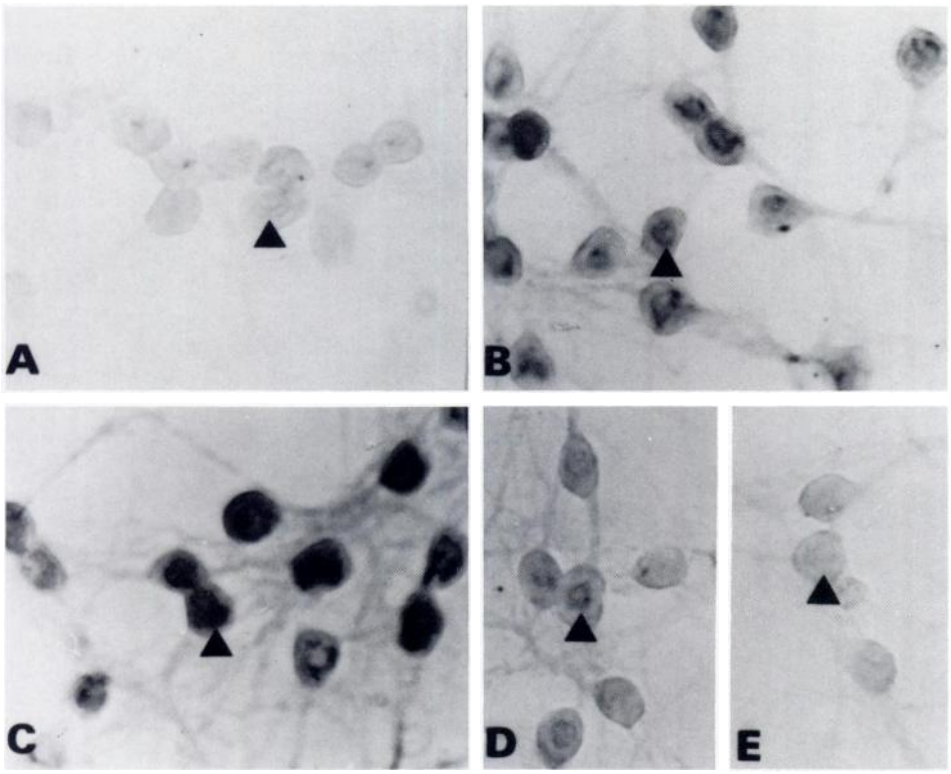

Fig. 7. Time course of the appearance of c-fos protein immunoreactivity in cerebellar granule cells after treatment with glutamate. Control culture (A) shows weak sporadic nuclear immunoreactivity; culture treated with 10 μM glutamate for 20 min displays an increased staining (B); treatment for 40 min shows intense nuclear staining (C); coadministration of PCP (700 nM) with glutamate greatly reduces the c-fos immunoreactivity following a 40-min incubation (D); preabsorption of the antisera with synthetic M peptide (15 μg/ml) abolishes the glutamate-induced staining (E). *Bar* represents 10 μM. In each panel the *black arrowheads* point to the cell nucleus.

of the view that this glutamate receptor subtype selectively mediates c-fos mRNA induction.

The precise cascade of events following the NMDA-sensitive glutamate receptor activation that ultimately leads to the increase in c-fos mRNA expression is not completely known. In primary cultures of neonatal rat cerebellar granule cells, the signal transduction mechanisms coupled to the activation of NMDA-sensitive glutamate receptors are 1) enhanced PI hydrolysis, 2) activation of high-conductance cationic channels, and 3) increase in arachidonic acid formation (35). The phospholipase C activation provides two second messengers, i.e., inositol-1,4,5-triphosphate, which brings about a rapid increase in cytosolic free Ca^{2+}, and diacylglycerol, which in the presence of Ca^{2+} activates PKC. The stimulation of the receptor-operated high-conductance cationic channels leads to a sustained increase of free cytosolic Ca^{2+}, predominantly due to Ca^{2+} influx. The NMDA receptor selectivity mediating the c-fos mRNA increase suggests that simultaneous stimulation of Ca^{2+} influx and the PI turnover activation, perhaps through the intervening PKC activation, may be necessary signals for a c-fos mRNA increase. In contrast, the activation of PI turnover by carbachol and quisqualate, in the absence of extracellular Ca^{2+} influx and translocation of PKC (22), appears to be insufficient to induce c-fos mRNA expression. That quisqualate fails to induce an important increase of Ca^{2+} influx is consistent with the incapacity of quisqualate to cause neuronal death in cerebellar granule cells (36). It seems that an interaction between Ca^{2+} influx, activation of diacylglycerol formation, and PKC translocation may be required for the increase of c-fos mRNA transcription. This possibility is supported by recently published data showing that, in isolated hippocampal neurons, both glutamate and NMDA application evoke a summation of two successive intracellular Ca^{2+} gradients; such a summation fails to occur when PKC activation and translocation are inhibited by sphingosine (37). The glutamate effect on c-fos expression is inhibited by the NMDA-sensitive glutamate receptor blockers PCP and Mg^{2+}, whereas it is potentiated by glycine, a positive allosteric modulator of glutamate-operated cationic channels (21, 25). It is also important to consider that nitrendipine, at a concentration that in granule cells blocks at least 50% of the voltage-sensitive Ca^{2+} channels (L type) (38), fails to inhibit the glutamate induction of c-fos mRNA. These observations suggest that Ca^{2+} entering through high-conductance cationic channels regulated by glutamate, together with other messengers formed following this transmitter stimulation, mediate the increase of c-fos mRNA. A role of Ca^{2+} in promoting c-fos mRNA expression is further suggested by the experiment with ionomycin. To prove that the effect of glutamate depends on Ca^{2+} influx, the obvious step is to repeat the experiment with glutamate in Ca^{2+}-free medium. Unfortunately, this condition is not suitable for the stimulation of NMDA-sensitive glutamate receptors, because the simultaneous removal of Ca^{2+} and Mg^{2+} from the medium results in neuronal degeneration and death. More work is necessary to construct the appropriate experimental model to study the role of Ca^{2+} in c-fos mRNA induction in cerebellar granule cells. That the ultimate step may be the PKC activation is consistent with the finding that DOG and TPA also evoke c-fos mRNA induction, whereas the addition of lipophilic cAMP or cGMP analogs fails to change the c-fos mRNA level, suggesting that PKC but not cyclic nucleotide-dependent kinases may be involved in c-fos mRNA induction in granule cells. This is in contrast to the induction of c-fos mRNA expression in nonneuronal cells (39) or astrocytoma cell lines.[2] Taken together, these

[2] This laboratory, unpublished observation.

data imply that Ca^{2+}-dependent PKC activation and translocation may be the specific intracellular signal involved in the glutamate-stimulated increase of c-fos mRNA expression.

The glutamate-evoked induction of c-fos mRNA is associated with active translation, as documented by a parallel increase in the nuclear c-fos protein immunoreactivity. A relationship between *in vivo* perturbation of neuronal activity and increase of c-fos expression has been previously suggested. i) c-fos immunoreactivity is increased in subsets of rat spinal cord neurons in response to afferent sensory stimulation (9). ii) c-fos immunoreactivity is increased in discrete brain regions following administration of convulsant agents (11) or following electrical stimulation evoking seizure activity (10, 12). In rats, pharmacologically induced convulsions increase the c-fos protein immunostaining in hippocampal regions, which are particularly rich in NMDA-sensitive glutamate receptors (40). The involvement of these receptors in enhanced synaptic activity elicited by repetitive electrical stimulation during kindling also has been reported (41). The present data raise the appealing possibility that the increased c-fos immunoreactivity following seizure activity is a consequence of the activation of the NMDA-sensitive glutamate receptors. Thus, the c-fos protein may act as a third messenger by interacting with other nuclear protein(s) to form a complex that may bind to regulatory elements of a set of target genes, coding for proteins mediating long-term neuronal changes in response to NMDA-sensitive glutamate receptor stimulation.

The present data are particularly interesting in light of the suggested involvement of the NMDA-sensitive glutamate receptor in *in vivo* and *in vitro* models of learning processes and memory consolidation (13, 15, 42, 43), where a role of Ca^{2+} and Ca^{2+}-dependent enzyme (such as PKC) signaling has been proposed (44–46). Thus, the present experiments have contributed to the elucidation of one of the processes whereby, stimulating the synthesis of a nuclear protein acting as third messenger, a transmitter initiates the synthesis of proteins necessary for the analysis, categorization, or storage of incoming information in neurons. Moreover, our results not only suggest that the induction of the *c-fos* proto-oncogene is part of the signal transduction cascade elicited by stimulation of the NMDA-sensitive glutamate receptors but also propose a new approach to the study of intrinsic processes operative in neuronal function.

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