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Differential Postreceptor Signaling Events Triggered by Excitotoxic Stimulation of Different Ionotropic Glutamate Receptors in Retinal Neurons

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The aim of this work was to investigate whether excitotoxicity induced by overstimulation of different ionotropic glutamate receptors could trigger different intracellular signaling cascades. Cultured chick neuronal retina cells, essentially amacrine-like, were particularly sensitive to the toxicity induced by non-NMDA glutamate receptor agonists. One hour stimulation with 100 μM kainate induced a reduction of cell viability of about 44%, as assessed by the MTT test 24 hr after stimulation. Kainate-induced toxicity was mediated through AMPA receptors. Glutamate (100 μM, 1 hr) reduced cell viability by 26%, essentially acting through N-methyl-D-aspartate receptors. Five hours after stimulation, neuronal retina cells had an apoptotic-like nuclear morphology. In retinal neurons, the excitotoxic stimulation, with either glutamate or kainate, induced a calcium-dependent enhancement of the DNA-binding activity of the activating protein-1 (AP-1) transcription factor, which was maximal 2 hr after stimulation. Glutamate induced a greater increase in the AP-1 DNA-binding activity than did kainate. Supershift assays using antibodies directed against different members of the Fos and Jun protein families showed that the AP-1 complex in retinal neurons includes proteins of the Fos family, namely, Fra-2, c-Jun, and Jun D. The DNA-binding activity of the nuclear factor-κB transcription factor was not significantly changed upon excitotoxic stimulation with any agonist. Stimulation of glutamate receptors with 100 μM kainate or 100 μM glutamate for 2 min was sufficient to induce the activation of the extracellular signal-regulated kinase (ERK). Inhibition of the ERK activation with the MEK inhibitors U 0126 and PD 98059 increased the toxicity induced by kainate but was without effect on the toxicity induced by glutamate. These results indicate that, although stimulation with both glutamate receptor agonists increased ERK phosphorylation, only kainate-induced ERK activation correlates with the activation of a survival signaling pathway. Our results suggest that, in chick embryo retinal neurons, the signaling pathways that mediate excitotoxic cell death and neuroprotection are stimulus specific. J. Neurosci. Res. 66:643–655, 2001.

Key words: AMPA receptors; AP-1 transcription factor; extracellular signal regulated-kinase; neuroprotection; apoptosis

Excitotoxic neurodegeneration involves overactivation of ionotropic glutamate receptors (Olney, 1978; Choi, 1988), which consist of the N-methyl-D-aspartate (NMDA) and non-NMDA receptors, including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) receptors (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). Excitotoxicity has been implicated in several neuronal disorders, such as cerebral ischemia, epilepsy, and Huntington's, Parkinson's, and Alzheimer's diseases (Bittigau and Ikonomidou, 1997; Martin et al., 1998; Doble, 1999; White et al., 2000). Ischemia or excitotoxic insults to the rabbit or chick retina point to a high sensitivity of retinal amacrine cells to overstimulation of the ionotropic glutamate receptors (Osborne and Herrera, 1994; Zeevalk and Nicklas, 1994; Osborne et al., 1995, 1996; Chen et al., 1999; for review see Duarte et al., 1998).

The overactivation of glutamate receptors can induce apoptotic cell death by a mechanism involving calcium influx (Ankarcrona et al., 1995; Glazner et al., 2000), and such toxicity may occur in acute neurodegenerative conditions, such as ischemia, trauma, and severe epileptic seizures, as well as in Alzheimer's disease and motor system disorders (Choi, 1996; Mattson, 2000). Often, the intensity of the same initial insult decides the prevalence of either apoptosis or necrosis (Bonfoco et al., 1995).

Apoptosis can be attenuated, in some cases, by inhibitors of protein synthesis, suggesting that transcription and translation of new products could be important during

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cell death. The induction of the activator protein-1 (AP-1) transcription factor has been associated with apoptosis in neuronal cells (Kaminska et al., 1994; Finiels et al., 1995; Kasof et al., 1995; Wenzel et al., 2000). AP-1 is a homodimeric and/or heterodimeric protein complex composed of members of the Fos and Jun families (Angel and Karin, 1991; Herdegen and Leah, 1998; Pennypacker, 1998). These proteins are able to modulate transcription of target genes, following formation of the dimeric AP-1 complex, and DNA-binding through their "leucine-zipper" motifs. Stimulation of ionotropic glutamate receptors has been shown to induce an increase of the AP-1 DNA-binding activity in a variety of neuronal systems (Condorelli et al., 1994; Kasof et al., 1995; Hou et al., 1997; Schwarzschild et al., 1997; Kitayama et al., 1999; Kovács et al., 1999; Yoneda et al., 1999). Furthermore, activation of glutamate receptors can increase the activity of mitogen-activated protein kinases (MAPKs), in a calcium-dependent manner, through stimulation of the NMDA receptors (Bading and Greenberg, 1991; Kurino et al., 1995; Xia et al., 1996; Schwarzchild et al., 1999; Jiang et al., 2000). MAPKs are a family of serine/threonine kinases that include p44/42 extracellular signal-regulated kinases, ERK 1 and ERK 2, respectively, c-Jun N-terminal protein kinases (JNKs), and p38 MAP kinase, which can contribute to the induction of AP-1 activity in response to a diverse array of extracellular stimuli (Karin, 1995).

Among the ionotropic glutamate receptor agonists, kainate is the most potent excitotoxin both for the intact chick embryo retina (Chen et al., 1999) and for cultured chick amacrine-like neurons (Ferreira et al., 1996, 1998). The kainate-induced toxicity in cultured chick amacrine-like neurons is due to excessive activation of AMPA receptors. Moreover, the blockade of AMPA receptor desensitisation with cyclothiazide potentiates cell damage induced by AMPA (Ferrreira et al., 1998). Several reports from other neuronal systems also show an increase in AMPA receptor-mediated neurotoxicity and Ca^{2+} influx when the desensitisation of AMPA receptor is blocked with cyclothiazide (Cebers and Liljequist, 1995; Cebers et al., 1997; Ohno et al., 1997, 1998; Ferreira et al., 1998; Jensen et al., 1998; Ambrósio et al., 2000).

In cultured chick amacrine-like cells, small Ca^{2+} loads through NMDA or AMPA receptor-associated channels have different effects on cell viability (Ferreira et al., 1996). This evidence, together with the fact that cultured amacrine cells are particularly sensitive to excitotoxic activation of AMPA receptors (Ferreira et al., 1998), points to the need for further studies to clarify the different intracellular mechanisms mediated by different ionotropic glutamate receptors upon an excitotoxic stimulus. Furthermore, cultured chick amacrine-like cells, which contain Ca^{2+}-permeable AMPA receptors (Duarte et al., 1996, 1998), are a relevant model for studying toxicity mediated by AMPA receptors. We investigated eventual differences in postreceptor events mediated by different ionotropic glutamate receptors upon excitotoxic stimulation of cultured chick amacrine-like neurons and found that overactivation of ionotropic glutamate receptors induces apoptotic-like cell death and activation of the transcription factor AP-1, but not of the nuclear factor (NF)-κB transcription factor. Moreover, we show that stimulation of either AMPA or possibly NMDA receptors increases ERK activity, but only kainate-induced toxicity is protected by activation of the ERK pathway, suggesting stimulus-specific neuroprotection pathways in retinal neurons.

MATERIALS AND METHODS

Materials

Fetal calf serum was purchased from Biochrom KG (Berlin, Germany), trypsin and gentamicin from Gibco BRL Life Technologies (Paisley, United Kingdom). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and cyclothiazide (CTZ) were obtained from Tocris Cookson Ltd. (Bristol, United Kingdom). LY 303070 was a gift from Eli Lilly Co. (Indianapolis, IN). MK-801 was obtained from Merk Sharp & Dohme Reseach Laboratories (Rahway, NJ). The MAPKs inhibitors PD 98059, U 0126, and SB 203580 were from Calbiochem-Novabiochem Corp. (Darmstadt, Germany). The DNA stains propidium iodide and Syto-13 were from Molecular Probes Europe (Leiden, The Netherlands). The oligonucleotides with consensus binding motifs for the AP-1 and NF-κB transcription factors were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). T4 polynucleotide kinase was from USB Corporation (Cleveland, OH), and Sephadex G-50 spin column, poly-(dIdC), and [γ-^{32}P]ATP, 10 mCi/ml, were purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). The rabbit antiactive MAPK antibody was from Promega (Madison, WI). The alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG were from Amersham Pharmacia Biotech. The mouse monoclonal anti-α-tubulin was obtained from Zymed Laboratories, Inc. (San Francisco, CA). The BCA assay kit was from Pierce (Rockford, IL). The following antibodies, raised against members of the AP-1 transcription factor, were purchased from Santa Cruz Biotechnology, Inc., and were used for supershift analysis: c-Fos (sc-253-G), Fra-2 (sc-604), Fos B (sc-48), Jun B (sc-46-G), c-Jun/AP-1 (sc-45-G), Jun D (sc-74-G), and ATF-2 (sc-187). The c-Fos, Fra-2, and c-Jun/AP1 antibodies are chicken reactive according to the manufacturer's specification. All other reagents were from Sigma Chemical Co. (St. Louis, MO) or from Merck (Darmstadt, Germany). Stock solutions of CNQX, LY 303070, PD 98059, U 0126, SB 203580, and Syto-13 were made in dimethyl sulfoxide. All the other chemicals were kept in aqueous stocks.

Culture of Chick Retina Cells

Primary cultures of chick retinal neurons were prepared from 8-day-old chick (white leghorn) embryos as described previously (Duarte et al., 1992). Retinas were dissected free from other ocular tissues and incubated for 15 min at 37°C in Ca^{2+},Mg^{2+}-free Hank's balanced salt solution, supplemented with 0.1% trypsin. The digested tissue was centrifuged at 140*g* for 1 min, and the pellet was resuspended in Eagle's basal medium (Earl's salts; BME) buffered with 25 mM HEPES and

10 mM $NaHCO_3$ and supplemented with 5% heat-inactivated fetal calf serum and gentamicin (60 μg/ml). After mechanical dissociation, cells were seeded at 0.6×10^6 cells/cm^2 in 12-well plates for neuronal injury assays or at 0.94×10^6 cells/cm^2 in 100 mm dishes for the transcription factors or the MAPK activation studies. The retinal neurons were cultured for 5 days in BME supplemented as described above, without medium changes during the culture period. Cells were cultured at 37°C, in a humidified atmosphere of 95% air/5% CO_2. It was confirmed by immunocytochemistry, using antibodies against choline acetyltransferase and against γ-aminobutyric acid (GABA), that our cultures of retinal neurons are highly enriched in amacrine-like neurons (Santos et al., 1998).

Exposure to Excitatory Amino Acids

On the fifth day in culture, cells were washed and exposed to 100 μM glutamate or 100 μM kainate, at 37°C, in Na^+ buffer with the following composition: 132 mM NaCl, 4 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.4, in the presence of 1 mM $CaCl_2$. When (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate (MK-801), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), LY 303070 [the active isomer of 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine; GYKI 53655], or cyclothiazide was used, a preincubation of 5 min was performed before stimulation with the agonists of the glutamate receptors. In the studies using the inhibitors of the MAPKs, a preincubation of 1 hr was performed prior to stimulation with the glutamate receptor agonists.

To study the Ca^{2+} dependence of the activation of the transcription factor AP-1 induced by excitotoxic stimulation, the cells were washed and exposed in Na^+ buffer with the following composition: 132 mM NaCl, 4 mM KCl, 6 mM glucose, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM HEPES, pH 7.4. The basal free calcium concentration in this buffer was 50 nM.

Assessment of Neuronal Injury

Cultures were exposed to the agonists of glutamate receptors, during 1 hr at 37°C, in the presence or not of other drugs, as indicated in the figures. After the incubation period, the stimulation media were removed and the cells were further washed in Na^+ buffer. The cells were returned to the incubation chamber in serum-free culture medium until assessment of neuronal injury, by a colorimetric assay for cell survival, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT), or by analysis of the nuclear morphology. The MTT assay was a modification of the method of Mosmann (1983). MTT (0.75 mg/ml) in Na^+ buffer was added to the cultures and incubated for 3 hr at 37°C in the incubation chamber. MTT when taken up by live cells is converted to a water-insoluble blue product. The precipitated dye was dissolved in 0.04 M HCl in isopropanol and colorimetrically (absorbance at 570 nm) quantitated. All experiments were performed in triplicate.

The nuclear morphology of retina cells was analyzed by fluorescence microscopy of live cells using the DNA stains propidium iodide (2 μg/ml), which fluoresces red and enters cells only when the membrane presents ruptures, and Syto-13 (0.5 μM), which fluoresces green and enters cells with intact cytoplasmatic membrane (Ankarcrona et al., 1995). Viable neurons display a normal nuclear size and green fluorescence. Necrotic neurons manifest red fluorescence without chromatin condensation, and apoptotic neurons display pyknotic nuclei with condensed or fragmented chromatin and fluoresce green. Secondary necrosis was also observed, with the nucleus displaying condensed or fragmented chromatin fluorescing red. These neurons were scored as apoptotic neurons, because the process of secondary necrosis may reflect an insufficient removal of apoptotic cells in in vitro systems, and in this case secondary processes may cause cell disintegration. Cells were examined and scored with a Nikon Diaphot TMD microscope. All experiments were performed in duplicate, and as a minimum 300 cells were scored for each coverslip.

Preparation of Nuclear Extracts and Electophoretic Mobility Shift Assay

Nuclear extracts were prepared either immediately after the excitotoxic stimulation or after incubation in culture medium lacking serum for the indicated poststimulation period. Cells were washed with ice-cold phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and solubilized in buffer 1 [10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EGTA, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 20 μg/ml benzamidine, and 25 μg/ml pepstatin A]. Cell clumps were scraped into Eppendorf tubes and incubated on ice for 40 min. The nuclei were pelleted by centrifugation at 2,400*g* for 10 min at 4°C and resuspended in buffer 2 (25 mM HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 20% glycerol, pH 7.4, 1 mM DTT, 1 mM PMSF, 10 μM E64, 20 μg/ml benzamidine, and 25 μg/ml pepstatin A). After incubation on ice for 60 min, the lysates were centrifuged at 12,000*g* for 20 min at 4°C. The supernatants (nuclear extracts) were collected and stored at –70°C until use. Protein concentration of the extracts was measured using the BCA assay kit.

Double-stranded oligonucleotides with consensus binding motifs for the AP-1 and NF-κB transcription factors (AP-1, 5′-CGCTTGA*TGACTCA*GCCGGAA- 3′; NF-κB, 5′-AGTTGA*GGGGACTTTCCC*AGGC- 3′) were end-labelled with [γ-^{32}P]ATP with the T4 polynucleotide kinase and purified through a Sephadex G-50 spin column. Eight or twenty micrograms of nuclear proteins, for the DNA-binding assay of AP-1 or NF-κB, respectively, were incubated for 30 min at room temperature in 20 μl of binding reaction buffer [20 mM HEPES, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 4% Ficoll, 2 μg poly(dIdC), 20 μg bovine serum albumin (BSA), pH 7.9] with 150,000 cpm of [γ-^{32}P]-labelled oligonucleotide probe. The DNA–protein complexes were resolved by electrophoresis on 4% native polyacrylamide gels. The electrophoresis was performed in TBE buffer (44.5 mM boric acid, 44.5 mM Tris, 1 mM EDTA, pH 8.0). After drying, the DNA–protein complexes in the gel were visualized by autoradiography. For supershift analysis, 8 μg of nuclear extracts were incubated on ice for 2 hr with 1.5 μg of antibodies before the addition of the radiolabelled oligonucleotide. Specificity of binding was assessed

by competition with a 100-fold excess of unlabelled oligonucleotide probe. The scanned digital images were quantified using ImageQuant software (Molecular Dynamics).

Western Blot Assay

Retinal cells in culture were solubilized and sonicated in a buffer solution containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 2 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF, pH 7.4, 1 mM DTT, 1 mM PMSF, 10 μM E64, 20 μg/ml benzamidine, and 25 μg/ml pepstatin A. The lysates were centrifuged at 12,000*g* for 20 min at 4°C, the supernatant was collected, and the protein concentration was determined using the BCA assay kit. Thirty micrograms of protein were resolved by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) on 12% polyacrilamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking in 0.5% milk/0.1% Tween 20 in TBS (20 mM Tris, 137 mM NaCl, pH 7.6), the membranes were probed during 1 hr with the antiphospho-ERK antibody (1:5,000) in blocking buffer. After multiple washes (five times for 5 min each with blocking buffer) membranes were incubated with 1:10,000 alkaline phosphatase-conjugated anti-rabbit IgG in blocking buffer for 1 hr. After additional washes (5 × 5 minutes) in the blocking buffer, membranes were processed with ECF chemifluorescence substrate (Amersham) for 5 min. The membranes were dried between filter papers, and scanned in Storm 860 (Molecular Dynamics). The phosphorylation-specific antibody was stripped off the membranes by incubation in 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol at 50°C for 30 min. The membranes were then reprobed with anti-α-tubulin antibody (1:1,500) to control for protein loading.

Other Methods

Results are presented as means ± SEM of the number of experiments indicated. Statistical significance was determined by ANOVA analysis followed by the Dunnett's and/or Bonferroni's tests.

RESULTS

Glutamate Receptor Overstimulation Induces Retinal Neuron Death

Primary cultures of chick retinal neurons, enriched in amacrine-like cells (Santos et al., 1998), were challenged for 1 hr at 37°C with 100 μM glutamate, or 100 μM kainate, in Mg^{2+}-free sodium buffer with 1 mM of Ca^{2+}. The cell viability, determined 20–22 hr after stimulation using the MTT assay, was reduced (Fig. 1A), and fragmentation of the neurites could be observed by phase-contrast microscopy 1 hr after stimulation (not shown). As shown in Figure 1A, stimulation of retinal neurons with glutamate or kainate decreased cell viability by 25.5% or 44.1%, respectively. Under these experimental conditions, kainate was a more potent toxin than glutamate.

The toxicity induced by the glutamate receptor agonists was reverted by the antagonists of the ionotropic glutamate receptors. As can be observed in Figure 1A, the decrease in cell viability induced by glutamate was com-

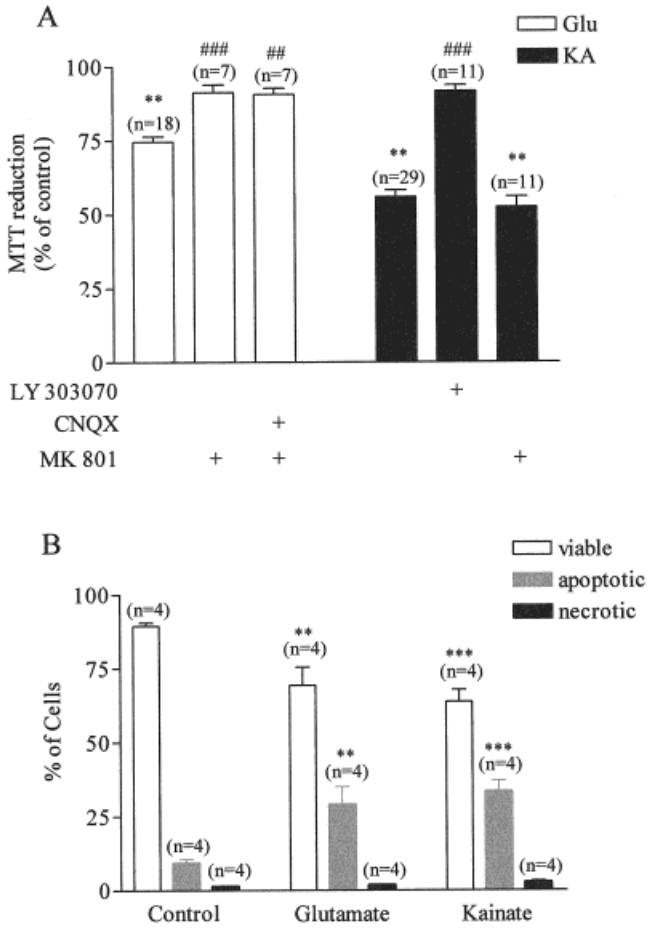


Fig. 1. Glutamate and kainate induce retinal cell death through activation of different receptors. **A:** Cells were exposed for 1 hr to 100 μM glutamate or 100 μM kainate in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}, with or without the glutamate receptor antagonists. A preincubation of 5 min was carried out with the glutamate receptor antagonists. After a washing step, the cells were returned to the incubator chamber for 20–22 hr in serum-free culture medium, and cell viability was assessed by the MTT assay as described in Materials and Methods. Control cells were incubated under conditions identical to those of cells submitted to excitotoxic stimulation but in the absence of drugs, and their MTT value was taken as 100% for each preparation (100% ± 5.83%, n = 30). Bars represent mean ± SEM values of the indicated number of experiments performed in triplicate in independent preparations. Asterisks indicate significant difference from control; pound signs indicate significant difference from glutamate agonists (two symbols, $P < 0.01$; three symbols, $P < 0.001$). **B:** Retinal neuron death induced by glutamate or kainate is apoptotic. Cells were exposed for 1 hr to 100 μM glutamate or 100 μM kainate in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}. After a wash step, the cells were returned to the incubator chamber for 5 hr in serum-free culture medium. Control cells were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs. The cells were then incubated with 0.5 μM Syto-13 and 2 μg/ml propidium iodide (PI) in saline buffer to visualize nuclei morphology and were scored with a fluorescence microscope. Bars represent mean ± SEM values of the indicated number of experiments performed in duplicate in independent cultures. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

pletely reverted by 10 μM MK-801, a selective antagonist for the NMDA receptors, without any additional protective effect of 20 μM CNQX, an antagonist of the non-NMDA ionotropic glutamate receptors, suggesting that glutamate toxicity is essentially mediated by the NMDA receptors. The toxicity induced by kainate was completely reverted by the 2,3-benzodiazepine LY 303070 (15 μM), which is the active isomer of the noncompetitive AMPA receptor antagonist GYKI 53655 (Donevan and Rogawski, 1993; Paternain et al., 1995). MK-801 (10 μM) was without any protective effect, suggesting that in this case the toxicity was mediated only through the activation of the AMPA receptors (Fig. 1A). The antagonists of the ionotropic glutamate receptors were without toxic effect under our experimental conditions (data not shown).

To determine whether retinal neurons die by apoptosis or necrosis upon excitotoxic stimulation, we analyzed the nuclear morphology of cells challenged for 1 hr at 37°C with 100 μM of glutamate or 100 μM of kainate in Mg^{2+}-free sodium buffer with 1 mM of Ca^{2+}. Five hours after stimulation, retinal neurons were loaded with the DNA stains propidium iodide and Syto 13, and the nuclear morphology was observed by fluorescence microscopy. Control cultures of neuronal retina cells had about 9.3% ± 1.3% dead cells, with condensed and/or fragmented chromatin in pyknotic nuclei. Cells with this type of morphology were scored as apoptotic neurons (Fig. 1B). In control cultures, about 1.4% of necrotic cells were also present. In retinal neurons exposed to excitotoxic stimuli, there was a significant increase in the number of neurons displaying condensed and/or fragmented chromatin in pyknotic nuclei, suggesting that retinal neurons exposed to the glutamate agonists die by apoptosis. Cultures stimulated with glutamate or kainate displayed about 29.0% ± 6.1% or 33.4% ± 3.7% of cells with apoptotic-like morphology, respectively. The number of necrotic cells did not increase significantly in cultures exposed to the excitotoxic stimuli.

We studied whether desensitisation of the AMPA receptors could influence the glutamate-induced toxicity in cultured retinal neurons using cyclothiazide, an inhibitor of AMPA receptor desensitisation. In the presence of 30 μM cyclothiazide, there was a significant increase in the toxicity induced by 100 μM glutamate, which reached a level similar to that observed with kainate. Under these experimental conditions, the MTT reduction was about 51.5% relative to the control (Fig. 2). Our results show that, in the presence of cyclothiazide, the glutamate-induced toxicity is mediated essentially through the AMPA receptors, because 73.5% of toxicity was reverted by LY 303070. However, a contribution of the NMDA receptors was also observed; we were able to revert 91.2% of the toxicity when the excitotoxic stimulation was performed in the presence of LY 303070 plus MK-801, even though MK-801 alone could not decrease the toxic effect of glutamate. Cyclothiazide was without toxic effect under our experimental conditions (data not shown).

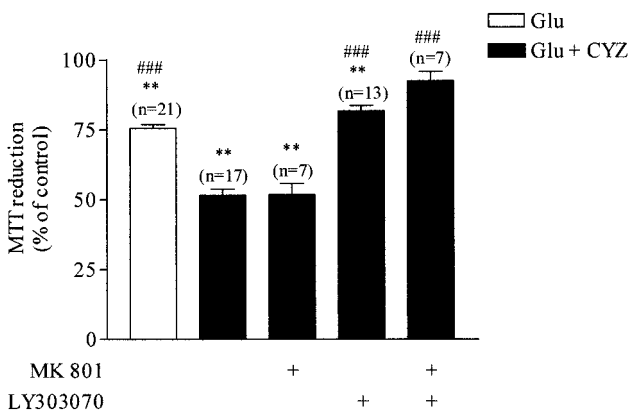


Fig. 2. Effect of AMPA receptor desensitization. Cells were exposed for 1 hr to 100 μM glutamate in the presence or in the absence of 30 μM cyclothiazide, with or without the glutamate receptor antagonists. After a washing step, the cells were returned to the incubator chamber for 20–22 hr in serum-free culture medium, and cell viability was assessed by the MTT assay as described in Materials and Methods. Cells were exposed to the drugs in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}. A preincubation of 5 min was carried out with the glutamate receptor antagonists and cyclothiazide. Control cells were incubated under conditions identical to those of cells submitted to excitotoxic stimulation but in the absence of drugs, and their MTT value was taken as 100% for each preparation (100% ± 5.83%, n = 30). Bars represent mean ± SEM values of the indicated number of experiments performed in triplicate in independent cultures. Asterisks indicate significant difference from control; pound signs indicate significant difference from glutamate plus cyclothiazide (two symbols, $P < 0.01$; three symbols, $P < 0.001$).

AP-1 DNA-Binding Activity Increases Upon Retinal Neuron Stimulation With Glutamate or Kainate

Retinal neurons were exposed to 100 μM of glutamate or 100 μM of kainate for 1 hr at 37°C in Mg^{2+}-free sodium buffer with 1 mM of Ca^{2+}. Nuclear extracts were prepared immediately after stimulation or after incubation at 37°C in BME without serum for different periods, as indicated. To determine the AP-1 DNA-binding activity, 8 μg of nuclear protein were incubated with a radiolabelled oligonucleotide containing the consensus binding motif for the transcription factor AP-1 and were analyzed by electrophoretic mobility shift assay (EMSA; Fig. 3A). Excitotoxic stimulation caused an increase in the AP-1 DNA-binding activity, which was maximal 2 hr after stimulation. Glutamate induced a greater increase in the AP-1 DNA-binding activity than kainate (Fig. 3A,B). The antagonists of the ionotropic glutamate receptors (MK-801 plus CNQX and LY 303070) caused a nonspecific increase of AP-1 activity on their own. Therefore, we could not test whether glutamate receptor antagonists revert the increase of AP-1 activity induced by glutamate and kainate.

To determine the specificity of AP-1 activation by glutamate and kainate, we studied the calcium dependence of the enhancement of the AP-1 DNA-binding activity

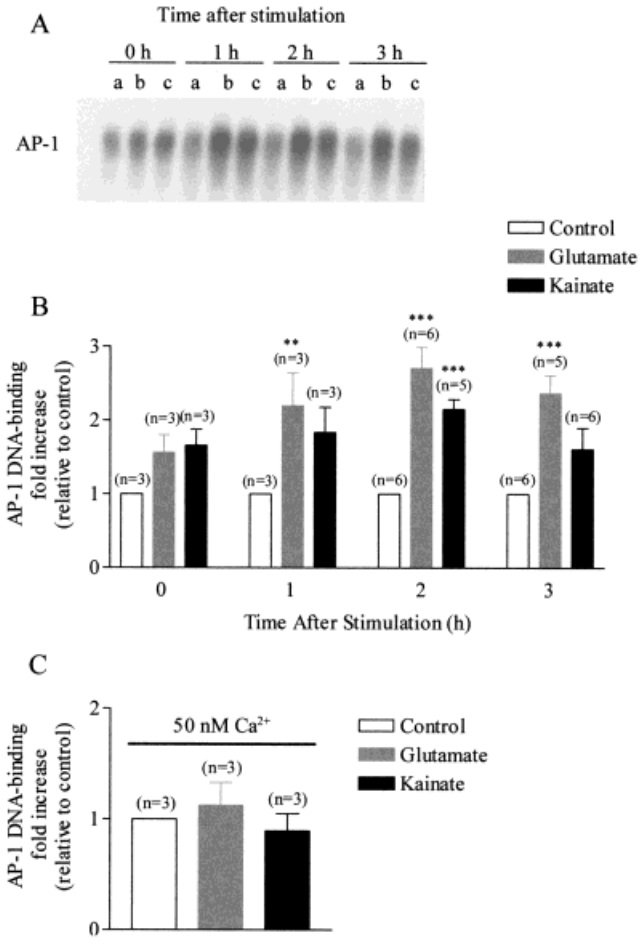


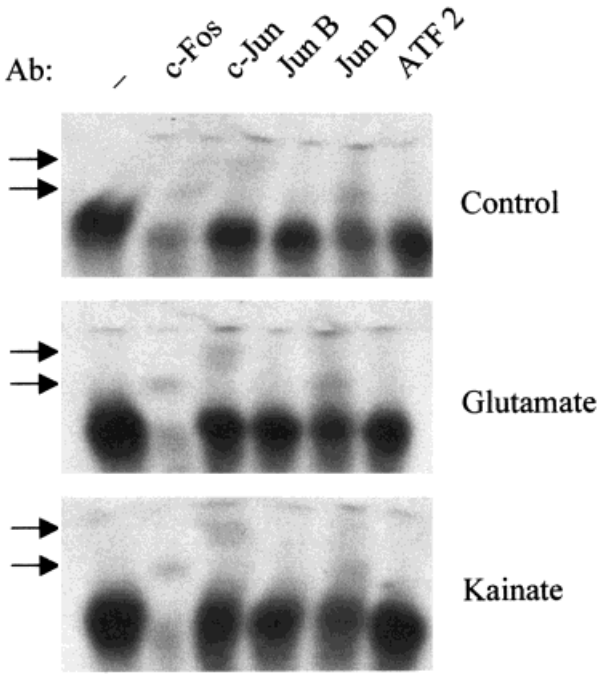
Fig. 3. AP-1 DNA-binding activity is increased in retinal neurons stimulated with glutamate or kainate. **A:** Representative autoradiogram showing a time course of the DNA-binding activity of AP-1 in retinal neurons exposed for 1 hr to 100 μM glutamate (b) or 100 μM kainate (c) in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}. Control cells (a) were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs. The nuclear extracts were prepared immediately after stimulation or 1, 2, or 3 hr after stimulation. Eight micrograms of nuclear protein were used for EMSA. **B:** Quantification of the AP-1 DNA-binding activity of retinal neurons stimulated as described for A using ImageQuant software (Molecular Dynamics). **C:** Calcium dependence of glutamate receptor agonists-induced activation of AP-1. Retinal neurons were exposed for 1 hr to 100 μM glutamate or 100 μM kainate in sodium buffer with a composition of 132 mM NaCl, 4 mM KCl, 6 mM glucose, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM HEPES, pH 7.4, and a basal free calcium concentration of 50 nM. The nuclear extracts were prepared 2 hr after the stimulation period, and AP-1 DNA-binding activity was examined by EMSA. Bars represent mean ± SEM values of the indicated number of experiments performed in independent cultures. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

induced by excitotoxic stimulation of chick retinal neurons (Fig. 3C). Cells were exposed for 1 hr at 37°C to 100 μM of glutamate or 100 μM of kainate in sodium buffer with a free calcium concentration of 50 nM. When cells were exposed to excitotoxic stimulation in the absence of calcium, none of the glutamate agonists could induce an increase in the AP-1 DNA-binding activity.

Because the AP-1 complex can be constituted by different Fos and Jun family members, we investigated the composition of the transcription factor following excitotoxic stimulation. Supershift experiments with several antibodies directed against different Fos and Jun proteins were performed to determine the contribution of each protein to the AP-1 DNA-binding activity in retinal neurons (Fig. 4). In control neurons a faint supershift was detected with the c-Fos antibody, which reacts with all members of the Fos family, and there was a decrease on the specific band of AP-1 DNA binding (Fig. 4). The supershift with this antibody became more evident in nuclear extracts from neurons exposed to excitotoxic stimulation. We also observed a supershift with the antibody against Fra-2 in extracts from control and stimulated neurons (Fig. 4). When we used the antibody against Fos B, we did not observe either a supershift or a decrease in the specific band for AP-1 DNA binding under either control or stimulated conditions. The absence of an effect with the Fos B antibody is not due to a lack of reactivity with our biological preparation; we observed a specific band for Fos B, using the same antibody, in a Western blot assay (data not shown). Additionally, we detected a supershift with the antibodies for c-Jun and Jun D and a decrease in the specific band of AP-1 DNA binding under both control and stimulated conditions. When the antibodies against Jun B and ATF 2 were used, a decrease in the specific band of AP-1 DNA binding in both control and stimulated situations was detected. The antibodies against Jun B and ATF 2 may block the DNA-binding site of these proteins. In that case, the decrease in the specific AP-1 DNA binding observed with those antibodies would suggest the participation of these proteins in the AP-1 complex. The results show that the AP-1 complex includes proteins of the Fos family, namely, Fra-2, and of the Jun family, namely, c-Jun and Jun D. Jun B and ATF 2 may also participate in the complex. In cultured amacrine neurons, for the stimulation conditions used, Fos B is not present in the AP-1 complexes. The pattern of AP-1 protein composition induced by excitotoxic stimulation was similar to the pattern observed for control neurons. Furthermore, we did not observe differences in the pattern of AP-1 proteins for the complexes present on retinal neurons 1, 2, or 3 hr after stimulation (data not shown).

Excitotoxic Stimulation of Retinal Neurons Does Not Change NF-κB DNA-Binding Activity

We also studied whether the excitotoxic stimulation of retinal neurons could induce the activation of the NF-κB transcription factor, which is sensitive to intracellular stress in many systems (Tong et al., 1998). Cells were stimulated with 100 μM glutamate or 100 μM kainate for 15, 30, 60, or 120 min, and the nuclear extracts were prepared immediately after the stimuli. With our preparation of retinal neurons, we did not observe a significant increase in the DNA-binding activity of NF-κB, either with glutamate or with kainate stimulation (Fig. 5).



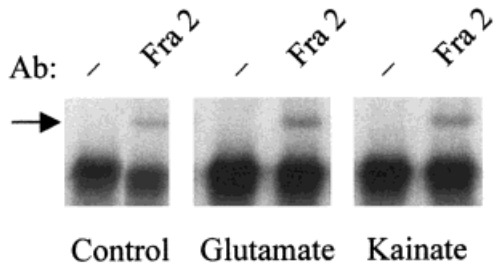


Fig. 4. Subunit composition of AP-1 dimers in cultured neuronal retina cells. Representative autoradiogram showing the supershift of the AP-1 subunits of retinal neurons exposed for 1 hr to 100 μM glutamate or 100 μM kainate in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}. Control cells were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs. The nuclear extracts were prepared 2 hr after the stimulation period. Eight micrograms of nuclear protein were incubated with 1.5 μg of antibody (Ab) for 2 hr at 4°C before incubation with the radiolabelled oligonucleotide. Arrows indicate positions of the bands representing antibody–protein–DNA complexes.

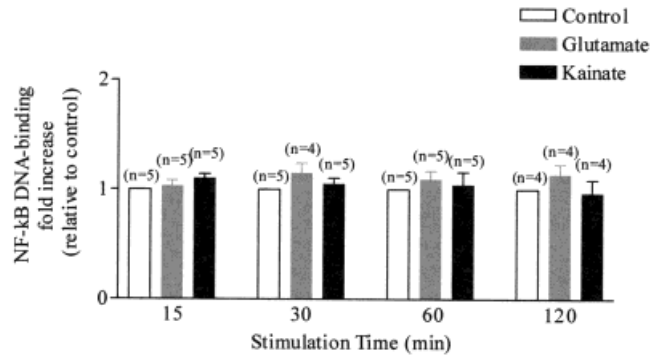


Fig. 5. NF-κB DNA-binding activity of retinal neurons stimulated with glutamate or kainate. Retinal neurons were exposed to 100 μM glutamate or 100 μM kainate in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} for the indicated times. Control cells were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs. The nuclear extracts were prepared immediately after stimulation, and 20 μg of nuclear protein were used for EMSA. The quantification of NF-κB DNA-binding activity was performed using ImageQuant software (Molecular Dynamics). Bars represent mean ± SEM values of the indicated number of experiments performed in independent preparations.

Glutamate and Kainate Increased the ERK Activity

Previous work in cultured rat hippocampal neurons and in rat striatal neurons has shown that neuronal ERK can be activated upon glutamatergic stimulus, in a calcium-dependent manner (Bading and Greenberg, 1991; Schwarzschild et al., 1999). Furthermore, ERK can regulate the expression of some of the proteins present on the AP-1 complex (Karin, 1995). Therefore, we investigated whether the activation of ionotropic glutamate receptors on cultured chick retinal neurons induces the activation of ERK. In previous studies in our laboratory, it was shown that in cultured chick retinal neurons stimulation of the ionotropic glutamate receptors leads to an increase in the intracellular free calcium concentration (Duarte et al., 1996; Carvalho et al., 1998). Cultured cells were exposed to 100 μM glutamate or 100 μM kainate, and total extracts were prepared 2 or 5 min after stimulation. We observed that, in retinal neurons, 2 min of stimulation with either glutamate or kainate was sufficient to increase the phosphorylation of ERK (Fig. 6), indicative of ERK activation. The increase of ERK phosphorylation was greater in neurons stimulated with kainate.

Effect of the MAPKs Inhibitors on the Neuronal Cell Death

Because glutamate and kainate activate ERK in retinal neurons, we investigated the contribution of the MAPK pathway to the excitotoxic response. Retinal neurons were preincubated for 1 hr with the MEK inhibitors 20 μM PD 98059 and 250 nM U 0126 (Favata et al., 1998; Encinas et al., 1999) and then challenged for 1 hr with 100 μM glutamate or 100 μM kainate in the presence of the MEK inhibitors. After stimulation, cells were washed and incubated 20–22 hr in BME without serum before the MTT assay was performed. The inhibition of MEK did not change the toxicity induced by glutamate (Fig. 7A) but increased the toxicity induced by kainate (Fig. 7B). These results suggest that the MAPK pathway normally contributes to the survival of retinal neurons exposed to kainate. When cells were preincubated with the p38 inhibitor SB 203580 (10 μM; Kawasaki et al., 1997), no change was observed in the excitotoxic response to either glutamate or kainate (Fig. 7). We did not observe

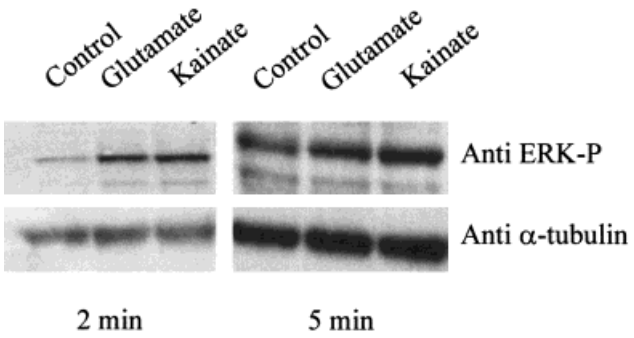


Fig. 6. ERK activation in retinal neurons stimulated with glutamate and kainate. Retinal neurons were incubated with or without 100 μM glutamate or 100 μM kainate in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} for the indicated times. Control cells were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs. Total cell extracts were prepared immediately after stimulation. Thirty micrograms of total proteins were used for immunoblot analysis with an antiphospho-ERK antibody. The membrane was reprobed with an antibody, anti-α-tubulin, to confirm an equal amount of protein loading in each gel lane. The experiments were performed in two independent preparations.

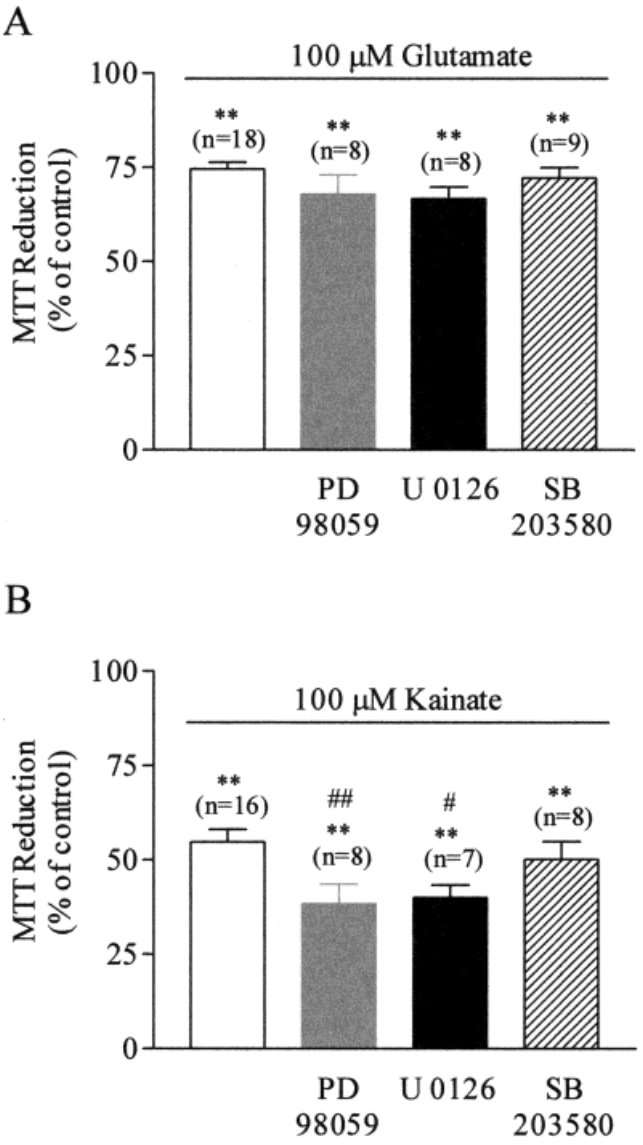


Fig. 7. Effect of the MAPK inhibitors on neuronal cell death. Retinal neurons were exposed for 1 hr to 100 μM glutamate (**A**) or 100 μM kainate (**B**) in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+}. The cells were preincubated for 1 hr in saline buffer with or without the MAPKs inhibitors 20 μM PD 98059, 250 nM U0126, and 10 μM SB 203580. The MAPKs inhibitors were also present during the stimulation with glutamate agonists. Cell viability was assessed using the MTT assay. Control cells were incubated in Mg^{2+}-free sodium buffer with 1 mM Ca^{2+} without drugs, and their MTT value was taken as 100% for each preparation (100% ± 5.83%, n = 30). Bars represent mean ± SEM values of the indicated number of experiments performed in triplicate in independent cultures. Asterisks indicate significant difference from control, pound signs indicate significant difference from glutamate agonists (one symbol, $P < 0.05$; two symbols, $P < 0.01$).

any toxic effects of the MAPKs inhibitors (data not shown).

DISCUSSION

The data presented show that, in chick embryo retinal neurons, the overstimulation of different ionotropic glutamate receptors triggers different intracellular signaling cascades. It was previously observed in our laboratory that in cultured amacrine-like cells, for small equivalent Ca^{2+} loads, the Ca^{2+} influx through the NMDA receptor-associated channel and through the AMPA receptor-associated channel triggers cell death to different extents (Ferreira et al., 1996), which suggests that different ionotropic glutamate receptors could be activating different signaling pathways upon excitotoxic stimuli. Accordingly, in the present work, we observed that stimulation of different ionotropic glutamate receptors induces different levels of toxicity. Stimulation of retinal neurons with either glutamate or kainate increased ERK phosphorylation, but only kainate-induced ERK activation was correlated with the activation of a survival signaling pathway. In contrast, glutamate induces a greater increase in the AP-1 DNA-binding activity than kainate.

Neurotoxicity Caused by Glutamate and Kainate in Cultured Retinal Neurons

In the present paper, we report a decrease in cell viability of cultured retinal neurons exposed to excitotoxic stimuli. The ionotropic glutamate receptor type mediating the toxic effect depends on the glutamate receptor agonist used. We observed that the toxicity induced by glutamate was mediated essentially through the NMDA receptors, because it could be completely reverted by MK-801, a selective noncompetitive antagonist of the NMDA receptors, without additional protective effect of CNQX, a competitive inhibitor of the AMPA/kainate receptors. With other neuronal systems, such as rat hippocampal neurons (Moudy et al., 1994) and rat cerebellar granule

cells (Cebers et al., 1997), it was also observed that glutamate-induced toxicity may be entirely mediated through NMDA receptors.

In cultured chick retinal neurons, excitotoxic stimulation with kainate induced a greater reduction of cell viability than stimulation with glutamate. The kainate-induced toxicity was mediated through AMPA receptors; it was completely blocked by LY 303070, and MK-801 was without effect on the MTT reduction by cells stimulated with kainate, excluding a contribution of the NMDA receptors. We have previously reported that, in cultured chick retinal neurons, kainate stimulates $^{45}Ca^{2+}$ uptake and decreases MTT reduction in an LY 303070-sensitive manner, which indicates that AMPA receptors are involved in kainate excitotoxicity (Ferreira et al., 1998). Other studies, in intact chick embryo retinas, showed that the toxicity induced by kainate is blocked by antagonists of the non-NMDA receptors, but not by MK-801 (Chen et al., 1999). These studies and our work point to the relevance of neuronal retina cells as a model to study toxicity mediated by AMPA receptors. The contribution of AMPA receptors to the toxicity induced by kainate has also been observed in other neuronal systems, such as embryonic rat hippocampal cultures (Ohno et al., 1997; Ambrósio et al., 2000) and cultured murine neocortical neurons (Jensen et al., 1999).

Glutamate and kainate treatment of cultured neuronal retina cells, essentially of the amacrine type, caused cell death with morphological characterisitcs of apoptosis, namely, an increase in the number of neurons presenting condensed and/or fragmented chromatin in pyknotic nuclei. Previous studies in rabbit or rat retina showed that kainate or NMDA induces cell death through apoptosis (Perez et al., 1997; Lam et al., 1999; Kwong and Lam, 2000), and activation of caspases was detected upon ischemia–reperfusion in the retina (Katai and Yoshimura, 1999).

Blockade of AMPA Receptor Desensitisation Increases the Excitotoxicity Caused by Glutamate

Activation of AMPA receptors, when AMPA or glutamate is used as an agonist, elicits a rapid and strong desensitisation of the receptor. However, kainate induces an unusually rapid but much weaker desensitisation, and, as a result, whole-cell currents induced by kainate appear nondesensitising (Patneau et al., 1993; Hollmann and Heinemann, 1994; Bettler and Mulle, 1995; Yamada, 1998). We show that, in the presence of cyclothiazide, the glutamate-induced toxicity increases to a level identical to that reached by kainate excitotoxic stimulation of cultured retinal neurons. Additionally, our results show that, in the presence of cyclothiazide, the glutamate-induced toxicity is mediated essentially through AMPA receptors, although a contribution of NMDA receptors was also observed; we obtained greater neuroprotection when MK-801 was added together with LY 303070, even though MK-801 alone could not decrease toxicity. The involvement of the NMDA receptors in the toxic response to glutamate when AMPA desensitisation is blocked could be due to a direct action of the agonist on the NMDA receptor and to an indirect component of AMPA receptor toxicity, which would involve activation of the NMDA receptor as a consequence of glutamate being released from neurons by AMPA receptor-mediated depolarization.

In cultured retinal neurons, a correlation between the Ca^{2+} influx through the NMDA and AMPA/kainate receptor-associated channels and cell death was established (Ferreira et al., 1996). Furthermore, in retinal neurons, cyclothiazide enhanced both the $^{45}Ca^{2+}$ uptake and the neurotoxicity induced by AMPA (Ferreira et al., 1998). Additionally, in our preparation of cultured retinal neurons, containing essentially amacrine-like cells, highly Ca^{2+}-permeable AMPA receptors are expressed (Duarte et al., 1996, 1998; Carvalho et al., 1998), enriched in the GluR3 and GluR4 subunits (A.L. Carvalho and C.B. Duarte, unpublished observations). These observations suggest that the increased glutamate toxicity that we observed in the presence of cyclothiazide is probably due to an increase in the intracellular Ca^{2+} concentration. In previous work with rat hippocampal neurons (Moudy et al., 1994), rat cerebellar granule neurons (Cebers et al., 1997), and human NT2-N neurons (Itoh et al., 1998), it was shown that cyclothiazide greatly enhances the glutamate-induced toxicity, essentially through activation of the AMPA receptors. A relationship between the increase in $[Ca^{2+}]_i$, measured in single cerebellar granule cells, and an enhancement of the toxicity induced by glutamate following a blockade of AMPA receptor desensitization, in the presence of NMDA receptor blockers, was previously reported (Cebers et al., 1997). Our results provide further support for the notion that AMPA receptors may play a significant role in the neurotoxic effects produced by glutamate or kainate in retinal neurons.

Activation of the AP-1 Transcription Factor by Excitotoxic Stimulation of Retinal Neurons

We provide evidence that, in primary cultures of chick embryo retinal neurons, enriched in amacrine cells, overstimulation of glutamate receptors significantly increases the DNA-binding activity of AP-1 but not the DNA-binding activity of NF-κB, two transcription factors sensitive to the intracellular redox conditions. In other neuronal systems, glutamate receptor activation was shown to enhance the AP-1 DNA-binding activity, but not NF-κB DNA-binding activity. In cultured rat forebrain cells, glutamate activated AP-1 (Condorelli et al., 1994), but not NF-κB (Lukasiuk et al., 1995), and, in nuclear extracts from whole brains of mice intracerebroventricularly injected with NMDA, no significant alterations were found in the binding activity of NF-κB, although an increase in the DNA-binding for AP-1 was observed (Yoneda and Ogita, 1994).

In this study, we observed that glutamate induces a greater increase of the DNA-binding activity of AP-1 than does kainate, and the increase was maximal 2 hr after stimulation. In the absence of calcium, neither glutamate nor kainate was able to increase the AP-1 DNA-binding activity, which shows that the glutamate/kainate-induced

increase in AP-1 DNA-binding activity is a receptor-mediated event. In cultured retinal neurons, the agonists of the ionotropic glutamate receptors increase the $[Ca^{2+}]_i$ through a composite effect, comprising Ca^{2+} permeating the receptor-associated channels and Ca^{2+} entering through voltage-gated Ca^{2+} channels activated upon cell depolarization after receptor activation (Duarte et al., 1996; Carvalho et al., 1998). Results from previous investigations of cultured neurons support the notion that Ca^{2+} plays a key role in the glutamate receptor agonist-induced activation of immediate early genes (IEG) and transcriptional activity (Lerea et al., 1992; Lerea an McNamara, 1993; Bading et al., 1993, 1995; Griffiths et al., 1998). The involvement of the ionotropic glutamate receptors in AP-1 DNA-binding activation has been demonstrated in other systems, such as rat cerebellar granule cells and striatal neurons (Hou et al., 1997; Schwarzschild et al., 1997; Kovács et al., 2000; Lidwell and Griffiths, 2000).

In previous work performed with cortical neurons, a sustained increase of the transcription factors that bind to the AP-1 consensus region was observed prior to the emergence of neuronal apoptotic-like cell death, suggesting that AP-1 binding factors could be involved in this process (Finiels et al., 1995). In cultured retinal neurons, the increase in the DNA-binding activity of AP-1 was observed early after the excitotoxic stimulation and precedes cell death, suggesting that activation of the AP-1 transcription factor could be involved in the retinal neuron death processes.

Different Fos/Jun heterodimers will have different transactivating potentials (Angel and Karin, 1991; Herdegen and Leah, 1998; Pennypacker, 1998). Consequently, different dimerization patterns of Fos and Jun proteins, induced by different stimuli, will cause functionally distinct effects on gene regulation. In this work, supershift experiments with several antibodies directed against different members of Fos and Jun protein families indicate that the AP-1 complex in retinal neurons includes proteins of the Fos family, namely, Fra-2, c-Jun, and Jun D. Additionally, Jun B and ATF 2 could also participate in the AP-1 complex. The pattern of AP-1 composition, after excitotoxic stimulation, was similar to that observed for control neurons. However, we cannot exclude the possibility that at least some other members of the Fos family participate in the AP-1 complex or that they might be differentially expressed relative to the control upon excitotoxic stimuli. A similar situation was previously described for cerebellar granule cells exposed to PDC, an inhibitor of glutamate reuptake; it was observed that PDC-induced AP-1 dimers, through the activation of NMDA receptors, which had the same composition as controls (Kovács et al., 1999).

ERK Activation as a Cell-Intrinsic Neuroprotection Against Kainate Stimulation

We demonstrate that, in primary cultures of chick embryo neuronal retina cells, there is an increase in ERK phosphorylation upon stimulation with glutamate or kainate. Neuronal ERKs can be activated in primary cultures of hippocampal neurons (Bading and Greenberg, 1991; Kurino et al., 1995), striatal neurons (Schwarzschild et al., 1999), and cortical neurons (Xia et al., 1996; Jiang et al., 2000) upon glutamatergic stimulation, through activation of the NMDA receptors, in a calcium-dependent manner. Although AMPA and kainate do not appear to activate ERK in hippocampal neurons (Kurino et al., 1995), for striatal neurons it was reported that both AMPA and kainate can activate ERK as a result of the direct influx of Ca^{2+} through the AMPA receptor itself (Perkinton et al., 1999).

In this study, we found that ERK activation by different glutamate agonists triggers a differential effect on cell viability of cultured retinal neurons. We observed that inhibition of MEK did not alter the toxicity induced by glutamate. However, when the toxic stimulation was performed with kainate, MEK inhibition induced a decrease in cell viability, suggesting that the ERK pathway could contribute to the survival of the retinal neurons when they are challenged with kainate. ERK was activated when retinal neurons were treated with kainate, and inhibition of ERK activation further increased kainate-induced cell death. These findings suggest that ERK does not actively contribute to the cell death caused by kainate but may be activated as a neuroprotective mechanism. Glutamate and kainate induce toxicity through the activation of different ionotropic glutamate receptors, so the observed differential effect of ERK activation on cell survival could be due to the different levels of toxicity mediated by NMDA and AMPA receptors or to the activation of different biochemical pathways by each receptor. A different localization of NMDA and AMPA receptors could also contribute to the different effect of ERK activation on cell survival upon excitotoxic stimulation. Other studies with neuronal cells have found that ERK activation functions as a cell-intrinsic survival pathway (Hetman et al., 1999; Irving et al., 2000). For example, ERK is activated in cortical neurons treated with camptothecin (Hetman et al., 1999), and ERK pathway inhibition increases camptothecin-induced apoptosis. In several cell lines, ERK is activated by H_2O_2 and low doses of radiation and provides protection against these stimuli (Guyton et al., 1996; Carter et al., 1998). However, evidence that ERK activation can be involved in a neurotoxic pathway has also been found (Murray et al., 1998; Jiang et al., 2000). Moreover, in other studies, MEK inhibition did not affect neuronal survival (Perkinton et al., 1999; Sugino et al., 2000).

The inhibitor of p38 did not change cell viability, suggesting that in retinal neurons this pathway is not involved in the excitotoxic response. In striatal neurons, glutamate failed to activate p38 (Scharzschild et al., 1997), but there are some reports with other neuronal systems implicating p38 in the toxic glutamatergic or ischemic response (Kawasaki et al., 1997; Irving et al., 2000; Sugino et al., 2000).

We propose that, in chick embryo retinal neurons, the excitotoxic responses induced by glutamate and kai-

nate are mediated by different intracellular signaling cascades. We observed that stimulation of different ionotropic glutamate receptors induces different levels of toxicity. Furthermore, stimulation of retinal neurons with either glutamate or kainate causes an increase in ERK phosphorylation, although only kainate-induced ERK activation correlates with the activation of a survival signaling pathway. Probably glutamate-induced ERK activation subserves another functional role(s) in this system. In contrast, glutamate induces a greater increase in the AP-1 DNA-binding activity than kainate. Our results suggest that signaling pathways that mediate excitotoxic cell death and neuroprotection are stimulus specific in cultured chick amacrine-like cells.

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