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Excitotoxicity mediated by Ca^{2+}-permeable GluR4-containing AMPA receptors involves the AP-1 transcription factor

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Abstract

Cells preferentially expressing GluR4-containing α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are particularly sensitive to excitotoxicity mediated through non-*N*-methyl-D-aspartate receptors. However, the excitotoxic signalling pathways associated with GluR4-containing AMPA receptors are not known. In this work, we investigated the downstream signals coupled to excitotoxicity mediated by Ca^{2+}-permeable GluR4-containing AMPA receptors, using a HEK 293 cell line constitutively expressing the $GluR4_{flip}$ subunit of AMPA receptors (HEK-GluR4). Glutamate stimulation of GluR4-containing AMPA receptors decreased cell viability, in a calcium-dependent manner, when the receptor desensitisation was prevented with cyclothiazide. The excitotoxic stimulation mediated through GluR4-containing AMPA receptors increased activator protein-1 (AP-1) DNA-binding activity. Inhibition of the AP-1 activity by overexpression of a c-Jun dominant-negative form protected HEK-GluR4 cells against excitotoxic damage. Taken together, the results indicate that overactivation of Ca^{2+}-permeable GluR4-containing AMPA receptors is coupled to a death pathway mediated, at least in part, by the AP-1 transcription factor.

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Keywords: excitotoxicity; glutamate; Ca^{2+}-permeable AMPA receptors; GluR4 subunit; activator protein-1 (AP-1) transcription factor

Abbreviations: HEK, human embrionic kidney; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; KA, kainate; NMDA, *N*-methyl-D-aspartate; GluR, glutamate receptor; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CTZ, cyclothiazide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium bromide; PBS, phosphate buffered saline; AP-1, activator protein-1; dn c-Jun, c-Jun dominant negative; JNK, c-Jun N-terminal kinase; MLK, mixed lineage kinase; ATF2, activating transcription factor 2; PSD, postsynaptic density; ALS, amyotrophic lateral sclerosis

Introduction

Glutamate signalling plays a key physiological role in synaptic plasticity, growth and differentiation. However, excessive stimulation of glutamate receptors induces toxicity, a process defined as excitotoxicity. Excitotoxicity is considered to be a major mechanism of cell death in diseases such as stroke, central nervous system trauma, epilepsy and chronic degenerative disorders. Although the molecular pathways subjacent to excitotoxicity are not completely understood, there is a general agreement that excitotoxicity is largely dependent on Ca^{2+}. It was recently suggested that Ca^{2+} toxicity is not simply a function of increased Ca^{2+} concentration but is rather coupled to the route of Ca^{2+} entry and the distinct second messenger pathways that are activated as a result. The 'source specificity hypothesis' proposes that key molecules for the excitotoxic signalling are physically linked or colocalised with glutamate receptors, and constitute a target for the development of selective neuroprotective therapies.[1]

Much of the toxicity associated with the overstimulation of glutamate receptors is due to activation of ionotropic receptors. The ionotropic glutamate receptors consist of ligand-gated ion channels that can be pharmacologically and physiologically divided into three distinct subtypes: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate (KA) receptors.[2] In agreement with the 'source specificity hypothesis', there are evidences showing that the downstream mechanisms coupled to the activation of different ionotropic glutamate receptors are distinct.[3,4] In fact, in primary cultures of retinal neurons, the route of Ca^{2+} entry, through NMDA or AMPA receptors, determines the magnitude of the effect on cell viability,[3] and may also determine the activation of specific survival pathways.[4] Moreover, an extrasynaptic *versus* synaptic location of a receptor also influences the signalling pathway which will be activated by Ca^{2+} entry through the receptor channel. In primary cultures of hippocampal neurons, the extrasynaptic NMDA receptors oppose the survival pathways coupled to the synaptic NMDA receptors by triggering cAMP response element binding protein (CREB) shut-off and cell death pathways.[5]

AMPA receptors are tetrameric structures that are assembled from GluR1, -2, -3, and -4 subunits.[6] The GluR2

subunit dictates Ca^{2+}-permeability of AMPA receptor channels, its presence decreasing Ca^{2+}-permeability due to an arginine residue in the Q/R editing site of the second membrane-inserted domain responsible for the pore.[7] Recently, Ca^{2+}-permeable AMPA receptors were shown to be crucial for the selective excitotoxic death of CA1 pyramidal neurons observed after transient global ischemia,[8,9] and for the selective neuronal death of spinal motor neurons in amyotrophic lateral sclerosis (ALS).[10,11] Also, in the cholinergic neurons of the basal forebrain, which may be affected in Alzheimer's disease, the Ca^{2+} influx through AMPA/KA receptors was shown to be a factor of preferential vulnerability to KA excitotoxicity when compared with most cortical or basal forebrain neurons.[12] It is interesting to notice that in several systems where Ca^{2+}-permeable AMPA receptors are involved in cell death,[3,10,12] AMPA receptors are particularly enriched in the GluR4 subunit.[13–15]

The activator protein-1 (AP-1) is a transcription factor sensitive to stress conditions, and induced by diverse stimuli, including glutamatergic stimulation. AP-1 consists of a variety of dimers constituted by proteins of the Jun and Fos families. The Jun proteins (c-Jun, JunB and JunD) can both homodimerize and heterodimerize with Jun or Fos proteins, while the Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) can only heterodimerize with the Jun family members to form transcriptionally active complexes.[16] AP-1 activation is mediated, in part, by the phosphorylation of c-Jun by the c-Jun N-terminal kinases (JNKs).[16] In the Jun family, c-Jun is the most potent activator of transcription. There are direct[17] and indirect evidences[18–20] that the JNK/c-Jun signalling pathway is important for neuronal death induced by excitotoxicity.

As neuronal cells preferentially expressing the GluR4 subunit of AMPA receptors are particularly vulnerable to AMPA-induced toxicity,[13] it is important to determine the postreceptor signalling events coupled to cell death under these conditions. In the present work, we investigated the role of the AP-1 transcription factor in excitotoxic cell death mediated by Ca^{2+}-permeable GluR4-containing AMPA receptors. We utilised a HEK 293 cell line constitutively expressing the $GluR4_{flip}$ subunit of AMPA receptors (HEK-GluR4), which allowed us to distinguish the contribution of GluR4 to the activation of an excitotoxic pathway. Our work shows that activation of the AP-1 transcription factor, mediated through Ca^{2+}-permeable GluR4-containing AMPA receptors, is involved in excitotoxicity-induced cell death.

Results

Ca^{2+}-permeable GluR4-containing AMPA receptors mediate cell death

In order to clarify the contribution of the GluR4 subunit of AMPA receptors to the excitotoxic response, we used an HEK 293 cell line constitutively expressing the $GluR4_{flip}$ subunit, which assembles into functional Ca^{2+}-permeable GluR4-homomeric AMPA receptors (HEK-GluR4).[21] The electrophysiological characteristics of $GluR4_{flip}$ channels expressed in HEK 293 cells are similar to those observed in a subpopulation of AMPA receptors present in cerebellar granule cells.[22] HEK-GluR4 cells were stimulated for 1 h at 37°C, with 100 μM or 1 mM glutamate in sodium buffer containing 2.5 mM $CaCl_2$. Glutamate did not significantly affect cell viability, assessed by the MTT test 20–22 h after stimulation, unless AMPA receptor desensitization was blocked with cyclothiazide (CTZ). The allosteric modulator CTZ strongly attenuates desensitization in flip variants of AMPA receptors, enhancing agonist-induced steady-state current changes.[2] Therefore, the use of CTZ allowed us to study AMPA receptor-mediated toxicity in experimental conditions more similar to those observed in several pathological conditions, such as in global ischemia and in ALS, where the molecular structure of AMPA receptors is changed,[8,9,11,23] leading to an increased Ca^{2+} permeability[7] or a slower desensitization kinetic.[24] In the presence of 100 μM CTZ, glutamate toxicity was concentration-dependent and sensitive to 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), indicating that toxicity was mediated through GluR4-homomeric AMPA receptors (Figure 1a and b). Additionally, we investigated whether excitotoxicity was dependent on the extracellular Ca^{2+} concentration in HEK-GluR4 cells. This was performed by exposing the cells to glutamate in sodium buffer without Ca^{2+} or, alternatively, in sodium buffers with 1 or 2.5 mM Ca^{2+} (Figure 1c). Cell viability studies showed that toxicity induced by 1 mM glutamate stimulation in the presence of 100 μM CTZ increased with the raise in the extracellular Ca^{2+} concentration.

The nuclear morphology of HEK-GluR4 cells submitted to excitotoxic stimulation was investigated with the DNA fluorescent dye Hoechst 33342. At 13 h after stimulation with 1 mM glutamate plus 100 μM CTZ, in sodium buffer with 2.5 mM Ca^{2+}, cultures were fixed and incubated with 15 μg/ml Hoechst 33342 (Figure 1d). Excitotoxic stimulation induced the formation of bright pyknotic nuclei with condensed or fragmented chromatin in $20.9 \pm 2.3\%$ of HEK-GluR4 cells. These changes in the nuclear morphology were inhibited by 50 μM CNQX, which is in keeping with the cell viability results obtained with the MTT assay. Moreover, the excitotoxicity-induced changes in the HEK-GluR4 cells nuclear morphology were similar to those described for neuronal excitotoxic death.[4,25]

The AP-1 DNA-binding activity is increased upon excitotoxic stimulation

To investigate whether the AP-1 transcription factor, which is sensitive to intracellular stress conditions, could participate in the excitotoxic response mediated through GluR4-homomeric AMPA receptors, we analysed the AP-1 DNA-binding activity. HEK-GluR4 cultures were stimulated with glutamate, in sodium buffer containing 2.5 mM $CaCl_2$, and nuclear extracts were prepared 2, 4, or 6 h after stimulation. In all 8 μg of nuclear protein were analysed by EMSA (Figure 2). Stimulation with 1 mM glutamate did not increase the activity of AP-1. However, when glutamate stimulation was performed in the presence of 100 μM CTZ there was a significant increase in the AP-1 activity, which was maximal 4 h after stimulation (Figure 2a and c), whereas 100 μM CTZ by itself did not induce any significant changes. Therefore, increases in the AP-1

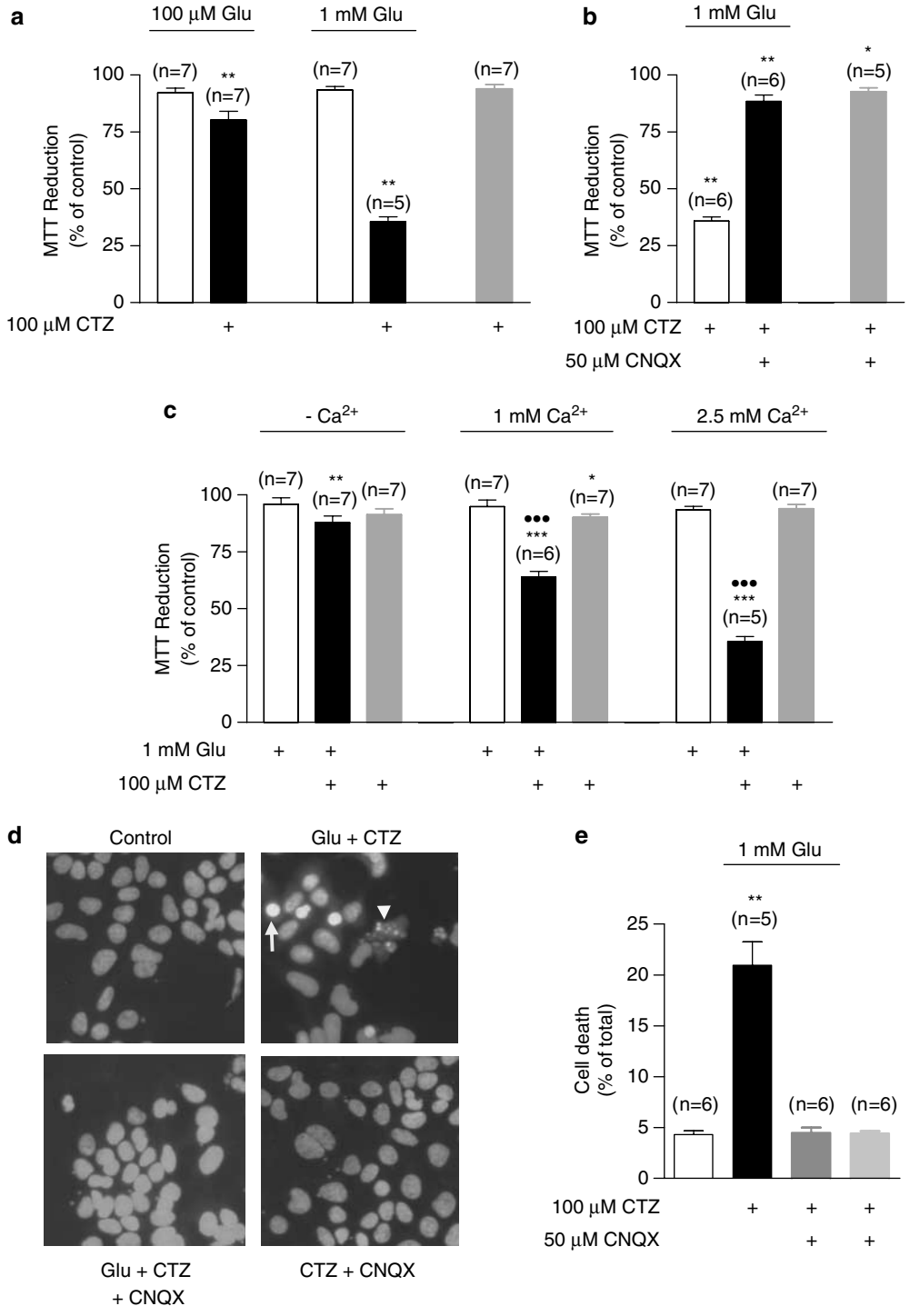

Figure 1 Overstimulation of GluR4-homomeric AMPA receptors induces HEK-GluR4 cell death. HEK-GluR4 cultures were exposed to 100 μM or 1 mM glutamate in sodium buffer with 2.5 mM $CaCl_2$, for 1 h at 37°C (**a**, **b**). Cell death, assessed using the MTT reduction assay, was observed when the receptor desensitization was blocked with 100 μM CTZ (**a**), and was prevented by the AMPA receptor antagonist CNQX (50 μM) (**b**). (**c**) Effect of the extracellular Ca^{2+} concentration on the viability of cells exposed to glutamate and CTZ. (**a**–**c**) Control cells were submitted to sodium buffer incubation in the absence of drugs and their MTT reduction considered as 100%. Bars represent the mean ± S.E.M. of the indicated number of independent experiments. *,**Statistically different from control (*$P<0.05$; **$P<0.01$), ●●● statistically different from stimulation with glutamate alone ($P<0.001$). (**d**) Nuclear morphology of HEK-GluR4 cultures exposed to glutamate and CTZ as described above (**a**, **b**). After incubation with the DNA stain Hoescht 33342, the cells were examined by fluorescence microscopy. The arrow indicates a nucleus with condensed chromatin while the arrow head indicates a nucleus with fragmented chromatin. The AMPA receptor antagonist CNQX (50 μM) inhibited the appearance of pycnotic nuclei. (**e**) Cell death was evaluated by scoring cells with condensed or fragmented chromatin. Bars represent the mean ± S.E.M. of the indicated number of independent experiments. **Statistically different from control ($P<0.01$)

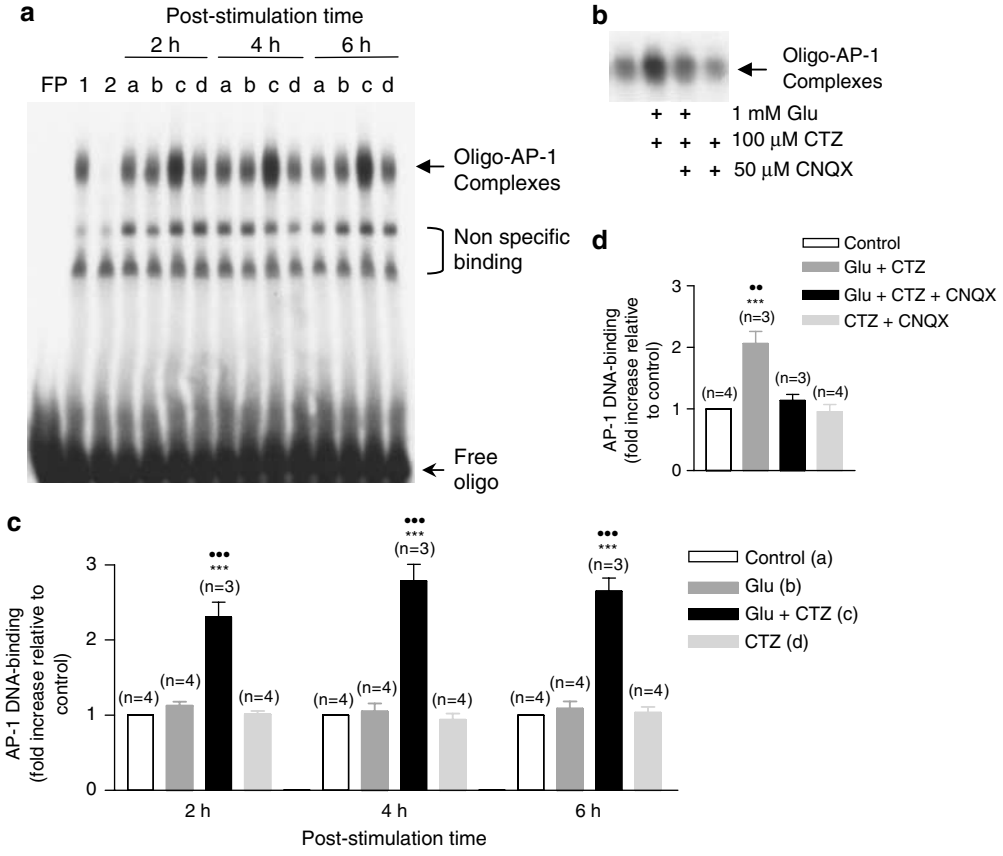

Figure 2 The AP-1 activity is increased in HEK-GluR4 cells submitted to excitotoxicity. (**a**) Representative autoradiogram showing a time course of the DNA-binding activity of AP-1 in HEK-GluR4 cells exposed to 1 mM glutamate (Glu, b), 1 mM glutamate plus 100 μM CTZ (Glu + CTZ, c), or 100 μM CTZ alone (d), in sodium buffer with 2.5 mM $CaCl_2$, for 1 h at 37°C. Control cells (a) were incubated in sodium buffer in the absence of drugs. The nuclear extracts were prepared 2, 4, or 6 h after stimulation. Nuclear protein (8 μg) was used for EMSA. In the autoradiogram FP is the free probe, 1 and 2 represent competition assays with unlabelled oligonucleotides with a consensus region for the Oct-1 (1) and AP-1 (2) transcription factors. (**b**) Inhibition of the excitotoxicity-induced AP-1 DNA-binding activity by CNQX. HEK-GluR4 cells were exposed to 1 mM glutamate plus 100 μM CTZ in the presence, or the absence, of 50 μM CNQX, as described above. Nuclear extracts were prepared 4 h after stimulation. (**c**, **d**) Quantification of the DNA-binding activity of AP-1 in HEK-GluR4 cells stimulated as described for (**a**) or as described for (**b**) respectively, using ImageQuant software. Bars represent the mean ± S.E.M. of the indicated number of independent experiments. The intensity of the control band was arbitrarily considered 1. ***Statistically different from control ($P<0.001$), ●●,●●●statistically different from stimulation with glutamate alone (**c**) (●●● $P<0.001$), or different from stimulation with glutamate plus CTZ in the presence of CNQX (**d**) (●● $P<0.01$)

activity correlated with the excitotoxic effects in HEK-GluR4 cells. The excitotoxicity-induced increase in AP-1 activity was completely inhibited by 50 μM CNQX, indicating that GluR4-homomeric AMPA receptors mediated the activation of the AP-1 signalling pathway (Figure 2b and d). CNQX plus CTZ by themselves did not affect the AP-1 DNA-binding activity (Figure 2b and d).

In neuronal systems, the activation of immediate-early genes, such as *c-fos*, is Ca^{2+} dependent.[26,27] Thus, we investigated whether activation of AP-1 induced by over-stimulation of GluR4-homomeric AMPA receptors also requires Ca^{2+} in HEK-GluR4 cells. The cultures were exposed to 1 mM glutamate plus 100 μM CTZ in the presence or absence of Ca^{2+}, and the nuclear extracts were analysed for the AP-1 DNA-binding activity 4 h after stimulation. The increase in the AP-1 DNA-binding activity, induced by glutamate plus CTZ stimulation, was higher when Ca^{2+} was present on the extracellular medium (Figure 3), which indicates that GluR4-containing AMPA receptors activate AP-1 in a Ca^{2+}-dependent manner in HEK-GluR4 cells. The basal DNA-binding activity of AP-1 was not sensitive to differences in the extracellular Ca^{2+} concentration.

Subunit composition of the AP-1 transcription factor in HEK-GluR4 cells

To study the subunit composition of the AP-1 transcription factor, we performed supershift assays using antibodies raised against proteins of the Fos and Jun families, and also against the activating transcription factor 2 (ATF2). The AP-1 composition was investigated in HEK-GluR4 cells submitted to excitotoxic stimulation, with 1 mM glutamate plus 100 μM CTZ, and in control cultures. The nuclear extracts were prepared 4 h after stimulation. The results showed the appearance of supershift bands, both in control and stimulated cells, with the c-Jun, JunD or Fra-2 antibodies (Figure 4). In stimulated cells, the bands were more intense, indicating a

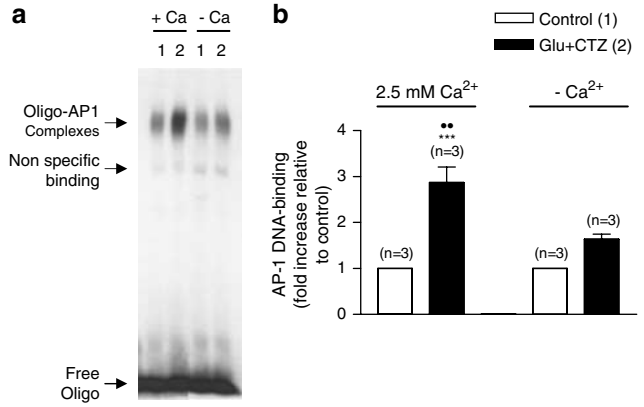

Figure 3 The GluR4-homomeric AMPA receptor-mediated AP-1 activity is Ca^{2+} concentration-dependent. (**a**) Representative autoradiogram showing the DNA-binding activity of AP-1 in HEK-GluR4 cells exposed, for 1 h at 37°C, to 1 mM glutamate plus 100 μM CTZ (Glu + CTZ, 2), in sodium buffer without Ca^{2+} or, alternatively, with 2.5 mM Ca^{2+}. Control cells (1) were submitted to sodium buffer incubation in the absence of drugs. Nuclear extracts were prepared 4 h after stimulation. (**b**) Quantification of the DNA-binding activity of AP-1 in HEK-GluR4 cells stimulated as described for (**a**) using ImageQuant software. Bars represent the mean ± S.E.M. of the indicated number of independent experiments. The intensity of the control band was arbitrarily considered 1. ***Statistically different from control ($P<0.001$), ●● statistically different from stimulation with glutamate plus CTZ in the absence of Ca^{2+} (●● $P<0.01$)

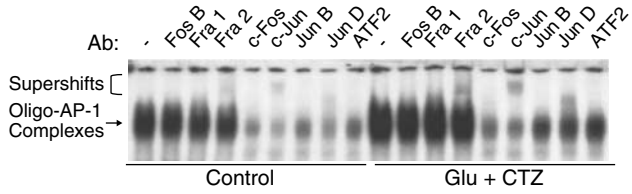

Figure 4 AP-1 transcription factor composition in HEK-GluR4 cells. Representative autoradiogram with the supershift assay for the AP-1 subunits in HEK-GluR4 cells exposed to 1 mM glutamate plus 100 μM CTZ (Glu + CTZ), in sodium buffer with 2.5 mM Ca^{2+}, for 1 h at 37°C. Control cells were submitted to sodium buffer incubation in the absence of drugs. Nuclear extracts were prepared 4 h after stimulation. In all, 12 μg of nuclear protein were incubated with 3 μg of antibody (Ab), for 2 h at 4°C, previously to the incubation with the labelled AP-1 oligonucleotide. Bracket indicates supershifts bands

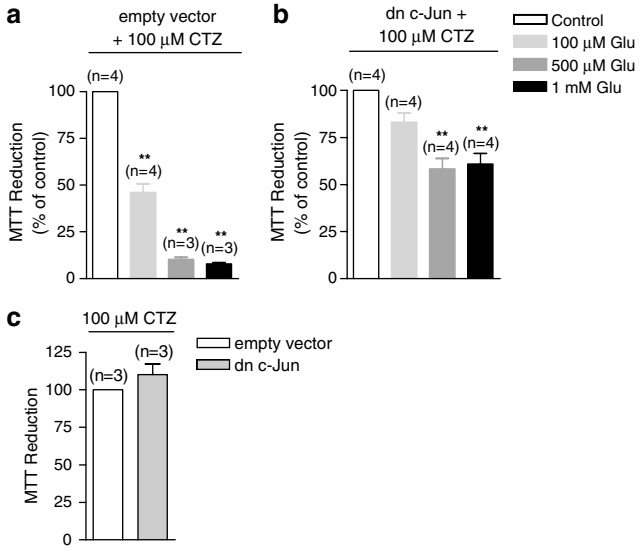

Figure 5 Inhibition of the AP-1 transcription factor protects the HEK-GluR4 cells against excitotoxic cell damage. HEK-GluR4 cultures were transfected with the empty vector (**a**) or the c-Jun dominant negative construct (dn c-Jun) (**b**) using lipofectamine. At 40 h post-transfection, the cultures were exposed to 100 μM, 500 μM or 1 mM glutamate plus 100 μM CTZ, in sodium buffer with 2.5 mM Ca^{2+}, for 1 h at 37°C. Control cells were submitted to sodium buffer incubation in the presence of CTZ and their MTT reduction considered as 100%. (**c**) MTT reduction in cells transfected with the empty vector (considered as 100% of cell viability), or the dn c-Jun, which were incubated for 1 h at 37°C in sodium buffer with 2.5 mM Ca^{2+} and 100 μM CTZ. Bars represent the mean ± S.E.M. of the indicated number of independent experiments. ** Significantly different from control ($P<0.01$)

higher AP-1 DNA-binding activity. With the antibodies against c-Fos (which is pan-reactive for the Fos family members), JunB and ATF2 we observed a decrease in the specific band for the AP-1 complexes, without the appearance of a clear supershift band, both in control and stimulated cells, which may be due to an antibody-induced obstruction to the binding of the transcription factor to the oligonucleotide. These results indicate that the AP-1 dimers in HEK-GluR4 cells included proteins of the Fos family, namely Fra-2, and the c-Jun and JunD proteins of the Jun family. Furthermore, JunB and ATF2 proteins may also be present on the AP-1 transcription factor. We did not observe differences in the assemblage of proteins that constituted the AP-1 complexes present in HEK-GluR4 control or stimulated cells. However, we cannot exclude the possibility of different partner combinations on the AP-1 dimers present in control and stimulated cells.

The AP-1 transcription factor is involved in excitotoxic pathways coupled to Ca^{2+}-permeable GluR4-containing AMPA receptors

To clarify whether the activation of the AP-1 transcription factor, coupled to overstimulation of Ca^{2+}-permeable GluR4-containing AMPA receptors, is contributing to a cell death or survival pathway we analysed the effect of a c-Jun dominant-negative form (dn c-Jun), which inhibits the transcriptional activity of AP-1,[28] on the viability of HEK-GluR4 cells exposed to an excitotoxic stimulus. One day after plating, HEK-GluR4 cultures were transfected with the dn c-Jun or the empty vector, and 40 h later they were exposed, for 1 h at 37°C, to 100 μM, 500 μM or 1 mM glutamate plus 100 μM CTZ, in sodium buffer with 2.5 mM Ca^{2+}. Cell viability was determined by the MTT assay 24 h after stimulation. Cell transfection with the dn c-Jun form did not alter cell viability (Figure 5c), but, after excitotoxic stimulation, cells transfected with the dn c-Jun presented higher values of MTT reduction than cells expressing the empty vector (Figure 5a and b). Indeed, 100 μM glutamate plus 100 μM CTZ did not affect significantly the viability of cells transfected with the dn c-Jun, while it caused a significant decrease of MTT reduction of 53.9 ± 4.7% in cells transfected with the empty vector. Stimulation with higher glutamate concentrations induced approximately 40 or 90% toxicity in cells transfected with the

dn c-Jun or the empty vector, respectively. These results indicate that Ca^{2+}-permeable GluR4-containing AMPA receptors are associated with a cell death pathway regulated, at least partially, by the AP-1 transcription factor.

Discussion

Recent studies revealed a crucial role for Ca^{2+}-permeable AMPA receptors in excitotoxicity-induced neuronal death, namely following transient forebrain ischemia[8,9] and in ALS.[10,11] However, the molecular mechanisms of excitotoxicity associated with Ca^{2+}-permeable AMPA receptors are not completely understood. In this work, we investigated the contribution of the GluR4 subunit of AMPA receptors to the excitotoxic response. We showed that glutamate stimulation of Ca^{2+}-permeable GluR4-containing AMPA receptors decreased HEK-GluR4 cell viability, in a Ca^{2+}-dependent manner, when the receptor desensitisation was prevented with CTZ. Moreover, GluR4-containing AMPA receptor activation increased the DNA-binding activity of the AP-1 transcription factor exclusively under excitotoxic conditions. A dn c-Jun form protected HEK-GluR4 cells against excitotoxic damage, suggesting an involvement of AP-1 on excitotoxic signalling pathways coupled to the GluR4 subunit of AMPA receptors.

Neuronal cells preferentially expressing the GluR4 subunit of AMPA receptors, such as the neurons of the reticular nucleus of the thalamus and the cholinergic neurons of the basal forebrain, are particularly vulnerable to AMPA-induced excitotoxicity.[13,29] Spinal motor neurons, which also possess GluR4-containing AMPA receptors,[14] are more sensitive to Ca^{2+}-permeable AMPA receptor-mediated toxicity than dorsal horn neurons.[10] Chicken amacrine cells that, among the retinal neurons, are particularly sensitive to non-NMDA receptor-mediated excitotoxicity,[30] have AMPA receptors constituted by the GluR3 and GluR4 subunits,[15] characterized by a high Ca^{2+} permeability.[31] Taken together these studies suggest that GluR4-enriched AMPA receptors may play an important role in AMPA receptor-mediated excitotoxicity.

To investigate excitotoxic signalling pathways coupled to the GluR4 subunit of AMPA receptors, we used the HEK-GluR4 cell line which constitutively expresses functional Ca^{2+}-permeable $GluR4_{flip}$-homomeric AMPA receptors.[21] In HEK-GluR4 cells, stimulation of the GluR4-containing AMPA receptors induced toxicity when the receptor desensitization was blocked with CTZ (Figure 1). The intensity of the toxic insult was dependent on the desensitisation degree of the receptors (data not shown). Importantly, toxicity mediated by GluR4-homomeric AMPA receptors increased when the extracellular calcium concentration was raised, suggesting the activation of a Ca^{2+}-dependent excitotoxic mechanism. In HEK-GluR4 cells, the increase in $[Ca^{2+}]_i$, after GluR4-homomeric receptor activation, is primarily due to Ca^{2+} influx through the receptor channel. In fact, studies with HEK 293 cells expressing AMPA receptors showed that stimulation with KCl (40 mM) did not change the $[Ca^{2+}]_i$.[32] These results suggest that, *in vivo*, Ca^{2+}-permeable GluR4-containing AMPA receptors may contribute to the excitotoxic response in the neuronal and oligodendroglial cells that express these receptors. Hence, it is important to study the excitotoxic signalling cascades coupled to Ca^{2+}-permeable GluR4-containing AMPA receptors as this may be relevant in several pathological situations involving excitotoxicity, namely in Alzheimer's disease which, among others, affects basal forebrain cholinergic neurons, and in ALS, where a selective loss of spinal motor neurons is observed.

In this work, we showed that activation of the AP-1 transcription factor, mediated through Ca^{2+}-permeable GluR4-containing AMPA receptors, correlates with excitotoxicity (Figure 2). Moreover, inhibition of the transcriptional activity of AP-1 with a c-Jun dn form[28] protected HEK-GluR4 cells against excitotoxicity (Figure 5). These findings are the first evidences showing that overactivation of Ca^{2+}-permeable GluR4-containing AMPA receptors is associated with a death signalling pathway modulated, at least partially, through the AP-1 transcription factor. An increase in the DNA-binding activity of AP-1 following glutamate receptor activation was previously observed *in vivo*,[33] as well as in primary cultures of striatal neurons,[34] cerebellar granule cells[35] and cortical neurons.[36] Although the activation of the AP-1 transcription factor was correlated to neuronal death in some of these studies, its role in the excitotoxic response is not completely understood. It was found that mice lacking the neuron-specific isoform of JNK, JNK3, display high resistance to KA excitotoxicity, which was associated with a strong decline in c-Jun phosphorylation and a decreased activity of AP-1.[18] Moreover, KA-induced hippocampal neuronal death requires functional c-Jun phosphorylation sites in mice,[17] suggesting that in this excitotoxicity model the transcription factor c-Jun/AP-1 is involved in a death pathway. Recently, JNK3-mediated cell death was also implicated in cerebral ischemia-hypoxia and its apoptotic downstream mechanisms may include the induction of *Bim* and *Fas*, and the mitochondrial release of cythocrome *c*.[20] However, it remains to be elucidated whether c-Jun/AP-1 transcriptional activity is involved in JNK3-mediated gene induction. *Bim* expression was also observed in sympathetic neurons deprived from trophic support. In this apoptotic model, a dn c-Jun protein protected sympathetic neurons from death, which was associated with an inhibition of cytochrome *c* release from the mitochondria and a decrease in *Bim* expression.[37] Despite the considerable progress made towards the understanding of the JNK/c-Jun signalling pathway, the molecular links between glutamate receptors and JNK activation are not completely known. Interestingly, a recent report showed that postsynaptic density protein 95 (PSD-95) links the GluR6 KA receptor subunit to JNK activation by anchoring to the receptor complex the mixed lineage kinase (MLK)2/3, which is an upstream activator of JNKs. The disturbance of these specific molecular connections reduced both KA-induced JNK activation and cell toxicity.[38] Likewise, in rats submitted to focal ischemia, the specific disruption of the interaction between the NR2B subunit of NMDA receptors and the PSD-95, which couples NMDA receptors to neuronal nitric oxid synthase (nNOS), reduced brain damage and improved the neurological function, without the negative consequences associated with blocking NMDA receptors.[39] Thus, dissection of the molecular pathway which couples Ca^{2+}-permeable GluR4-containing AMPA receptors to c-Jun/AP-1 will allow

the development of selective therapeutic strategies aiming at the disruption of specific intracellular interactions coupled to glutamate receptors while maintaining the physiological actions of the receptor.

Materials and Methods

Material

Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), fetal calf serum was purchased from Biochrom KG (Berlin, Germany), and trypsin and geneticin (G418) from Gibco BRL Life Technologies (Paisley, UK). CNQX and CTZ were obtained from Tocris Cookson Ltd (Bristol, UK). The DNA stain Hoechst 33342 was from Molecular Probes Europe (Leiden, Netherlands). Both oligonucleotides, with the consensus binding motif for the AP-1 and the Oct-1 transcription factor, were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The T4 polynucleotide kinase was from USB Corporation (Cleveland, OH, USA). Sephadex G-50 spin column, poly(dIdC), and [γ-^{32}P]ATP (10 mCi/ml) were purchased from Amersham Biosciences, Part of GE Healthcare (Buckinghamshire, UK). The BCA assay kit was from Pierce (Rockford, USA). The protease inhibitors cocktail Complete Mini was from Roche Applied Science (Penzberg, Germany). The following antibodies, raised against members of the AP-1 transcription factor, were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany): c-Fos (sc-253-G), FosB (sc-48), Fra-1 (R-20), Fra-2 (sc-604), JunB (sc-46-G), c-Jun/AP-1 (sc-45-G), JunD (sc-74-G) and ATF-2 (sc-187). The dn c-Jun form was generously provided by Eisai London Research Laboratories Ltd (London, UK). The QIAGEN Plasmid maxi kit was obtained from QIAGEN (QIAGEN GmbH, Germany). Lipofectamine™ Reagent, and OptiMEM® I were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck (Darmstadt, Germany). Stock solutions of CNQX and CTZ were made in dimethyl sulfoxide. All the other chemicals were kept in aqueous stocks.

Cell culture and transfection

HEK293 cells, constitutively expressing the GluR4$_{flip}$ subunit of AMPA receptors, were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS), in the presence of geneticin (G418), and kept at 37°C, in a humidified incubator with 5% CO_2/95% air.[21] The GluR4 clone, obtained from HEK293 cells transformed with a vector containing the human GluR4 cDNA, was selected with G418.[21] In the transient expression studies, 1 day before transfection, cells (0.5×10^5 cells/cm^2) were plated in 48-well plates coated with poli-D-lysine (0.1 mg/ml) and cultured in DMEM without G418. Transfections were carried out with Lipofectamine™ Reagent, OptiMEM® I and 0.15 μg DNA/cm^2, for 3 h at 37°C. After transfection, the medium containing the DNA–lipofectamine complexes was replaced with the HEK-GluR4 conditioned medium, and 40 h later cells were submitted to excitotoxic stimulation. In the transient transfection experiments, G418 was absent from the media.

Exposure to excitatory amino acids

At the second day *in vitro*, subconfluent cultures of HEK-GluR4 cells, plated in multiwell plates coated with poli-D-lysine (0.1 mg/ml), were washed and exposed to 100 μM or 1 mM glutamate, in sodium buffer (132 mM NaCl, 4 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.4) with 2.5 mM $CaCl_2$, for 1 h, at 37°C. When CNQX or CTZ was used, a preincubation of 5 min was performed. After stimulation, cells were washed, placed on serum-free DMEM, with G418 and 2.5 mM $CaCl_2$, and kept at 37°C in the incubator. To investigate Ca^{2+}-dependent events, glutamate stimulation was performed in Na^+ buffers with 0–2.5 mM Ca^{2+}. In the absence of calcium, 1 mM $MgCl_2$ was added to the Na^+ buffer.

Assessment of cell viability

Assessment of cell viability was performed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium bromide (MTT), or by analysis of the nuclear morphology. MTT (0.5 mg/ml) in sodium buffer with 1 mM $CaCl_2$ was added to the cultures, 20–24 h after stimulation, and incubated for 30 min (12-multiwell plates) or 3 h (48-multiwell plates) at 37°C. Formazan, the water-insoluble blue-coloured product of the MTT reduction by live cells, 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 HEK-GluR4 cells was analysed by fluorescence microscopy using the DNA stain Hoechst 33342, which fluoresces blue and is cell permeable.[40] Viable cells display a normal nuclear size and a diffuse blue fluorescence, while excitotoxicity damaged cells display bright blue pyknotic nuclei with condensed or fragmented chromatin. At 13 h after excitotoxic stimulation, HEK-GluR4 cells were fixed at room temperature with 4% paraformaldehyde in phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), for 20 min, washed with PBS and incubated with Hoechst 33342 (15 μg/ml) for 10 min. Cells were examined with a Zeiss Axioskop microscope. The experiments were performed in duplicates.

Preparation of nuclear extracts and electophoretic mobility shift assay (EMSA)

Nuclear extracts of HEK-GluR4 cells were prepared 2, 4 or 6 h after stimulation with glutamate, as previously described.[4] Briefly, cells were washed with ice-cold PBS and solubilized in ice-cold buffer 1 (10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EGTA, pH 7.5) for 30–40 min. The nuclei were pelleted by centrifugation at 2400 $\times$ *g*/10 min/4°C, resuspended in ice-cold buffer 2 (25 mM HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 20% glycerol, pH 7.4) for 60 min, and then centrifuged at 12 000 $\times$ *g*/20 min/4°C. The supernatants (nuclear extracts) were collected and stored at -70°C until use. Both buffers were supplemented with 1 mM dithiothreitol (DTT) and with the protease inhibitors cocktail *Complete Mini* immediately before use. Protein concentration of the extracts was measured using the BCA assay kit.

For the AP-1 DNA-binding assay, a double-stranded oligonucleotide, with a consensus binding motif for the AP-1 transcription factor (5′-CGCTTGATGACTCAGCCGGAA- 3′), was end-labelled with [γ-^{32}P]ATP by the T4 polynucleotide kinase and purified through a Sephadex G-50 spin column. Eight μg of nuclear proteins were incubated, for 30 min at room temperature, with 150 000 cpm of [γ-^{32}P]-labelled oligonucleotide probe, 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).[4] 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 gel, the DNA–protein complexes were

visualized by autoradiography. The specificity of binding was assessed by competition with a 100-fold excess of unlabelled oligonucleotides selective for AP-1 and Oct-1 (Oct-1, 5′-TGTCGAATGCAAATCACTAGAA-3′). For supershift analysis, before the addition of the radiolabelled oligonucleotide, 12 μg of nuclear proteins were incubated with 3 μg of antibody, for 2 h at 4°C. The scanned digital images were quantified using the ImageQuant software (Amersham Biosciences, Part of GE Healthcare).

Other methods

Results are presented as means $\pm$ S.E.M. of the number of experiments indicated. Statistical significance was determined by ANOVA analysis followed by the Dunnett's and/or Bonferroni's test, or unpaired students *t*-test.

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