ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1994, 54: 65-72

Seizures-evoked activation of transcription factors

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Abstract. Chemically provoked seizures have proved to serve as useful model to investigate long term neuronal responses collectively termed as neuronal plasticity. In particular, rapid, transient activation of immediate early gene expression induced by such chemoconvulsants like pentylenetetrazole (PTZ) and kainic acid (KA) drew a great attention. These genes code for transcription factors, known to influence gene expression, and therefore able to orchestrate genomic responses to extracellular stimuli. In our studies reviewed herein and reported in detail eslewhere, we have investigated PTZ- and KA-dependent activation of a functional feature of transcription factors i.e. their DNA-binding activity. We have found that only AP-1 DNA-binding activity was elevated in the rat hippocampus, entorhinal and sensory cortices 2-6 h after the PTZ administration, and only in the hippocampus and entorhinal cortex at similar times following KA injection. The AP-1 response to PTZ was strikingly enhanced in aged (18-24 months old) animals when compared to young (3 months old) ones. KA, apart from this early phase of AP-1 DNA-binding activity increase, evoked also the late one (reaching a peak value at 72 h). The protein composition of the latter differed from the former mostly by substitution of Jun B with Jun D protein and lack of c-Fos. Because the KA treatment leads not only to the seizures but to apoptosis (programmed cell death) as well, our results indicate that various AP-1 complexes may be involved in both of these phenomenona.

Key words: kainic acid, metrazole, epilepsy, apoptosis, *c-fos, c-jun,* gel shift

INTRODUCTION

It has been well proven that glutamate or, more generally, excitatory amino acids (EAA) are of critical importance for development and maintenance of seizure activity (Meldrum 1991). In particular, the role of stimulation of such subtypes of EAA receptors as those recognized by either N-methyl-D-aspartate (NMDA) or kainate (KA) is well documented in initiation and propagation of seizures (Meldrum 1991). Obviously, other neuronal inputs are also of paramount significance as e.g. an excessive synaptic excitation is partially prevented by close coupling of EAA (i.e. glutamatergic) excitation and inhibitory amino acid (GABA-ergic) inhibition (Meldrum 1991).

Kainic acid (KA) is an analog of glutamate which causes neuronal excitation as well as neuronal death following systemic or intracerebral injections (Schwob et al. 1980, Ben-Ari et al. 1981, Ben-Ari 1985). It was suggested that KA might induce neuronal loss both by a direct excitotoxic action and by the intense seizure activity (Schwob et al. 1980). As a convulsive agent which exerts severe neuropathological effects, KA may prove useful in establishing an experimental model of epilepsy. In particular, systemic injections of the drug produce generalized tonic-clonic seizures in association with widespread damage to forebrain structures.

Pentylenetetrazole (PTZ) is a potent chemoconvulsant and an useful agent in the screening of anticonvulsant drugs that are effective in the treatment of epilepsy (Woodbury et al. 1982). Probably by blocking inhibition provided by the opening of GABA-receptor-chloride channels, PTZ potentiates the excitatory responses generated at EAA receptors. PTZ evoked seizures can be reversed by EAA antagonists acting on different subtypes of receptors including the NMDA kind. Seizure activity elicited by administration of PTZ is not accompanied by neuronal damage (Meldrum 1991).

SEIZURES-EVOKED ACTIVATION OF TRANSCRIPTION FACTORS

Many studies have established that excitation of neurones results in adaptative and plastic alterations which require ongoing protein synthesis and lead to changes in gene expression (Morgan and Curran 1991a,b, Kaczmarek 1993a,b). Administration of the convulsant doses of KA and PTZ to rats and mice causes a dramatic increase of mRNA level of *c-fos* nuclear protooncogene (Morgan et al. 1987, Sonnenberg et al. 1989, Morgan and Curran 1991a). An increase of the mRNA level is followed by elevated c-Fos protein immunoreactivity (Le Gal La Salle 1988, Sonnenberg et al. 1989a,b, Popovici et al. 1990, Sakurai-Yamashita et al. 1991, Gass et al. 1993, Pennypacker et al. 1993).

The importance of these findings stems from the fact that c-Fos together with members of Jun family may form transcription factor AP-1 (activator protein-1) as shown on Fig.1 (for review see: Morgan and Curran 1991b). The AP-1 complex binds with high affinity to the DNA regulatory element (also termed AP-1) in promoter and enhancer regions of many genes and has been shown to be able to confer both positive and negative regulation of their expression (Chiu et al. 1989, Angel and Karin 1991). There are at least four Fos: c-Fos, Fos B, Fra 2, Fra 1 and three Jun proteins: c-Jun, Jun B and Jun D. A common features of these proteins is their "leucine zipper" region which allows for dimerization between Jun family proteins (including homodimers and heterodimers) and between Jun family proteins and those encoded by the *fos* family of genes (Fos-Jun heterodimers) (Gentz et al. 1989, Schutte et al. 1989, Bravo et al. 1990, Hai and Curran 1991). Understanding of the functional role of the each dimer presents a formidable challenge. Unfortunately, almost no data are available about the composition of AP-1 complexes *in vivo* at different physiological situations.

Increased expression of at least of some of AP-1 components has been found to be induced by all

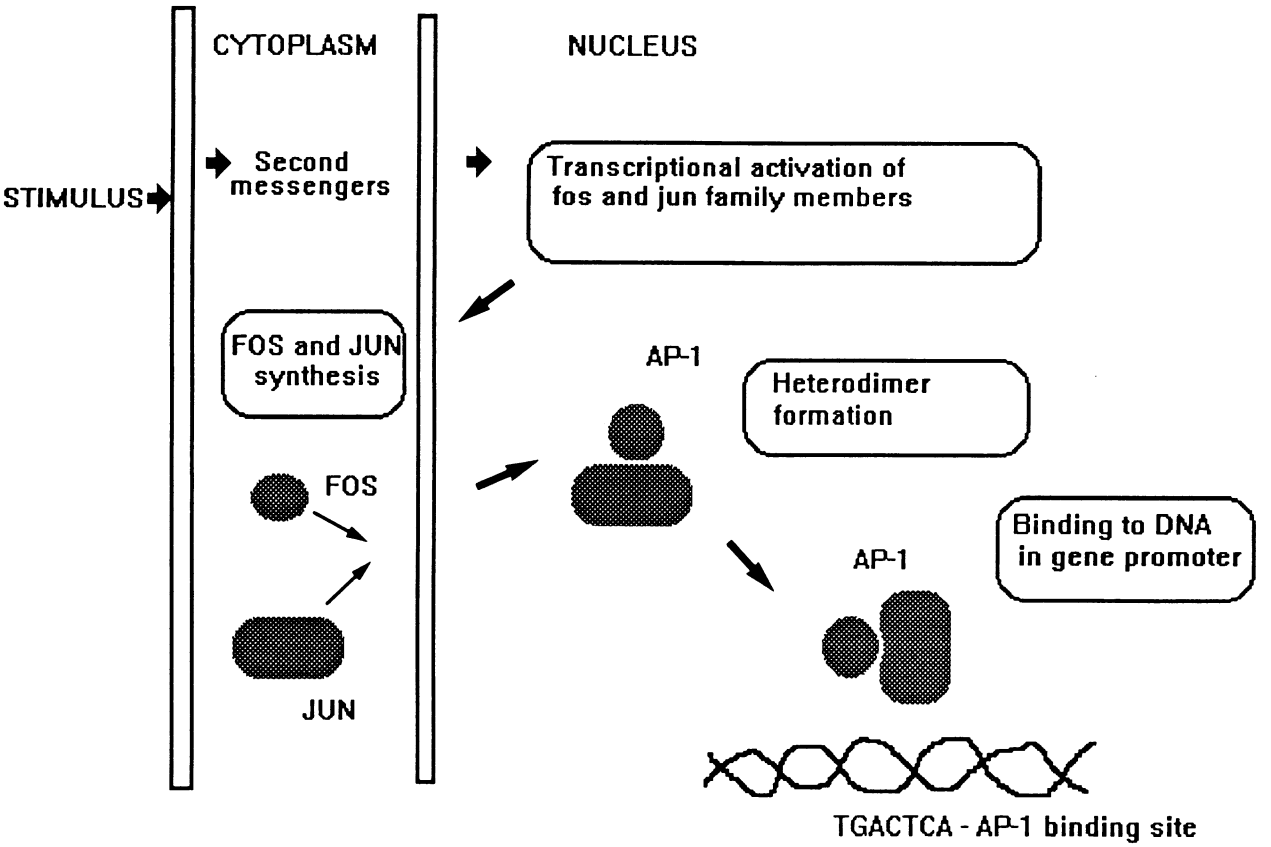

Fig. 1. Diagram illustrating an activation of AP-1 transcription factor.

chemoconvulsants studied so far like kainate, pentylenetetrazol (PTZ), NMDA, picrotoxin, flurothyl, bicucculline, as well as by other epileptogenic treatments, including electrical stimulation and specific lesions (for review see: Morgan and Curran 1991a,b). The elevated gene and protein expression has been repeatedly found to follow similar transient kinetics. The PTZ evoked seizures offer the best studied example in this regard. Within an hour after the seizures there is an increase of AP-1 DNA binding activity stemming apparently from an increase of *c-fos* mRNA and protein as well as expression of other members of *fos* and *jun* gene families (Morgan at el. 1987, Saffen et al. 1988, Sonnenberg et al. 1989a). These increases are transient and AP-1 levels are low again within 6-8 h after the PTZ seizures. Similar kinetics occurs after a plethora of physiological situations (Kaczmarek and Kamińska 1989, Angel and Karin 1991).

For transcription factors acting as dimers, the expression of any of their selected components cannot be taken as a sufficient measure confirming the presence of active complex in the cell. There are pieces of evidence that under some circumstances the expression of c-Fos and c-Jun protein does not occur in the same cells, as revealed by double immunocytochemical staining of neurons in the suprachiasmatic nuclei (Takeuchi et al. 1993). Since such colocalization is not uniformly present probably Fos- and Jun-expressing cells comprise distinct cell populations. Thus, we addressed the question about the expression of functional form of transcription factors.

In general, transcription factors (TF's) have two distinct functional activities. A DNA-binding activity referred to as its ability to bind the specific DNA sequence present in regulatory element of genes is responsible for directing TF to the vicinity of the

promoter. A transactivating potential is the capability to stimulate gene expression through interaction with general transcription machinery (Fig.2). We decided to study the DNA-binding activity of selected TF's using the electrophoretic mobility shift assay (EMSA), as the studies of transactivating potential are technically much more difficult. EMSA is a rapid, simple and extremely sensitive method for the detection of sequence-specific DNA-binding protein. Either crude cellular extracts or nuclear protein fractions can be used in this method to study the binding specificity and to determine the abundance, affinity, etc. of the TF. An end-labelled DNA fragment (containing the binding site for the protein) is incubated with the protein extracts and later electrophoresed through a native polyacrylamide gel which is then dried and exposed onto X-ray film for the autoradiography. Proteins that bind to the DNA fragment retard its mobility during electrophoresis resulting in discrete bands corresponding to distinct protein-DNA complexes (Kamińska and Kaczmarek 1993).

We have introduced several control procedures to standardize the EMSA method (Kamińska and Kaczmarek 1993, Łukasiuk and Kaczmarek, in press, Kamińska et al., submitted). One important control was to ensure that the protein amounts used in the binding reaction were equal in all samples. The protein concentration was colorimetrically determined and confirmed by Coomassie staining of SDS-polyacrylamide gels. Specificity of the retarded bands was demonstrated by their disappearance when 15-fold excess of unlabelled sequence was added to the binding reaction.

In our first studies we found that using this method we could easily distinquish the activation of two different transcription factors in the same nuclear extract from the rat brain following PTZ-induced seizures. We investigated the abundance of AP-1 and CREB/ATF (cAMP responsible element binding proteins) transcription factors by determining their DNA-binding activities. Surprisingly, after the seizures the AP-1 DNA-binding activity was overinduced in the hippocampus of aged (2 years old) rats when compared to young (3-months old) animals (Kamińska and Kaczmarek 1993). This finding is important as potential target for AP-1 is the gene coding for β-amyloid presursor protein (APP) whose participation in pathogenesis of Alzheimer disease has been well proven (Ashall and Goate 1994).

During last two years our major efforts were focused on systematic studies on the DNA-binding activities of different transcription factors in the rat brain after seizures elicited by either kainate or PTZ. Figure 3 shows an activation of the AP-1 com-

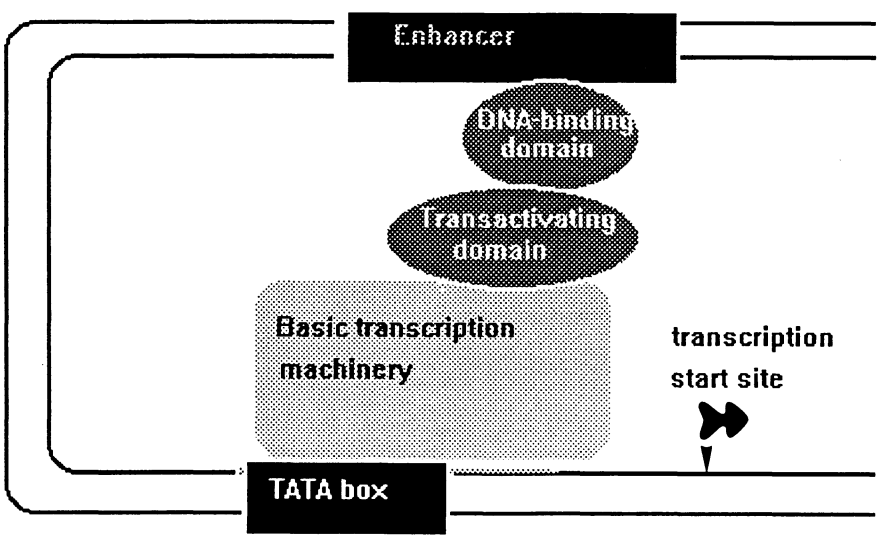

Fig. 2. Schematic model of a transcription factor complex.

plex in the hippocampi of rats injected with KA or PTZ. Because it has been shown that even weak stressors like handling (Campeau et al. 1991) can induce an AP-1 component, *c-fos* expression, all experiments were carried out after 6 days of habituation to injection of physiological saline. Indeed, we excluded the role of injection itself as an inducer, showing no effects of injection alone on the DNA-binding activities.

Our studies revealed significant differences in the patterns of TF activation following different kinds of seizures. After PTZ the AP-1 was induced equally well in the hippocampus as in the entorhinal and sensory cortices (Łukasiuk and Kaczmarek, in press). Kainate-induced seizures enhanced the AP-1 DNA-binding activity only in the hippocampus and entorhinal cortex (Kamińska et al., submitted) suggesting a correlation between the sites of AP-1 expression and the behavioral phenotype of seizures, known to differ between the PTZ and KA treatments (Morgan and Curran 1991a). These increases of AP-1 DNA-binding activity were selective and did not reflect the general increase in transcription factor activities after seizures. We demonstrated that DNA-binding activities of other transcription factors, like CREB/ATF and Octamer, did not change after PTZ or KA elicited seizures (Kamińska et al., submitted; Łukasiuk and Kaczmarek, in press).

KAINATE-INDUCED NEURONAL DEATH

Elucidating of molecular basis of neurodegeneration is among major challenges of the present research. It is well recognized that the knowledge of these processes should prevent and help to fight the most disabling brain diseases including Alzheimer's, Parkinson's, ischemia, etc. It has been recently suggested that excitotoxicity, i.e. neuronal cell death evoked by an excessive action of excitatory amino acids (EAA) could be involved in all of these disorders (Choi 1988).

In our studies we have focused on EAA-dependent neurotoxicity, using kainate treatment as a model system. Kainate is known to interact with non-NMDA EAA receptors leading to neuronal depolarization. Moreover kainate activates neurons both directly as well as transsynaptically. Therefore, final effects of kainate are complex, and dependent not solely on its own receptors but also on transsynaptically activated other subtypes of EAA receptors .

The main effects of kainate on the brain are induction of seizures, which last up to a few hours after the treatment, and neuronal cell death, well visible several days after the onset of the seizures. Traditional thinking in toxicology and pharmaco-

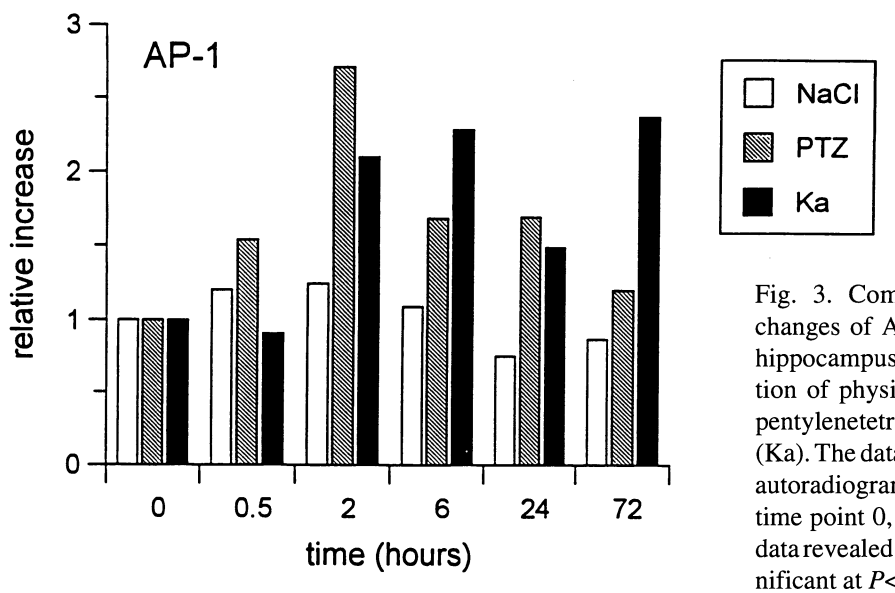

Fig. 3. Comparison of time-course of relative changes of AP-1 DNA binding activity in the rat hippocampus following intraperitoneal administration of physiological saline (NaCl), 50 mg/kg of pentylenetetrazole (PTZ), or 10 mg/kg of kainate (Ka). The data are based on densitometer-reading of autoradiograms. Results were normalized for the time point 0, taken as 1. Statistical analysis of the data revealed that increases at 2, 6 and 72 h were significant at $P<0.05$ (Mann-Whitney, two-tailed).

logy considered cell death as a passive degenerative event consequent to toxic injury. Kerr, Wyllie and Currie (1972) introduced the concept of apoptosis describing a nontoxic type of cell death, a genetically programmed event that requires active gene transcription and translation. Molecular mechanisms of apoptosis are not well understood but it is clear that they involve a sequence of events, starting from cell surface receptor activation, through increased second messenger levels, with elevation of Ca^{+2} being of great importance, then turning on transcription factors and finally including activation of specific endonucleases fragmenting the genetic material (Martin et al. 1994).

The question whether *in vivo* excitotoxicity is mediated by mechanisms of programmed cell death - apoptosis remains the matter of controversy. Ignatowicz et al. (1991) suggested that hippocampal neuronal cell death induced by neurotoxic doses of kainate or quinolinic acid injected into the hippocampus was not mediated by apoptosis. They did not find any signs of DNA fragmentation at 8 and 16 h after the intrahippocampal injection of the neurotoxins. On the other hand, Smeyne et al. (1993) has proposed that kainate evoked neuronal loss bears characteristics of programmed cell death, however no proof for this notion was provided.

More recently we have found a direct evidence that kainate-induced neuronal death goes through an apoptotic pathway. Early in apoptosis nuclear DNA is cleaved at intranucleosomal sites and the DNA fragments are released to the cytoplasm. On an agarose gel, DNA from apoptotic cells displays a ladder which ascends in multiples of 180 base pairs representing DNA released from the nucleosomes and nucleosome oligomers (Bursh et al. 1992, Cohen 1993). This pattern of DNA degradation is believed to be diagnostic feature for apoptosis. We have found the characteristic ladder-like pattern of DNA fragmentation in the hippocampi, entorhinal and sensory cortices at 72 h after kainate treatment. The appearence of DNA fragmentation was in a good spatio-temporal correlation with the neurodegeneration.

A growing body of evidence suggests a possible involvement of protein synthesis in excitotoxin-mediated brain damage. It is then of great importance that recently Schreiber et al. (1993) observed that cycloheximide, an inhibitor of protein biosynthesis, blocked KA-evoked neuronal degeneration in the rat hippocampus. Interestingly, the same authors also demonstrated that kainate-induced neuronal death correlated with a prolonged expression of *c-fos* gene and both these events were prevented by cycloheximide. These results led to the idea that prolonged c-fos expression could be a marker of neuronal death.

Moreover, Smeyne et al. (1993), using *c-fos-lacZ* transgenic mice, expressing β-galactosidase under control of *c-fos* regulatory elements, observed that β-gal levels were increased transiently within few hours after the KA treatment, then declined and raised again at several days following KA injection. These results were interpreted as indicating elevated *c-fos* expression to correlate with KA-evoked programmed cell death. Although intriguing and suggestive, these studies were based on a gene construct and they did not address the functional form of c-Fos i.e. AP-1. Unfortunately, previous studies on KA-evoked elevation of AP-1 DNA binding activity, which can be viewed as a reflection of a functional form of transcription factor, have not proceeded beyond several hours after the seizures (Sonnenberg et al. 1989b, Pennypacker et al. 1993).

In another recently described model of electrically induced *status epilepticus* coupled to neurodegeneration, an increase of c-Jun immunoreactivity and to much smaller extent of c-Fos and Jun B was demonstrated (Dragunow et al. 1993). These results suggested that not only *c-fos* but AP-1 activation as well could be involved in a programmed cell death. Similar suggestions could be also drawn from studies on other cell systems. An increase of c-fos and *c-jun* as well as AP-1 expression during programmed cell death of thymocytes has been also reported (Colotta et al. 1992, Sikora et al. 1993). Moreover, blocking of either *c-fos* or *c-jun* expression by anti-sense oligonucleotidess prevented apoptosis of thymocytes (Colotta et al. 1992).

Thus, enhanced expression of certain AP-1 components in correlation with neuronal cell death led us to suggest that AP-1 itself could also be elevated. To prove this hypothesis, we analyzed the kinetics of KA-elevated AP-1 DNA binding activity in various areas of rat brain, known to be engaged in KA-induced neurodegeneration, namely hippocampus, entorhinal cortex and sensory cortex. We noted two phases of elevated AP-1 DNA binding activity in limbic structures (hippocampus and entorhinal cortex) at 2-6 hours and 72 h after the kainate treatment. Then we found it was of particular interest to investigate the protein composition of AP-1 at early (seizure-related) and late (linked to neurodegeneration) phases of AP-1 activation. Interestingly, apparent composition of the AP-1 changed over this time. During the early phase c-Fos, Fra-2, Fos B and Jun B proteins were detected while at 72 hours Fos B and Jun D were major participants in AP-1 DNA binding activity. Incidentally, the protein composition of AP-1 activated after the PTZ seizures greatly resembled the one obtained at early times after the KA treatment. In particular no participation of Jun D in the AP-1 complex was observed. Morevoer, no second phase of AP-1 elevation was detected at 72 h after the PTZ evoked seizures, further supporting specific involvement of late phase of AP-1 activation in KA-dependent neurodegeneration, as no such phenomenon occurs after the PTZ administration.

Our results not only demonstrated that kainate-induced programmed cell death correlates with the prolonged expression of AP-1 transcription factor. These findings raise an intriguing possibility that physiological differences in AP-1 complex may lie at the foundation of specific roles played by this TF in seizures vs. neurodegeneration.

ACKNOWLEDGEMENTS

This work was partially supported by KBN grant 2228/4/91. The authors would like to express their gratitude to prof. B. Grzelakowska-Sztabert for careful reading of the manuscript and helpful comments.

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Paper presented at the Conference on 75-th Anniversary of the Nencki Institute of Experimental Biology