Short communication

Phenidone blocks the increases of proenkephalin and prodynorphin gene expression induced by kainic acid in rat hippocampus: involvement of Fos-related antigene protein

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Abstract

To determine the possible role of cyclooxygenase/lipoxygenase pathway in the regulation of proenkephalin (proENK) and prodynorphin (proDYN) gene expression induced by kainic acid (KA) in rat hippocampus, the effects of esculetin, aspirin, or phenidone on the seizure activity, proENK and proDYN mRNA levels, and the level of fos-related antigene (Fra) protein induced by KA in rat hippocampus were studied. Esculetin (5 mg/kg), aspirin (15 mg/kg), or phenidone (50 mg/kg) was administered orally five times every 12 h before the injection of KA (10 mg/kg, i.p.). Seizure activity induced by KA was significantly attenuated by phenidone. However, neither esculetin nor aspirin affected KA-induced seizure activity. The proENK and proDYN mRNA levels were markedly increased 4 and 24 h after KA administration. The elevations of both proENK and proDYN mRNA levels induced by KA were inhibited by pre-administration with phenidone, but not with esculetin and aspirin. ProENK-like protein level increased by KA administration was also inhibited by pre-administration with phenidone, but not with esculetin and aspirin. The increases of proENK and proDYN mRNA levels induced by KA were well correlated with the increases of Fra protein level. Additionally, the induction of Fra protein was inhibited by pre-administration with phenidone, but not with esculetin and aspirin. The results suggest that blockade of both cyclooxygenase and lipoxygenase pathways appears to be responsible for increases of proENK and proDYN mRNA levels induced by KA via inhibiting the induction of Fra protein in rat hippocampus.

Keywords: Kainic acid; Cyclooxygenase; Lipoxygenase; Proenkephalin; Prodynorphin; Fra protein; Hippocampus

Kainic acid (KA), a unique neuroexcitatory/neurotoxic substance, produces seizure and neural degeneration in the mammalian CNS, and appears to provide a good model for some aspects of human temporal lobe epilepsy [3,17]. In the rat hippocampus, KA receptors are concentrated in the CA3/CA4 regions, with moderate levels in the other areas of the hippocampus [8,12].

Several types of proto-oncogene product, such as Fos and Jun family proteins, serve as the third messengers in the regulation of various types of genes [4,14]. These proteins interact with AP-1 domain in the promoter of various types of genes, in turn, modulate the transcriptional activity [16]. Convulsant doses of KA induce c-Fos, Fra, and Jun proteins in rat hippocampus and entorhinal cortex [13,19]. In addition, the increases of c-Fos, Fra, and Jun proteins induced by convulsant dose of KA correlate with AP-1 DNA binding activity [18,19]. The neurons of the hippocampus express both proENK and proDYN mRNA and their protein products [11,15]. These proENK and proDYN mRNA levels are also elevated by KA [9]. Both proENK and proDYN genes contain an AP-1-like domain in their promoter regions [7,10].

Cis-unsaturated fatty acids can be liberated by phospholipase A₂ or diacylglycerol lipase and serve as a precursor, and subsequently metabolized further via the action of cyclooxygenase and lipoxygenase [5]. Recently, Suh et al. [21,22] have reported that arachidonic acid or prostaglandin E₂ increases proENK mRNA levels in bovine chromaffin cells. However, the involvement of cyclooxygenase and lipoxygenase pathways in KA-induced increase of opioid peptides mRNA in the hippocampus has not been
characterized. In an attempt to examine the role of cyclo-
.oxygenase/lipoxygenase in the regulation of proENK and
proDYN mRNA expression, the effects of phenidone, es-
culetin, and aspirin on KA-induced proENK and proDYN
mRNA levels, or proENK and Fra proteins were studied in
the present study.

Male Sprague–Dawley rats weighing 200–280 g were
used. Animals were housed two per group in a room
maintained at 22 ± 5°C with an alternating 12 h light–dark
cycle. Animals were used only once. Esculetin (5 mg/kg),
aspirin (15 mg/kg), or phenidone (50 mg/kg) was admin-
istered orally five times every 12 h. Thirty minutes after
the last administration of each drug, KA (10 mg/kg) was
administered intraperitoneally. Then, animals were sacrifi-
ced 4 and 24 h after KA administration.

Seizure activity was rated during a 4-h period after the
KA challenge according to the scale devised by Baran et
al. [1]. The seizure rating scale were as follows: 0; normal,
rare wet-dog shakes (WDS), no convulsions; 1; intermedi-
ate numbers of WDS, rare convulsion type A, 2; frequent
WDS, frequent type A convulsions (no rearing or saliva-
tion); 3; frequent WDS, frequent type A convolution, ap-
pearance of type B convulsions with rearing (but without
falling), salivation; 4; WDS, type A convulsions, frequent
type B convulsions with falling, salivation; 5; continuous
generalized limbic seizures, death within 3 h. Type A
convulsions: focal convulsions affecting head and extremi-
ties, starting 30 min after KA injection. Type B convul-
sions: frequent generalized convulsions usually starting
60–90 min after KA injection. Surviving rats expressed
recurrent seizure behavior.

Animals from each group were anesthetized with 50
mg/kg pentobarbital and then perfused transcardially with
a 140 ml syringe containing saline (40 ml/100 g body
weight) followed by 4% paraformaldehyde (70 ml/100 g
body weight). The brains were then removed, stored in 4%
paraformaldehyde overnight and then cut at 40 μm in the
horizontal plane with a vibratome. Prior to overnight incu-
bation in primary antibody, sections were prewashed in
0.2% Triton X-100 for 15 min, followed by 4% normal
goat serum for 20 min. After a 24-h incubation with the
primary Fra antisera (1:1000 dilution) or proENK antisera
(1:1000 dilution), sections were then incubated with a
secondary biotinylated antiserum (1:500 dilution) for 1 h.
Sections were always washed three times with PBS (pH
7.4) between each incubation step. As the chromogen,
3,3′-diaminobenzidine was used.

Total cellular RNA and proteins were extracted from
pooled rat hippocampus (n = 3/group) using a rapid
guanidine thiocyanate–water saturated phenol/chloroform
extraction and subsequent precipitation with acidic sodium
acetate [6]. A total of 10 μg of total RNA were elec-
trophoresed and transferred on nylon membrane. After
baking for 1–2 h at 80°C, the membranes were pre-hy-
bridized at 68°C for 1 h in pre-hybridization buffer (5 ×
SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl
sarcosine, 2% blocking reagent), and hybridized overnight
in Dig-labeled proenkephalin and prodynorphin probes
containing prehybridization buffer at 68°C. Then mem-
branes were washed twice in 2 × wash solution (2 × SSC,
0.1% SDS) at room temperature, and further washed in
0.1 × wash solution (0.1 × SSC, 0.1% SDS) at 68°C for 1
h. After equilibrating in a buffer I (100 mM maleic acid,
150 mM NaCl, pH 7.5) for 1 min, the membranes were
gently agitated in buffer II (1% blocking reagent in buffer
I) for 30–60 min and immuno reacted with the diluted
anti-DIG-alkaline phosphatase [1:10,000 (75 mU/ml)] in a
buffer II for 30 min. After washing twice for 15 min per
wash in 0.3% Tween 20 (in buffer I), the membranes were
hybridized in a buffer III (100 mM Tris–HCl, pH 9.5,
100 mM NaCl, 50 mM MgCl₂) for 2 min and added 1:100
diluted CSPD (Boehringer Mannheim, Germany) solution,
a chemi-luminescent substrate solution, in buffer III. After
incubation at 37°C for 15–20 min, the membranes were
exposed to Hyperfilm-MP (Amersham, Buckinghamshire,
England). For re-hybridization, membranes were washed
for 20 min at room temperature in sterile millepore water,
then further washed for overnight at 65°C in 50 mM
Tris–HCl, pH 8.0, 50% dimethylformamide and 1% SDS,
and re-hybridized to Dig-labeled rat cyclophilin cRNA
probe as a internal loading control.

All data shown in the present study were repeated at
least three-times and showed the same tendency of results.
No animal received phenidone, esculetin or aspirin alone
exhibited seizure behavior (data not shown). The rats
receiving KA (10 mg/kg) manifested robust behavioral
seizures lasting 4 to 6 h (Fig. 1A). Increased seizure scores
induced by KA were significantly attenuated by the pre-
treatment with phenidone (Fig. 1A). However, neither
esculetin nor aspirin affected KA-induced response (Fig.
1A).

Both hippocampal proENK and proDYN mRNA levels
were not altered in the animals treated with saline, phenidone,
esculetin or aspirin alone (Fig. 1B). Both proENK and proDYN mRNA levels were remarkably in-
creased 4 and 24 h after KA administration. Six hours after
KA injection, in all nonseizing rats given KA plus
phenidone, esculetin or aspirin, the levels of proENK and
proDYN mRNA was sightly increased. However, these
levels were returned to the basal level after 24 h after KA
administration (Fig. 1B). Seizing rats pretreated with
phenidone, but not esculetin and aspirin, exhibited de-
creases in both proENK and proDYN mRNA levels at all
time points (Fig. 1B).

Hippocampal proENK-like immunoreactivity or Fra-im-
munoreactivity was not affected by the treatment with
phenidone, esculetin or aspirin alone (data not shown).
As shown in Figs. 2 and 3, KA significantly increased proENK
and Fra proteins, especially in the dentate gyrus area in the
hippocampus. The increases of proENK and Fra proteins
induced by KA was significantly attenuated by the pre-
treatment with phenidone. However, neither esculetin nor
Fig. 1. (A) Effects of phenidone, esculetin and aspirin on seizure activity induced by kainic acid (KA). (B) proENK and proDYN mRNA levels induced by KA in seizing (S) and nonseizing (NS) rats. (C) Rats were orally administered with either saline, phenidone (P, 50 mg/kg), esculetin (E, 10 mg/kg), or aspirin (A, 15 mg/kg) five times every 12 h. Thirty minutes after the last treatment of each compound, either PBS or 10 mg/kg of KA was administered intraperitoneally. Seizure activities were scored according to Baran et al. [1]. The statistical significance of the changes in seizure activity was determined by the One-way ANOVA with post-hoc. Each value is the mean ± S.E.M. * p < 0.05 compared with group of rats injected with KA alone. n = Number of animals used in each group. For measuring proENK and proDYN mRNA levels, the animals were sacrificed at indicated time points (4 and 24 h). Total RNA of 10 μg, which were extracted from pooled rat hippocampus (n = 3/group) using a guanidium thiocyanate/phenol/chloroform gradient method, were used for determination of proENK and proDYN mRNA levels. The unregulated mRNA encoding cyclophilin was used as an internal loading control. Nonseizing (NS) group indicates only the animals with a score of ‘0’.

aspirin affected KA-induced increases of proENK and Fra protein levels (Figs. 2 and 3).

The present study demonstrates that KA increases proENK-like protein and proDYN mRNA levels in rat hippocampus. Co-blockade of cyclooxygenase and lipoxygenase by phenidone was required for producing antagonistic effect against KA-induced responses. Furthermore, inhibition of Fra protein induced by KA by phenidone was well correlated with opioid peptides gene expression or proENK-like protein expression, suggesting that the blocking effect of phenidone against KA-induced increases of proENK and proDYN mRNA level appears to be mediated by reducing the expression of Fra protein, which binds to AP-1 regulatory domain of proENK and proDYN genes.

In Northern blot analyses, the levels of proENK and proDYN mRNA expression were markedly increased after 4 h and 24 h after KA administration. Previous studies have shown that dentate gyrus of hippocampus expresses proENK and proDYN gene, or their products, and KA administration elevates both proENK and proDYN mRNA levels in the dentate gyrus of hippocampus [11,15]. The results of the present study were partially in line with previous studies which showed that both proENK and proDYN mRNA levels begin to increase 3 or 4 h after KA administration [9,19]. Previous works have demonstrated that convulsant doses of KA causes the inductions of immunoreactivities of Fos and Jun-related proteins in mouse and rat hippocampus [19,20]. In a recent study, we and others have shown that Fra protein was induced after KA administration [19,23]. In the present study, we found that the KA-induced increase of Fra protein is significantly inhibited by the pre-administration with phenidone, but not esculetin and aspirin at all time points, suggesting that the
Fig. 2. The representative photomicrographs of immunostained sections with antibody against proENK-like protein 24 h after KA administration. Hippocampal sections of control (A) revealed low level of proENK-like immunostaining. Dense immunostaining for proENK-like protein was visualized in the dentate gyrus (DG) after KA injection (B). Seizing KA-treated rats with 50 mg/kg of phenidone showed clearly decreased proENK-like immunostaining in the DG (C). ProENK staining in the hippocampus from seizing KA-treated rats with 50 mg/kg of esculetin (D) or aspirin (E) was still apparent in the neuronal layer of the DG. Scale bar = 150 μm. The intensity of proENK-like immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1), very weak (grade 0.5) and not detectable (grade 0). n = Number of animals used in each group. Each value is the mean ± S.E.M. *p < 0.05 vs. control (A), #p < 0.05 vs. KA-treated rat (B) or the seizing rat pretreated with esculetin (D) or aspirin (E).
Fig. 3. The representative photomicrographs of immunostained sections with antibody against Fra protein 24 h after KA administration. Hippocampal sections of control (A) revealed very little Fra immunostaining. Dense immunostaining for Fra protein was visualized in the dentate gyrus (DG) after KA injection (B). Seizing KA-treated rats with 50 mg/kg of phenidone showed clearly decreased Fra immunostaining in the DG (C). Fra nuclear staining in the hippocampus from seizing KA-treated rats with 50 mg/kg of esculetin (D) or aspirin (E) was still apparent in the neuronal layer of the DG. Scale bar = 150 μm. The intensity of Fra immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1), very weak (grade 0.5) and not detectable (grade 0). n = Number of animals used in each group. Each value is the mean ± S.E.M. *p < 0.01 vs. control (A), #p < 0.01 vs. KA-treated rat (B) or the seizing rat pretreated with esculetin (D) or aspirin (E).
products of both cyclooxygenase and lipoxygenase pathways might be involved in inducing Fra protein, in turn, causing elevation of proENK and proDYN gene transcriptional activity. Won et al. [23] have recently reported that cycloheximide, a protein synthesis inhibitor, effectively inhibits KA-induced increases of proENK and proDYN mRNA, and Fra protein levels, suggesting that on-going protein synthesis is required for the increases of opioid peptides gene expression induced by KA in the hippocampus.

Neither esculetin nor aspirin treated alone affected the expressions of proENK and proDYN mRNA levels, or proENK and Fra proteins induced by KA, suggesting that the blockade of one pathway of arachidonic acid cascade may not be enough to exert an inhibitory action against KA-induced responses. The reasons for the requirement of blockade of both cyclooxygenase and lipoxygenase for the inhibition of KA-induced increases of proENK and proDYN mRNA levels, or proENK and Fra protein levels are not currently clear. Although not comparable directly, Baran et al. have previously reported that blockade of both cyclooxygenase and lipoxygenase, but not cyclooxygenase or lipoxygenase alone, are also required to show an antagonistic effect against seizure activity and neurotoxic effect induced by KA in the hippocampus [2].

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References