Regulation of Proenkephalin Gene Expression by Transcription Factors Fos and CREB: an Antisense Oligonucleotide Approach

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1. Introduction

The proenkephalin (PENK) gene codes for the precursor of enkephalins — neuropeptides which act on opioid receptors and play an important role in nociception and processes of drug dependence. In recent years, their putative role in epilepsy has also received much attention. Since enkephalins act to increase neuronal excitability in the hippocampus, they are regarded as endogenous pro-convulsive substances (6). In animal models of temporal lobe epilepsy, the hippocampal expression of the PENK gene increases dramatically (6-8,11). Rises in the PENK gene expression may confer vulnerability to seizures, and the released enkephalins may be involved in maintenance of convulsions.

It has been proposed that expression of the PENK gene might be regulated by Fos and other proteins belonging to the Fos and Jun families, which dimerize to form the transcription factor AP-1. The Fos-Jun complexes bind to the ENK-2 (CRE-2) element of the PENK gene promoter *in vitro* (10). Moreover, in several *in vivo* models, increases in the PENK gene expression in discrete regions of the central nervous system are preceded by induction of the c-fos gene (2,3,7-11). Such correlation occurs in the hippocampus where, during seizures induced by kainic acid (KA) or pentylenetetrazole, inductions of the c-fos and PENK genes show a striking correlation in terms of both their distribution and time-course (3,7,8,10,11). These observations suggest that Fos, as well as other inducible proteins from the Fos and Jun families, may play a role in activating the PENK gene transcription in the hippocampus.

On the other hand, evidence has emerged that there may be no causal link between induction of c-fos and up-regulation of the PENK gene, even if they occur in the same cells. This is the case in the striatum, where expression of both the c-fos and PENK genes is increased after administration of classical neuroleptics, but activation of the transcription factor CREB rather than Fos seems to be responsible for up-regulation of the PENK gene (5).

To address the question of Fos and/or CREB involvement in the induction of the PENK gene transcription in the hippocampus directly, we chose to use an antisense oligonucleotide approach in the kainic acid-induced seizure model in rats. Our aim was to ascertain whether the KA-produced increase in the hippocampal PENK mRNA levels could be blocked by antisense oligodeoxynucleotides (AS ODNs), directed against c-fos or CREB mRNA. As a reference *in vitro* system, we also studied the model of the PENK gene induction by noradrenaline in C6 glioma cells.

2. Methods

The *in vivo* experiments were carried out on male Wistar rats weighing 220-370 g. The rats were anaesthetized with hexobarbital, and antisense or control ODNs were injected unilaterally in a dose of 2 nmoles (2 µl of 1 mM aqueous solution) into the dorsal hippocampus (stereotaxic coordinates: AP: -3.3; L: +1.9 or -1.9; V: -3.5 from bregma). 2 µl of sterile water were injected into the contralateral hippocampus. Kainate (12 mg/kg) was administered intraperitoneally 7 h after intrahippocampal injection of c-fos AS ODN, or 20 h after injection of CREB AS ODN. The rats were killed 3 h thereafter. The PENK mRNA levels in the hippocampus were analysed by the Northern blot method and by *in situ* hybridization as described previously (8,13,14).

C6 glioma cells were incubated for 4 h with ODNs at 1, 5 and 10 µM concentrations. The medium was then replaced, and 10 µM noradrenaline and 1 µM dexamethasone were added. 8 h later, the cells were frozen at -70°C. Levels of the PENK mRNA were measured by Northern blot hybridization.

All the ODNs used were phosphorothioated in all positions. The AS ODNs against c-fos and CREB mRNA which were used in our experiments, had been shown by other authors to inhibit the synthesis of the respective proteins in the brain *in vivo* (c-fos antisense: 5'-GAA-CAT-CAT-GGT-CGT-3'; CREB antisense: 5'-TGG-TCA-TCT-AGT-CAC-CGG-TG-3') (1,4). Scramble ODNs and a mixture of random pentadecamer ODNs were used as controls.

3. Results

During seizures elicited by KA, strong induction of the PENK mRNA took place in the hippocampal dentate gyrus. The AS ODN against c-fos mRNA reduced this induction by about 60%, while the random ODN was without

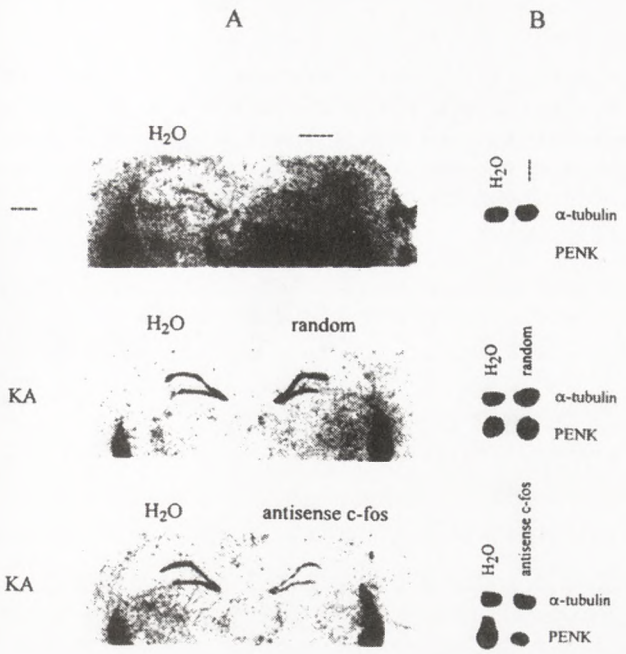

Fig. 1. (A) *In situ* hybridization autoradiograms showing the PENK mRNA signal in the brains of rats injected with water into the left hippocampus, and with antisense ODN to c-fos mRNA or random ODN into the right hippocampus (--- — no injection). KA or saline (---) was administered to the rats 7 h after intrahippocampal injections; the animals were killed 3 h after KA administration.

(B) Northern blot autoradiograms showing the α-tubulin and PENK mRNA bands in the hippocampal RNA extracts of rats treated as described in (A). Pairs of samples represent the left and right hippocampi of a single rat.

effect (Fig. 1). The levels of other mRNA species (the NMDA receptor R1 subunit, Gs protein α-subunit and α-tubulin mRNAs) in the dentate gyrus were not affected by the c-fos AS ODN, which suggests that this ODN did not non-specifically suppress gene expression (data not shown).

Preliminary results indicate that induction of the PENK gene by KA in the dentate gyrus may also be strongly inhibited by the AS ODN against CREB mRNA (Fig. 2).

In the C6 glioma cells, noradrenaline and dexamethasone produced an increase in the PENK mRNA levels by 500%. This increase was not reduced by the c-fos AS ODN. In contrast, the AS ODN against CREB mRNA inhibited the induction of the PENK gene by about 50% when applied at 10 μM concentration (lower concentrations were without effect) (Fig. 3).

Fig. 2. *In situ* hybridization autoradiogram showing the PENK mRNA signal in the brain of a rat injected with water into the left hippocampus, and with antisense ODN to CREB mRNA into the right hippocampus. The rat was injected with KA 20 h after ODN application, and was killed 3 h later.

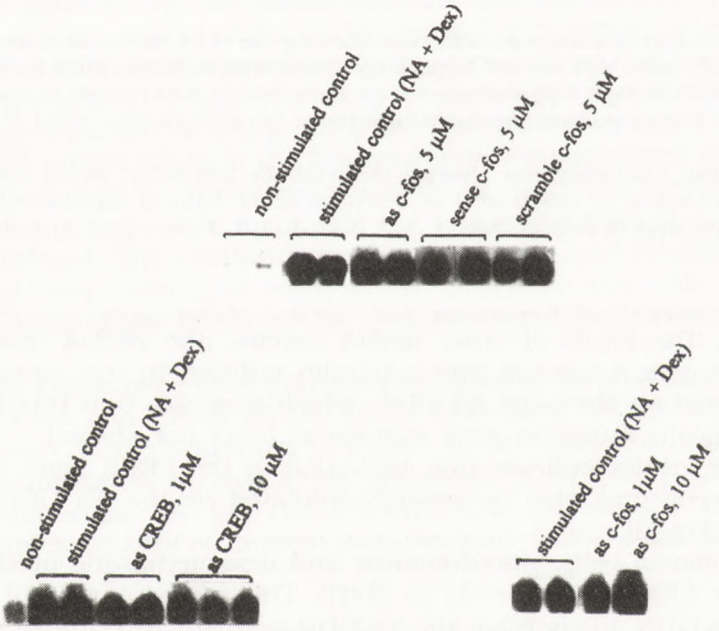

Fig. 3. Northern blot autoradiograms showing the PENK mRNA signal in the RNA extracts of C6 glioma cells. Antisense (as) and control (sense, scramble) ODNs were added to the culture medium at the indicated concentrations. 4 h later they were washed out, and the cells were stimulated by incubation with 10 μM noradrenaline (NA) and 1 μM dexamethasone (Dex) for 8 h.

4. Discussion

Our results suggest that the transcription factor Fos, and maybe also CREB, mediates the induction of the PENK gene in the hippocampus during KA-induced seizures. In contrast, CREB rather than Fos seems to be involved in the induction of the PENK gene by β-adrenergic agonists in C6 glioma cells.

The mechanism of the PENK gene induction in the hippocampus is not clear. During seizures the whole hippocampal neuronal circuit becomes activated. Stimulation (probably via NMDA receptors) of the granule cells of the dentate gyrus, which contain enkephalins, leads to rapid induction of numerous immediate early genes, including c-fos and other members of the Fos and Jun families (3). This, in turn, is followed by the formation of the Fos-Jun complexes, which may act directly at the PENK gene promoter to activate transcription of this gene. Alternatively, the AP-1 transcription factor may be indirectly involved in the regulation of the PENK gene expression by influencing other transcription factors. We have demonstrated that Fos is necessary for induction of the PENK gene in the dentate gyrus during seizures. However, the mechanism of its action remains to be elucidated. Our results also point to the involvement of the transcription factor CREB in the regulation of the PENK gene expression in the dentate gyrus.

In C6 glioma cells, the PENK gene expression appears to be regulated in a different way than in the hippocampus. The level of PENK mRNA in these cells can be increased several times by stimulation of the cells with agonists of β-adrenergic receptors (e.g. noradrenaline). Such induction is potentiated by glucocorticoids (e.g. dexamethasone). Since β-adrenergic receptors are positively coupled to adenylate cyclase and the cAMP pathway, CREB may be expected to regulate the expression of the PENK gene in this model (probably by acting at a CRE site of the PENK gene promoter). Indeed, the increase in the PENK mRNA levels was reduced by the AS ODN against CREB in our experiment. In contrast, such an inducible transcription factor as Fos seems unlikely to be involved in the induction of the PENK gene in glioma cells, since this induction does not require *de novo* protein synthesis (12). This view is supported by the fact that the AS ODN against c-fos was without effect in our *in vitro* model.

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References

1. Chiasson B. J., Hooper M. L., Murphy P. R., Robertson H. A., (1992), Eur. J. Pharmacol. -Mol. Pharmacol. Sect., 227, 451-453.
2 Draisci G., Iadarola M. J., (1989), Mol. Brain Res., 6, 31-37.
3. Kamińska B., Filipkowski R. K., Żurkowska G., Lasoń W., Przewłocki R., Kaczmarek L., (1994), Eur. J. Neurosci., 6, 1558-1566.
4. Konradi C., Cole R. L., Heckers S., Hyman S., (1994), J. Neurosci., 14, 5623-5634.
5. Konradi C., Kobierski L. A., Nguyen T. V., Heckers S., Hyman S. E., (1993), Proc. Natl. Acad. Sci. USA, 90, 7005-7009.
6. Morris B. J., Johnston H. M., (1995), Trends Neurosci., 18, 350-355.
7. Pennypacker K. R., Walczak D., Thai L., Fannin R., Mason E., Douglass J., Hong J. S., (1993), J. Neurochem., 60, 204-211.
8. Przewłocki R., Kamińska B., Łukasiuk K., Nowicka D. Z., Przewłocka B., Kaczmarek L., Lasoń W., (1995), Neuroscience, 68, 73-81.
9. Robertson G. S., Vincent S. R., Fibiger H. C., (1992), Neuroscience, 49, 285-296.
10. Sonnenberg J. L., Rauscher F. J. III, Morgan J. I., Curran T., (1989), Science, 246, 1622-1625.
11. White J. D., Gall C. M., (1987), Mol. Brain Res., 3, 21-29.
12. Yoshikawa K., Sabol S. L., (1986), Mol. Brain Res., 1, 75-83.
13. Young III, W. S., Bonner T. J., Brann M. R., (1986), Proc. Natl. Acad. Sci. USA, 83, 9827-9831.
14. Ziółkowska B., Hollt V., (1993), Neurosci. Lett., 156, 39-42.

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Summary

The present study investigated the effects of antisense oligonucleotides (AS ODNs) against c-fos and CREB mRNA in two models of the proenkephalin (PENK) gene induction.

AS ODNs to both c-fos and CREB mRNA markedly reduced induction of the PENK gene in the rat hippocampus *in vivo* during seizures produced by kainic acid (KA). In contrast, in an *in vitro* model of the PENK gene induction by noradrenaline and dexamethasone in C6 glioma cells, the AS ODN to c-fos was without effect, whereas the AS ODN to CREB reduced the increase in the PENK mRNA level.

The obtained results suggest that the transcription factors Fos and CREB may mediate induction of the PENK gene in the hippocampus, while induction of this gene in C6 glioma cells is mediated by CREB rather than Fos.

Key words:
antisense oligonucleotides, proenkephalin, gene induction, c-fos, CREB.

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