Korean J. Toxicol.
Vol. 9, No. 2, pp. 195-206, 1993

REGULATION OF PROENKEPHALIN GENE EXPRESSION AND MET-ENKEPHALIN SECRETION IN BOVINE ADRENAL MEDULLARY CHROMAFFIN CELLS AND C6 RAT GLIOMA CELLS

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(Received May 27, 1993
(Accepted August 2, 1993)

ABSTRACT: *The expression of proenkephalin (proENK) mRNA and Met-enkephalin (ME) secretion in C6 rat glioma cells and bovine adrenal medullary chromaffin (BAMC) cells were elucidated in the present study. The levels of proENK mRNA and ME secreted into the media in BAMC cells were measured in the presence of cycloheximide and 12-tetradecanoylphorbol-13-acetate (TPA). Cycloheximide (20 μM) abolished the induction of proENK mRNA expression, protein synthesis and ME secretion by TPA (1 μM), indicating that de novo protein synthesis was necessary for proENK gene expression and ME secretion. In protein-RNA gel mobolity retardation experiments, the induction of ENKCRE-2/AP-1 activity correlated well with the level of proENK mRNA induction. This ENKCRE-2/AP-1 complex could be inhibited by a specific c-Jun antiserum and was supershifted by Fos family of proteins. Furthermore, Western blot analysis suggests that c-Jun and Fos-related antigens rather than c-Fos per se were components of an ENKCRE-2/AP-1 complex. In C6 rat glioma cells, proENK mRNA level and ME secretion were stimulated by norepinephrine (10 μM) and markedly enhanced by dexamethasone (1 μM), although dexamethasone alone exihibited no significant increase in proENK mRNA and ME secretion. In contrast, the stimulation of either proENK expression and ME secretion was not observed with a PKC activator, TPA, which stimulated the expression of c-Fos and c-Jun mRNA and their proto-oncoproteins. In addition, an AP-1 activity was induced by TPA. The results suggest that activation of proENK gene in C6 cells is probably mediated mainly through the β-adrenergic agonist-elicited cAMP signal pathway, and induction of AP-1 binding activities by TPA appear not to participate in gene activation. In conclusion, proENK gene expression and ME secretion are differentially*

regulated in C6 rat glioma cells and BAMC cells; cAMP but not PKC pathway plays an important role in rat C6 glioma cells. However, in BAMC cells, both PKC and cAMP pathways appear to be involved in the regulation of proENK gene expression and the secretion of ME.

Key words: *Proenkphalin, Met-enkephalin, Gene expression, Secretion, Second messengers, Oncogene, C6 glioma cells, Bovine adrenal medullary cells.*

INTRODUCTION

Proenkephalin (proENK) mRNA and its protein products, and processed enkephalins are expressed in a wide variety of tissues including brain (Hughes *et al.*, 1975; Khachaturian *et al.*, 1985), lymphoid cells (Zurawski *et al.*, 1986; Monstein *et al.*, 1986; Roth *et al.*, 1989), the adrenal gland (Suh *et al.*, 1992a, 1992b, 1992 c, 1993; Eiden *et al.*, 1984; Quach *et al.*, 1984), and germ cells (Yoshikawa *et al.*, 1989a, 1989b; Kilpatrick *et al.*, 1990). This diverse tissue distribution of proENK mRNA and enkephalins implies that these endogenous enkephalins can function as neurotransmitters, neurohormones, or neuroimmunomodulators. The expression of proENK mRNA and enkephalins is regulated by several signal transduction pathways and by the cellular environment. The proENK appears to be an important participant in ligand-mediated signal transduction events. In fact, the activation of the proENK gene can be mediated through an increase in intracellular cyclic AMP (cAMP) levels (Suh *et al.*, 1993; Eiden and Hotchkiss, 1983; Yoshikawa and Sabol, 1986a, 1986b), the activation of protein kinase C (PKC) (Yoshikawa *et al.*, 1989a), and the depolarization of voltage-dependent CA^{2+} channels (Kley *et al.*, 1986; Naranjo *et al.*, 1986; Siegel *et al.*, 1985). These diverse ligand evoked gene activations have prompted studies to uncover the molecular mechanisms underlying the regulation of the proENK gene. Although several signal transduction pathways are involved in the regulation of the proENK gene expression and ME secretion, it is not well established whether the regulations of the proENK gene expression and ME secretion are tissue specific or not. Therefore, in this paper, the contrasting molecular mechanisms regulating proENK gene expression and ME secretion in bovine adrenal mecullary chromaffin (BAMC) cells and C6 rat glioma cells were emphasized.

Met-Enkephalin Secretion

ME, an opioid pentapeptide, is costored and coreleased with catecholamine in the BAMC cells (Viveros *et al.*, 1979; Livett *et al.*, 1981). Although the physiological function of ME in in the adrenal medulla is not fully understood, the regulation of ME secretion has been extensively studied. For example, insulin-induced hypoglycemia or stress alters ME levels as well as proENK mRNA levels in the adrenal medulla (Kanamatsu *et al.*, 1986; Weisinger *et al.*, 1990). In addition, increase of ME release and proENK mRNA levels were seen after the treatment of chromaffin cells with nicotine, arachidonic acid, PGE_2, histamine or angiotensin II (Suh

et al., 1992a, 1992b, 1993; Eiden *et al.*, 1984; Wan *et al.*, 1989, 1990a, 1990b; Farin *et al.*, 1990; Stachowiak *et al.*, 1990, 1991). In addition to these secretagogues-induced ME secretion, recent studies have demonstrated that the secretion of ME in BAMC cells is regulated by various second messengers. Calcium and calmodulin, cAMP and protein kinase C (PKC) are the major second messengers. For example, the treatment of chromaffin cells with a PKC activator such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), or an adenyl cyclase activator such as 8-bromo-cyclic AMP or forskolin, increases the secretion of ME as well as proENK

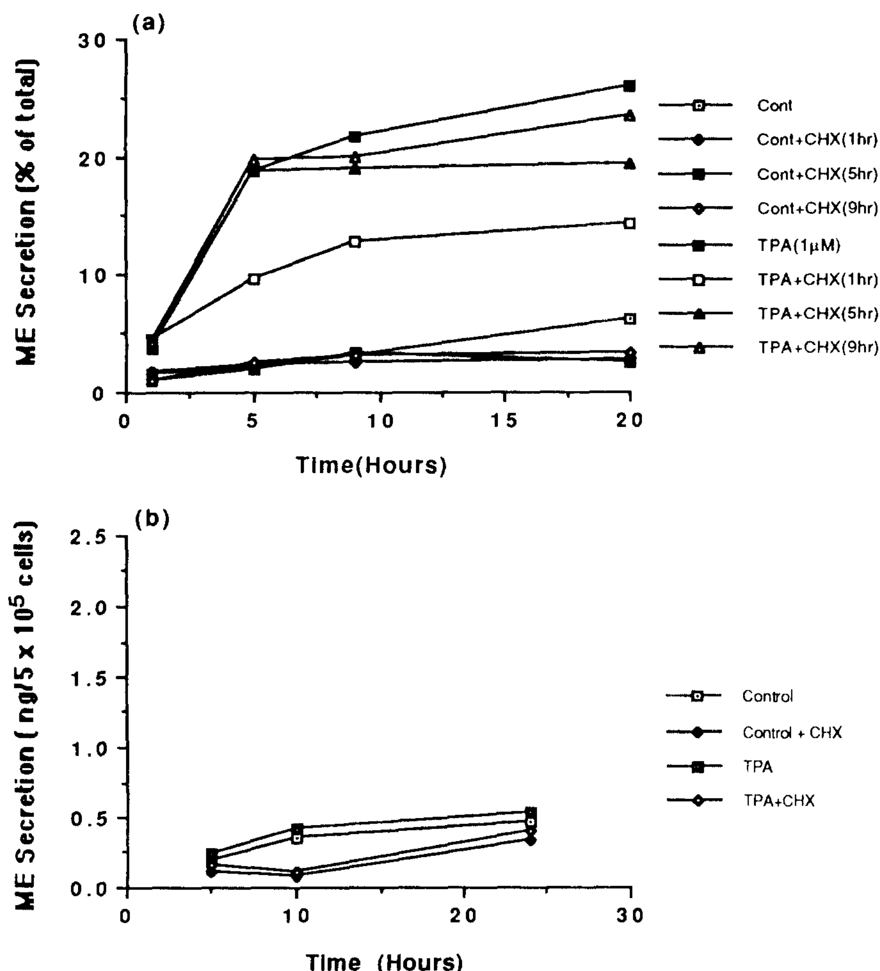

Figure 1. Time-dependent secretion of Met-enkephalin (ME) in BAMC cells (a) and C6 rat glioma cells (b) treated with TPA. In BAMC cells, after the addition of control medium or TPA (1 μM) to the media, 20 μM of cycloheximide (CHX) was added to the incubation media at 1, 5, and 9 hr after TPA treatment. C6 rat glioma cells were pretreated with 20 μM of cycloheximide (CHX) for 30 min and either control medium or the medium containing TPA (1 μM) was added into the medium. Incubation was terminated 20 and 24 hr in BAMC and C_6 rat glioma cells, respectively, after TPA treatment. ME levels in the media or cells were measured by radioimmunoassay. Each time point represents the mean of three sepresents the mean of three seperate experiments with triplicate samples for each experiment.

mRNA level (Suh *et al.*, 1992c, 1993; Farin *et al.*, 1990; Eiden *et al.*, 1984; Quach *et al.*, 1984). In addition, treatments with calcium channel blockers or with calmidazolium, a calmodulin antagonist, effectively inhibit the increased secretion of ME (Suh *et al.*, 1992c, 1993; Stachowiak *et al.*, 1987). In contrast to the effects in BAMC cells, the secretion of ME in C6 glioma cells has not been well characterized. Figure 1a and b show the effects of long-term exposure of TPA on the secretion of ME from BAMC cells and C6 rat glioma cells, respectiviely. In BAMC cells, small amount of ME was secreted as early phase and profound ME release was obserevd after 5, 9, and 12 hr whereas TPA had no significant effect on the secretion of ME from C6 glioma cells. However, ME secretion in C6 glioma cells was markedly increased in norpeinephrine (β-adrenergic agonist which stimulates adenyl cyclase pathway) plus dexamethasone (a glucocorticoid agonist)-treated cell medium during the time course of 10 to 24 hr as shown in Figure 2. The results suggest that cAMP but not PKC is involved in the regulation of ME secretion in C6 glioma cells. However, both cAMP and PKC are involved in the regulation of ME secretion in BAMC cells. To assess if delayed ME secretion induced by each drug was dependent upon protein synthesis, the temporal effect of cycloheximide (a protein synthesis inhibitor) on the ME secretion in BAMC cells and C6 glioma cells were also investigated. The cycloheximide (20 μg) was added 1, 5, and 10 hr after treatment of BAMC cells with TPA and 30 min before the treatment with norepinephrine plus dexamethasone in C6 glioma cells. The posttreatment of BAMC cells with cycloheximide effectively inhibited the secretion of ME induced by TPA in BAMC cells in proportion with time incubation (Figure 1a), suggesting that continuous protein synthesis is required for long-term ME secretion. In C6 glioma cells, pretreatment with cycloheximide inhibited secretion of ME induced by norepinephrine plus dexamethasone (Figure 2). Additionally, because cyclohe-

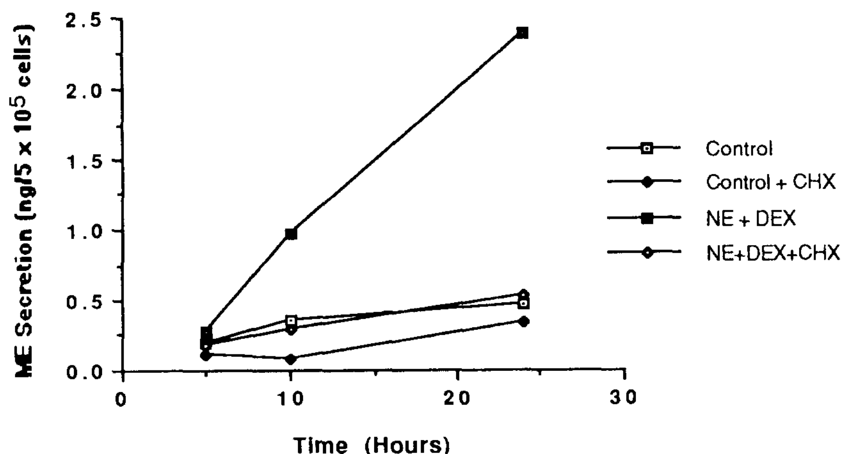

Figure 2. Time-dependent secretion of Met-enkephalin (ME) in C6 rat glioma cells treated with norepinephrine plus dexamethasone. C6 rat glioma cells were pretreated with 20 μM of cycloheximide (CHX) for 30 min and either control medium or the medium containing norepinephrine (10 μM) plus dexamethasone (1 μM) was added into the medium. Incubation was terminated 24 hr after TPA treatment. ME levels in the media were measured by radioimmunoassay. Each time point represents the mean of three seperate experiments with triplicate samples for each experiment.

Table 1. Met-enkephalin (ME) secretion in BAMC cells and C6 rat glioma cells.

	BAMC Cells	C6 glioma Cells	C6 glioma Cells
Drug used	TPA	TPA	NE[a]+DEX[b]
ME Secretion	Yes	No	Yes
CHX[C] effect on Me secretion	Inhibition	–	Inhibition

[a] indicates norepinephrine. [b] indicates dexamethasone. [c] indicates cycloheximide

Table 2. Proenkephalin (ProENK) mRNA regulation in BAMC cells and C6 rat glioma cells.

	BAMC Cells	C6 glioma Cells	C6 glioma Cells
Drug used	TPA	TPA	NE[a]+DEX[b]
ProENK mRNA level	Increase	No change	Increase
CHX[C] effect on proENK mRNA	Inhibition	–	No inhibition
Intracellular ME precursor Level	Increase	–	Slight increase
CHX effect on precursor level	Inhibition	–	–
Induction time of proENK mRNA	5~7 hrs	–	2~3 hrs

[a] indicates norepinephrine. [b] indicates dexamethasone. [c] indicates cycloheximide.

ximide also inhibited basal ME secretion, on-going protein synthesis may also be involved in basal ME secretion. The results of the ME secertion regulation in BAMC cells and C6 rat glioma cells were summarized in Table 1.

The Regulation of Proenkephalin Gene Expression

The effects of TPA and norepinephrine plus dexamethasone on proENK mRNA in BAMC cells and C6 rat glioma cells are summarized in Table 1. TPA in BAMC cells and norepinephrine plus dexamethasone in C6 glioma cells increased the level of proENK mRNA. However, long-term treatment of C6 glioma cells with TPA did not affect proENK mRNA level, suggesting that PKC pathway plays an important role in the regulation of proENK gene expression in BAMC cells but not in C6 glioma cells. The onset time for the induction of proENK mRNA is also different in BAMC cells and C6 glioma cells. Five to 7 hrs in BAMC cells and 2 to 3 hrs are required for an increase of proENK mRNA level. In the Western immunoblot, long-term treatment of BAMC cells with TPA increased intracellular level of precusor of ME, proenkephalin. However, no obvious increase in intracellular levels of proenkephalin in norpeinephrine plus dexamethasone-as well as TPA-treated C6 glioma cells. Using the radioimmunoassay technique, it was found that a slight increase by norepinephrine plus dexamethasone in proenkephalin and ME immunoreactivities in C6 glioma cells. These results and the fact that a marked increase of proenkephalin and ME immunoreactivities were found in culture medium suggest that most of proenkephalin newly synthesized by norepinephrine plus dexamethasone in C6 glioma cells are secreted into the media. Similarly, it has been demonstrated that astrocytes cultured from neonatal rat brain secreted mainly proenkephalin into the medium where it was subsequently cleaved to smaller peptide products (Batter *et al.*, 1991). The increases of proENK mRNA and precursor levels induced by TPA in BAMC cells are also cycloheximide dependent, suggesting that protein synthesis is required for the induction of proENK

Table 3. The effect of TPA on protooncogenes in BAMC cells and C6 rat glioma cells

	BAMC Cells	C6 glioma Cells
Drug used	TPA	TPA
c-Fos mRNA or c-fos protein	Increase	Increase
CHX[a] effect on c-fos mRNA	–	Prolonged
CHX effect on c-fos protein	No inhibition	Inhibition
c-Jun mRNA or c-jun protein	Increase	Increase
CHX effect on c-Jun mRNA	–	Prolonged
CHX effect on c-jun protein	Inhibition	Inhibition
Fra protein	Increase	Increase
CHX effect on Fra protein	Inhibition	Inhibition
Transcription factors	AP-1 incerase	AP-1 increase
CHX effect on AP-1 1 binding	Inhibition	Inhibition

[a] indicates cycloheximide.

mRNA and proenkephalin in BAMC cells. However, no inhibition of proENK mRNA level by cycloheximide in C6 glioma cells was observed, implying that the stimulation of proENK mRNA was independent of de novo synthesis, and hence mediated by preexisting proteins.

The Role of Proto-Oncogenes in the Regulation of Proenkephalin Gene Expression

Molecular analyses of the human proENK gene defined a cluster of responsive elements, namely, ENKCRE-1, ENKCRE-2, AP-2, and AP-4, involved in gene regulation (Comb *et al.*, 1988a; Hyman *et al.*, 1989). The transcriptional regulatory regions of the proENK gene display an extremely high degree of interspecies sequence conservation between humans (Hyman *et al.*, 1989; Comb *et al.*, 1983), rats (Kilpatrick *et al.*, 1990; Rosen *et al.*, 1984; Joshi and Sabol, 1992), and cows (L. MacAuthor and L. E. Eiden, personal communication). By DNase footprinting technique, a protein factor named ENKTF-1 from C6 rat glioma cells bound to the human ENKCRE-1 element (Comb, 1988b). Additionally, transfection studies demonstrated that the ENKCRE-2 element functioned independently of other neighboring auxiliary elements (Comb *et al.*, 1986; Kobierski *et al.*, 1991) and responded to activation by cAMP and phorbol esters (Comb *et al.*, 1986). However, the other auxiliary elements functioned to augment the activity of the ENKCRE-2 element (Hyman *et al.*, 1989). Furthermore, Yoshikawa *et al.* (1989a) reported that both the TPA and forskolin (an activator of adenylate cyclase) could markedly elevate the level of proENK mRNA in cultured peritubular cells prepared from the rat testes. Similarly, in a primary culture of BAMC cells, the synthesis of proENK mRNA and its protein was stimulated by TPA and forskolin (Suh *et al.*, 1992c, 1993; Kley, 1988). Oligonucleotide probe containing AP-1, CRE, ENK-CRE-1, ENKCRE-2, kB and AP-2 motifs was used to perform protein-DNA hand shift assays. In conjunction with the Western immunoblot technique, it was attempted to determined which transcription factors were involved in the regulation of proENK gene in BAMC cells and C6 rat glioma cells.

In BAMC cells, AP-2 and CRE DNA binding activities were detectable and ap-

peared not to be inducible (data not shown), although AP-2 binding activity could be demonstrably enhanced by TPA and agents that elevated cAMP levels in HeLa cells (Imagawa *et al.*, 1987). When ENKCRE-2 and AP-1 oligonucleotide probes were used, the electrophoretic mobility shift assay (EMSA) results revealed that the ENKCRE-2 element behaved like an AP-1 element rather than like a CRE element, although ENKCRE-2 has been demonstrated to function like either an AP-1 or a CRE element (Comb *et al*,, 1988a). The functional similarity between AP-1 and ENKCRE-2 elements could be further sustained by the findings that AP-1 and ENKCRE-2 binding activities, but not CRE binding activity, could be inhibited by an antiserum against Fos family antigens. In competition study, AP-1 oligonucleotide effectively blocked ENKCRE-2-DNA binding activity. In contrast, ENKCRE-2 oligonucleotides could not efficiently eliminate AP-1 binding activity. Interestingly, we also found that CRE oligonucleotides were able to block ENK-CRE-2-DNA binding activity, but not the converse. This is probably due to the fact that the ENKCRE-2 element basically harbors an imperfect consensus sequence for AP-1 and CRE elements. Although there is only one nucleotide difference between the AP-1 and CRE elements, these two elements intrinsically function specifically; no cross-competition between these two elements was observed. The inducibility of ENKCRE2/AP-1 binding activity was inhibited by cycloheximide. The Western immunoblot assays were performed using specific antisera against c-Jun and Fos family antigens. Three proteins were identified with molecular weights of 60, 42 and 32 kDa. This protein profile is very similar to that found in an *in vivo* study in which AP-1 activity was elevated in mouse brain during metrazole-induced seizures (Sonnenberg *et al.*, 1989a and 1989b). Basiclly, TPA-elicited induction of the 60 kDa protein was enhanced by cycloheximide. Thus, this protein is characteristic of an immediate-early antigen, the c-Fos protein (Sonnenberg *et al.*, 1989a and 1989b). In contrast, the other two smaller Fos-related antigens (42 and 35 kDa) were regulated by TPA and cycloheximide. Induction of c-Jun protein synthesis could also be seen with TPA although to a lesser extent compared to Fos family proteins. Similar to Fra proteins, c-Jun induction was inhibited by cycloheximide if this inhibitor was present no later than 5 hr following the addition of proENK modulator. Nevertheless, the induction of c-Jun correlated with AP-1 binding activity and the elevation of proENK mRNA and polypeptide. Furthermore, the EMSA experiments demonstrated that AP-1 binding activity could be altered by anti-Fos and anti-c-Jun sera. Taken together, c-Jun and Fra proteins appear most likely to be the critical determinant factors of the ENKCRE-2/AP-1 complex that act together to activate the proENK gene.

In C6 rat glioma cells, TPA induced a remarkable elevation in c-Jun and c-Fos mRNAs although TPA did not affect the proENK mRNA level. Additionally, the Western blot analysis indicates that TPA-mediated induction of the proto-oncoproteins, c-Fos (55 kDa) or Fra (36 kDa) and c-Jun (39 kDa), that can form a complex to bind the AP-1 motif (Lee *et al.*, 1987; Sonnenberg *et al.*, 1989a and 1989b) was entirely blocked by cycloheximide. Furthermore, the EMSA results showed an inducible AP-1 binding activity but little ENKCRE-2 or CRE-binding activity for TPA-treated C6 nuclear proteins; this AP-1 binding activity was also inhibited by cycloheximide. These results indicated that the induction of c-Fos, Fra, and

c-Jun proteins was attributable to the increase in AP-1 activity. The rat proENK enhancer/promoter region contains a kB-like domain named B2, and transfection studies revealed that the B2 region was up-regulated by the induction of NF-kB DNA-binding activity by concanavain A plus TPA in a human leukemic T cell line, Jurkat cells (Rattner *et al.*, 1991). Although an inducible nuclear NF-kB binding activity was found in the nucleus of C6 glioma cells after treatment with TPA plus cyclohiximide. However, no stimulation of proENK mRNA expression was seen. This suggests that, in contrast to BAMC cells, induction of the AP-1 complex (c-Fos or Fra and c-Jun) and the NF-kB factor appeared not to participate in the activation of the rat proENK gene in C6 cells.

Dexamethasone was demonsrated to potentiate the stimulatory effect of norepinephrine on proENK mRNA expression (Yoshikawa and Sabol, 1986a). However, it was not detectable any GRE-binding activity in either the nuclear fraction or the cytoplasmic compartment in C6 cells treated with norepinephrine plus dexamethasone, even with dexamethasone in the EMSA mixture to transform the inactive glucocorticoid receptor molecule into the active species (data not shown). This raises as to whether the rat proENK gene contains any GRE responsible for augmentation of the gene activity. A search of the rat proENK gene and 5′-flanking region (Kilpatrick *et al.*, 1990; Rosen *et al.*, 1984; Joshi and Sabol, 1991) for sequences resembling the positive GRE consensus sequences did not reveal any good matches in the 5′-flanking region, three GRE-like sequences were found in intron A (Joshi and Sabol, 1991). The studies with proENK/CAT (chloramphenicol acetyltransferase) chimeric genes failed to demonstrate a strong positive GRE, or even one cooperating positively with the cAMP-inducible enhancer, within 5800 bp of the 5′-flanking sequence, exon 1, or intron A of the rat proENK gene (Joshi and Sabol, 1991). Nevertheless, several reports demonstrated that glucocorticoid agonists (dexamethasone and triamcinolone) stimulated the expression of β-adrenergic receptor mRNA and its receptor number in hamster cells (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Malbon and Hadcock, 1988); the increase in receptor number was found to correlate well with the activation of adenyl cyclase activity (Collins *et al.*, 1988). Furthermore, molecular studies (Malbon and Hadcock, 1988) inferred that GRE motifs in the 5′-noncoding regions of the hamster β-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels. It remains to be explored whether similar observations can be found in rat C6 glioma cells in which dexamethasone potentiates the activation of the proENK gene induced by norepinephrine. The results of the regulation of proto-oncogenes by TPA in BAMC cells and C6 rat glioma cells were summarized in Table 3.

CONCLUSION

The stimulation of proENK mRNA expression and ME secretion in the C6 rat glioma cells is mediated through the cAMP-dependent signal pathway and probably not through the PKC-dependent pathway. However expression of proENK mRNA and ME secretion could be stimulated by both PKC-and cAMP-dependent pathways in BAMC cells. In addition, Fos-related antigenes (Fra) but not c-Fos

per se form a complex with c-Jun that transactivates the proenkephalin gene in BAMC cells.

REFERENCES

Batter, D.K., Vilijin, M.-H. and Kessler, J. (1991): Cultured astrocytes release proenkephalin, *Brain Res.*, **563**, 28-32.

Collins, S., Caron, M.G. and Lefkowitz, R.J. (1988): β_2-Adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids, *J. Biol. Chem.*, **263**, 9067-9070.

Comb, M., Rosen, H., Seeburg, J.A. and Herbert, E. (1983): Primary structure of the human proenkephalin gene, *DNA*, **2**, 213-229.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., and Goodman, H.M. (1986): A cyclic AMP-and phorbol ester-inducible DNA element, *Nature*, **323**, 353-356.

Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. and Goodman, H.M. (1988): Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription, *EMBO J.*, **7**, 3793-3805.

Eiden, L.E. and Hotchkiss, A.J. (1983): Cyclic adenosine monophosphate regulates vasoactive intestinal polypeptide and enkephalin biosynthesis in cultured bovine chromaffin cells, *Neuropeptides*, **4**, 1-9.

Eiden, L., Giraud, P., Dave, J.R., Hotchkiss, A.J. and Affolter, H.-U. (1984): Nicotine receptor stimulation activates enkephalin release and biosynthesis in adrenal chromaffin cells, *Nature*, **312**, 661-663.

Farin, C.-J., Kley, N. and Hollt, V. (1990): Mechanisms involved in the transcriptional activation of proenkephalin gene expression in vovine chromaffin cells, *J. Biol. Chem.*, **265**, 19116-19121.

Hadcock, J.R. and Malbon, C.C. (1988): Regulation of β-adrenergic receptors by "permissive" hormones: Glucocorticoids increase steady state levels of receptor mRNA. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8415-8419.

Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1975): Identification of two related peptapeptides from the brain with potent opiate agonist activity, *Nature*, **258**, 577-580.

Hyman, S.E., Comb, M., Pearlberg, J. and Goodman, H.M. (1989): An AP-2 element acts synergistically with the cyclic AMP and phorbol ester-inducible enhancer of the human proenkephalin gene, *Mol. Cell. Biol.*, **9**, 321-324.

Imagawa, M., Chiu, R. and Karin, M. (1987): Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C and cAMP, *Cell*, **51**, 251-260.

Joshi, J. and Sabol, S.L. (1991): Proenkephalin gene expression in C6 rat glioma cells: Potentiation of cyclic adenosine 3',5'-monophosphate-dependent transcription by glucocorticoids, *Mol. Endocrinol*, **5**, 1069-1080.

Kanamatsu, T., Unsworth, C.D., Diliberto, E.J.J. Jr., Viveros, O.H. and Hong, J.-S. (1986): Reflex splanchnic nerve stimulation increases levels of proenkephalin A mRNA and proenkephalin A-related peptides in the rat adrenal medulla, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9245-9249.

Khachaturian, H., Lewis, M.E., Schafer, K.-H. and Watson, S.J. (1985): Anatomy of

CNS opioid receptors, *Trends Neurosci,* **8**, 111-119.
Kilpatrick, D.L., Zinn, S.A., Fitzgerald, M., Higuchi, H., Sabol, S.L. and Meyerhardt, J. (1990): Transcription of the rat and mouse proenkephalin genes is initiated at distinct sites in spermatogenic and somatic cells, *Mol. Cell. Biol.,* **19**, 3717-3726.
Kley, N. (1988): Multiple regulation of proenkephalin gene expression by protein kinase *C. J. Biol. Chem.,* **263**, 2003-2008.
Kley, N., Loeffler, J.P., Pittius, C.W. and Hollt, V. (1986): Pronekpehalin A gene expression in bovine adrenal chromaffin cells is regulated by changes in electrical activity, *EMBO J.,* **5**, 967-970.
Kley, N., Loeffler, J.P., Pittius, C.W. and Hollt, V. (1987): Involvement of ion channels in the induction of proenkephalin A gene expression by nicotine and cAMP in bovine chromaffin cells, *J. Biol. Chem.,* **262**, 4083-4089.
Kobierski, L.A., Chu, H.-M., Tan, Y. and Comb, M.J. (1991): cAMP-dependent regulation of proenkephalin by Jun D and Jun B: Positive and negative effects of AP-1 proteins, *Proc. Natl. Acad. Sci. U.S.A.,* **88**, 10222-10226.
Lee, W., Mitchell, P. and Tijan, R. (1987): Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements, *Cell,* **49**, 741-752.
Livett, B.G., Dean, D.M., Whelan, L.G., Udenfriend, S. and Rossier, J. (1981): Corelease of enkephalin and catecholamine from cultured adrenal chromaffin cells, *Nature,* **289**, 317-319.
Malbon, C.C. and Hadcock, J.R. (1988): Evidence that glucocorticoid response elements in the 5′-noncoding region of the hamster β_2-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels, *Biochem. Biophys. Res. Commun.,* **154**, 676-681.
Monstein, H., Folkesson, J.R. and Terenius, L. (1986): Proenkephalin A-like mRNA in human leukemia leukocytes and CNS tissues, *Life Sci.,* **39**, 2237-2241.
Naranjo, J.R., Mocchetti, I., Schwartz, J.P. and Costa, E. (1986): Permissive effect of dexamethesone on the increase of proenkephalin mRNA induced by depolariation of chromaffin cells, *Proc. Natl. Acad. Sci. U.S.A.,* **83**, 1513-1517.
Quach, T.T., Tang, F., Kageyama, H., Mocchetti, I., Guidctti, A., Meek, J.L., Costa, E. and Schwartz, J.P. (1984): Enkephalin biosynthesis in adrenal medulla: Modulation of proenkephalin mRNA content of chromaffin cells by 8-bromo-adenosine-3′,5′-monophosphate, *Mol. Pharmacol.,* **26**, 255-260.
Rattner, A., Korner, M., Rosen, H., Baeuerle, P.A. and Citri, Y. (1991): Nuclear factor kB activates proenkephalin transcription in T lymphocytes. *Mol. Cell Biol.,* **11**, 1017-1022.
Rosen, H., Douglass, J. and Herbert, E. (1984): Isolation and characterization of the rat proenkephalin gene, *J. Biol. Chem.,* **259**, 14309-14313.
Roth, K.A., Lorenz, R.G., Unanue, R.A. and Weaver, C.T. (1989): Nonopiate active proenkephalin-derived peptides are secreted by T helper cells, *FASEB J.,* **3**, 2401-2407.
Siegel, R.E., Eiden, L.E. and Affolter, H.-U. (1985): Elevated potassium stimultes enkephalin biosynthesis in bovine chromaffin cells, *Neuropeptides,* **6**, 543-553.
Sonnenberg, J.L., Macgregor-Leon, P.F., Curran, T. and Morgan, J.I. (1989a): Dynamic alterations occur in the levels and composition of transcription factor AP-1 comp-

lexes after seizure, *Neuron,* **3**, 359-365.
Sonnenberg, J.L.Mitchelmore, C., Macgregor-Leon, P.F., Hempstead, J., Morgan, J.I. and Curran, T. (1989a): Glutamate receptor agonists increase the expression of Fos, Fra, and AP-1 DNA binding activity in the mammalian brain, *J. Neurosci. Res.,* **24**, 72-80.
Suh, H., Mar, E.-C., Hudson, P.M., McMillian, M.K. and Hong, J.-S. (1992a): Effects of [Sar[1]] angiotensin II on proenkephalin gene expression and secretion of [Met[5]] enkephalin in bovine adrenal medullary chromaffin cells, *J. Neurochem.,* **59**, 993-998.
Suh, H., Hudson, P., Fannin, R., Boulom, K., McMillian, M.K., Poisner, A.M. and Hong, J.-S. (1992b): Prolonged stimulation of bovine adrenal chromaffine cells with arachidonic acid and prostaglandin E_2 increases expression of the proenkephalin gene and the secretion of [Met[5]]-enkephalin, *J. Pharmacol. Exp. Ther.,* **263**, 527-532.
Suh, H., Hudson, P., McMillain, M.K. and Hong, J.-S. (1992c): Pertussis toxin stimulates the secretion of [Met[5]]-enkephalin and the expression of proenkephalin A mRNA in bovine adrenal medullary chromaffin cells, *Biol. Signals,* **1**, 257-265.
Suh, H., McMillian, M.K., Hudson, P.M., Pennypacker, K.R. and Hong, J.-S. (1993): Expression of the proenkephalin A gene and [Met[5]]enkephalin secretion induced by prostaglandin E_2 in bovine adrenal medullary chromaffin cells: Involvement of second messengers, *Mol. Cell. Neurosci.m* **4**, 113-120.
Stachowiak, M.K., Hong, J.S. and Viveros, O.H. (1990): Coordinate and differential regulation of phenylethanolamine N-methyltransferase, tyrosine hydroxylase and proenkephalin mRNAs by neural and hormonal mechanisms in cultured bovine adrenal medullary cells, *Brain Res.,* **510**, 277-288.
Stachowiak, M.K., Poisner, A. Jiang, H.K., Hudson, P.M. and Hong, J.-S. (1991): Regulation of proenkephalin gene expression by angiotensin in bovine adrenal medullary cells. Molecular mechanisms and nature of the second messenger systems, *Mol. Cell. Neurosci.,* **2**, 213-220.
Viveros, O.H., Diliberto, Jr., Hazum, E.J. and Chang, K.-J. (1979): Opiate-like materials in the adrenal medulla: Evidence for storage and secretion with catecholamines, *Mol. Pharmacol.,* **16**, 1101-1108.
Wan, D.C.-D., Marley, P.D. and Livett, B.G. (1989): Histamine activated proenkephalin A mRNA but not phenylethanolamine N-methyltransferase mRNA expression in cultured bovine adrenal chromaffin cells, *Eur. J. Pharmacol.,* **172**, 117-129.
Wan, D.C.-D., Marley, P.D. and Livett, B.G. (1990a): Coordinate and differential regulation of proenkephalin A and PNMT mRNA expression in cultured bovine adrenal chromaffin cells: Response to secretory stimuli, *Mol. Brain Res.,* **9**, 103-111.
Wan, D.C.-C., Marley, P.D. and Livett, B.G. (1990b): Angiotensin II stimulates the expression of proenkephalin A mRNA in cultured bovine adrenal chromaffine cells, *Neuropeptides,* **16**, 141-147.
Weisinger, G., DeCristofaro, J.D. and La Gamma, E.E. (1990): Multiple proenkephalin transcriptional start sites are induced by stress and cholinergic pathways, *J. Biol. Chem.,* **265**, 17389-17392.
Yoshikawa, K., and Sabol, S.L. (1986a): Expression of the proenkephalin precursor gene in C6 rat cells: Regulation by β-adrenergic agonists and glucocorticoids, *Mol.*

Brain Res., **1**, 75-83.
Yoshikawa, K., and Sabol, S.L. (1986b) Glucocorticoids and cyclic AMP synergistically regulate the abundance of preproenkephalin messenger RNA in neuroblastoma ×glioma hybrid cells, *Biochem. Biophys. Res. Commun.*, **139**, 1-10.
Yoshikawa, K., Aizawa, T. and Nozawa, A. (1989a): Phorbol ester regulates the abundance of enkephalin precursor mRNA but not amyloid β-protein precursor mRNA in rat testicular peritubular cells, *Biochem. Biophys. Res. Commun.*, **161**, 568-575.
Yoshikawa, K., Maruyama, T., Aizawa, T. and Yamamoto, A. (1989b): A new species of enkephalin precursor mRNA with a distinct 5′-untranslated region in haploid germ cells, *FEBS Lett.*, **246**, 193-196.
Zurawski, G., Benedik, M., Kamb, B.J., Abrams, J.S., Zurawski, S.M. and Lee, F.D. (1986): Activation of mouse T-helper cells induces abundant preproenkephalin mRNA, *Science,* **232**, 772-775.