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# Enkephalin Gene Transcription in Bovine Chromaffin Cells Is Regulated by Calcium and Protein Kinase A Signal Transduction Pathways: Identification of DNase I-Hypersensitive Sites

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**SUMMARY**

The bovine enkephalin gene is responsive to multiple signaling pathways in primary chromaffin cell cultures. We examined the effects of activation of the calcium and protein kinase A pathways on accumulation of enkephalin peptide and mRNA, gene transcription, and chromatin structure in the 5′ region of the gene. We show here that the increase of enkephalin mRNA and peptide after depolarization of chromaffin cells with KCl or activation of adenylate cyclase with forskolin is preceded by an increase in enkephalin gene transcription. Both enkephalin peptide and mRNA were reduced by co-treatment of KCl- or forskolin-stimulated cultures with phorbol esters. Three enhancer sequences that were previously shown to be responsive to calcium, protein kinase A, and phorbol esters in the human gene *in vitro* were identified in the bovine enkephalin promoter, identifying a potential locus of control for these pathways *in vivo*. DNase I hypersensitivity mapping identified two tissue-specific sites that are associated with enkephalin gene expression in adrenal medulla and chromaffin cells; site 1 is in the promoter, which contains the three enhancer elements, and site 2 is in the first intron. These results suggest that regulation of the enkephalin gene in primary chromaffin cells by the calcium, protein kinase A, and protein kinase C signaling pathways occurs by modulation of transcription factor activity at several discrete loci on the enkephalin gene.

The proenkephalin A gene encodes a family of enkephalin peptides with opiate-like activity that are expressed primarily in neuroendocrine cells of the adrenal gland (1), neurons of the brain and spinal cord (reviewed in Refs. 2 and 3), and mesenchymal (4–8) and reproductive tissue (9). Primary cultures of bovine chromaffin cells are useful for the study of molecular mechanisms involved in enkephalin gene regulation (10). These adrenomedullary cells contain membrane receptors coupled to different messenger systems to which the enkephalin gene is responsive. *In vivo*, intracellular levels of the second messenger calcium are increased by membrane depolarization caused by splanchnic nerve stimulation and the release of acetylcholine. This is mimicked *in vitro* by the addition of nicotine, veratridine, or high concentrations of KCl to cells (11). Depolarization increases enkephalin peptide biosynthesis (12) and mRNA levels in chromaffin cells (13, 14), rat adrenal medulla (15), and fetal rat brain cells (16).

The protein kinase A pathway is activated by addition of vasoactive intestinal polypeptide (17) or agents such as forskolin or 8-Br-cAMP to cultured chromaffin cells. Activation of adenylate cyclase elevates enkephalin peptide (18, 19) and mRNA in chromaffin cells (20, 21) and neuroblastoma cells (22). Activation of protein kinase A or cAMP causes an increase in enkephalin mRNA at the level of enkephalin gene transcription in C6 glioma cells (23) and rat striatum (24).

Activation of protein kinase C occurs after stimulation of the histamine H1 receptor (25) or after the addition of phorbol esters to cultured cells *in vitro*. Addition of phorbol esters has been reported to increase enkephalin mRNA in chromaffin cells and rat striatum (26, 27) and has been reported by others to have no effect (28). Phosphoinositol turnover has been implicated in the histamine-stimulated elevation of enkephalin mRNA at the level of transcription (29). Paradoxically, phorbol esters inhibit the accumulation of enkephalin mRNA in chromaffin cells co-treated with KCl, possibly by inhibiting calcium entry through voltage-dependent calcium channels (26, 28).

Although regulation of the enkephalin gene by these pathways is well described, the means by which these signal transduction pathways regulate the transcription of the enkephalin gene in primary neuroendocrine cells have not been addressed. Comb *et al.* (30) have defined three *cis*-acting regulatory elements in the human enkephalin promoter. These elements act

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**ABBREVIATIONS:** PMA, phorbol myristate acetate; Menk, Met-enkephalin; PDBu, phorbol dibutyrate; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kb, kilobase(s); TAE, Tris-acetate EDTA.

synergistically in *in vitro* reporter assays to confer responsiveness to calcium, cAMP, and PMA in monkey CV-1 cells and rat C6 glioma cells (31), thereby identifying a putative locus for enkephalin regulation by these signal transduction pathways in chromaffin cells.

In this report we examine the effects of stimulation of the calcium and protein kinase A signal transduction pathways on enkephalin mRNA abundance, gene transcription, and chromatin structure and determine the effects of protein kinase C activation on these pathways, to define their locus of control, in primary cultures of nondividing neuroendocrine cells.

## Materials and Methods

**Cell culture.** Chromaffin cells were isolated from fresh bovine adrenal glands after digestion with 0.1% collagenase as described (10, 18). Chromaffin cells were maintained in 24-well plates at a concentration of $1 \times 10^6$ cells/ml/well in a humidified atmosphere of 37° and 5% $CO_2$. The medium was changed on day 4 and then replaced on day 5 with medium containing 40 mM KCl or 25 μM forskolin, with or without 100 nM PMA, or PMA alone. The calcium channel blocker D600 was added at a final concentration of 30 μM. Elevated potassium medium was obtained by isotonic replacement of NaCl with KCl. Only cells that gave a robust response to treatment with KCl or forskolin were included in the PMA co-treatment experiments.

**Peptides.** Chromaffin cells and medium were analyzed for peptide content by radioimmunoassay after 72 hr of drug treatment, to allow significant accumulation of Menk peptide, as described previously (11, 19).

**Northern analysis.** Total RNA was purified 24 hr after the addition of drugs, when enkephalin mRNA reaches maximal levels, as described (19). Equal amounts of denatured RNA were separated on 1% agarose-formaldehyde gels before electroblotting onto Nytran membranes according to the manufacturer's instructions (Schleicher and Schuell). RNA was immobilized with a UV Stratalinker (Stratagene), and membranes were prehybridized for 1 hr at 42° in hybridization buffer containing 5× SSPE (1X SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 0.1% SDS, 0.1 mg/ml tRNA, 5× Denhardt's solution (1X Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 50% formamide. Denatured, nick-translated probes ($2 \times 10^6$ cpm/ml) were added to hybridization buffer containing 5% dextran sulfate, and membranes were hybridized overnight at 42°. Membranes were washed at 42° in 2× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate)/0.2% SDS, followed by a more stringent wash in 0.2× SSC/0.1% SDS at 60°. The probe used for Northern analysis was a 400-base pair *Pst*I fragment of the bovine enkephalin cDNA (32). The loading of Northern blots was normalized by scanning a negative of 28 S rRNA or by using blots reprobed with an actin cDNA (19, 33). Actin was selected instead of cyclophilin or glyceraldehyde phosphate dehydrogenase as a loading control because only actin expression was not regulated by the drug treatments in chromaffin cells.

**Nuclear run-on assay.** The rate of gene transcription was determined by using the method of Farin *et al.* (29), with modifications. Time points between 1 and 8 hr were chosen to analyze early and late transcriptional effects. Chromaffin cells ($2 \times 10^7$) maintained in T-150 flasks were purified from adherant cells by re-plating in a fresh flask. The following day, cells were treated with drugs, shaken loose, and pelleted by centrifugation. The nuclei were prepared and the transcripts were labeled as described. After the addition of 50 μg of tRNA, transcripts were purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol and an equal volume of chloroform/isoamyl alcohol. The RNA was recovered by addition of ½ volume of 7.5 M ammonium acetate and 2 volumes of ethanol and incubation at −20° for 30 min. The samples were centrifuged in a microfuge for 20 min, and the RNA pellets were washed twice with 95% ethanol before resuspension in 100 μl of 0.5% SDS.

Equal counts of the transcripts were hybridized to either double-stranded plasmids or single-stranded M13 immobilized on Nytran membranes, with similar results. Filters were prehybridized with hybridization buffer for 1 hr at 42° before hybridization to transcripts in hybridization buffer, containing 5% dextran sulfate, for 48 hr. Filters were washed at 42° for 30 min in 2× SSC/0.2% SDS. To remove background counts, filters were washed at 37° for 15 min in 2× SSC containing 1 μg/ml RNase A, followed by a brief wash in 2× SSC at 60°.

**DNase I hypersensitivity mapping.** Limited digestion with DNase I and DNA purification were performed by the procedure of Breindl *et al.* (34), with slight modification. Briefly, bovine tissue obtained from a slaughterhouse was cut into aproximately 0.5-g pieces, frozen on dry ice, and stored in tubes over a layer of ice (to prevent tissue dehydration) at −70°. Frozen tissue was dispersed with a glass-Teflon homogenizer in 20 ml/g of tissue weight of buffer A (17.1% sucrose, 15 mM Tris·HCl, pH 7.4, 25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA) containing 0.1% Nonidet P-40, as described. After tissue dispersal and removal of aggregates with nylon mesh, nuclei were counted by diluting an aliquot of nuclei with an equal volume of crystal violet (0.05% in homogenization buffer) and counting with a hemocytometer.

Nuclei were resuspended in digestion buffer B (50 mM Tris·HCl, pH 7.5, 25 mM KCl, 2 mM $MgCl_2$) at a concentration of $6.3 \times 10^7$ nuclei/ml. The samples were divided into aliquots, and $CaCl_2$ (2 mM final concentration) and different amounts of DNase I (0–100 units/ml; Promega) were added. After incubation on ice for 10 min, the digestion was stopped by the addition of an equal volume of 20 mM EDTA/2% SDS and DNA was purified as described (34). All incubations were carried out on ice because this reduced endogenous DNase activity and gave greater reproducibility between experiments.

For the preparation of chromaffin cell nuclei, primary bovine chromaffin cells ($40 \times 10^6$ cells in 40 ml of medium) plated in T-150 flasks were pooled and divided into groups containing $80 \times 10^6$ cells. Cells were pelleted and resuspended in 40 ml of serum-free chromaffin cell medium containing 40 mM KCl, 100 nM PMA, both drugs together, or no drug. Chromaffin cell nuclei were prepared by homogenization of $80 \times 10^6$ cells in 2 ml of buffer A containing 0.2% Nonidet P-40, without filtration through nylon mesh.

Purified DNA was digested with restriction endonucleases and separated on agarose-TAE gels before electrophoretic transfer to Nytran membranes, as specified in the manufacturer's instructions (Schelicher & Schuell). λ DNA digested with *Hind*III and labeled with $^{32}P$ was used as a molecular weight standard. The Nytran membranes were prehybridized and hybridized using the same conditions as for Northern blotting.

## Results

**Effects of depolarization, cAMP, and PMA on enkephalin peptide and mRNA levels.** Exposure of chromaffin cells to 40 mM KCl or 25 μM forskolin significantly increased the total detectable Menk peptide (both intracellular and secreted) at 72 hr (Fig. 1). Pretreatment of cultures with either PMA or PDBu for 30 min before stimulation with KCl or forskolin resulted in at least 50% reduction of Menk, compared with cells treated with KCl or forskolin alone. Treatment of cells with PMA or PDBu alone reduced Menk levels to slightly below the control levels obtained with the inactive analogs α-PDBu and α-PMA. Therefore, the KCl- or forskolin-stimulated synthesis of Menk in bovine chromaffin cells is blocked by phorbol esters.

Total RNA was isolated from cells treated as in Fig. 1 and was analyzed for enkephalin expression by Northern analysis (Fig. 2). Chromaffin cells treated with 40 mM KCl had increased levels of enkephalin mRNA, ranging from 1.49- to 4.15-fold greater than those in untreated cells. Similarly, 25 μM forskolin

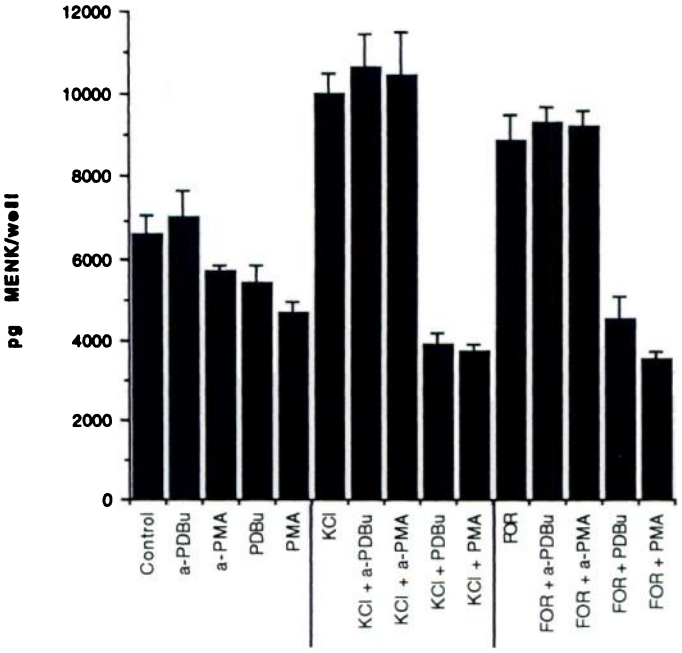


**Fig. 1.** Menk peptide in chromaffin cell cultures. Chromaffin cells were maintained in 24-well plates as described in Materials and Methods. Cells were treated with 40 mM KCl or 25 μM forskolin (*FOR*), with and without 100 nM PMA or PDBu, or with phorbol esters alone. Controls for phorbol ester treatment were the inactive analogs α-PDBu and α-PMA. Cells and media were harvested for determination of total Menk peptide at 72 hr. *Bars*, mean and standard deviation of three wells/experiment.

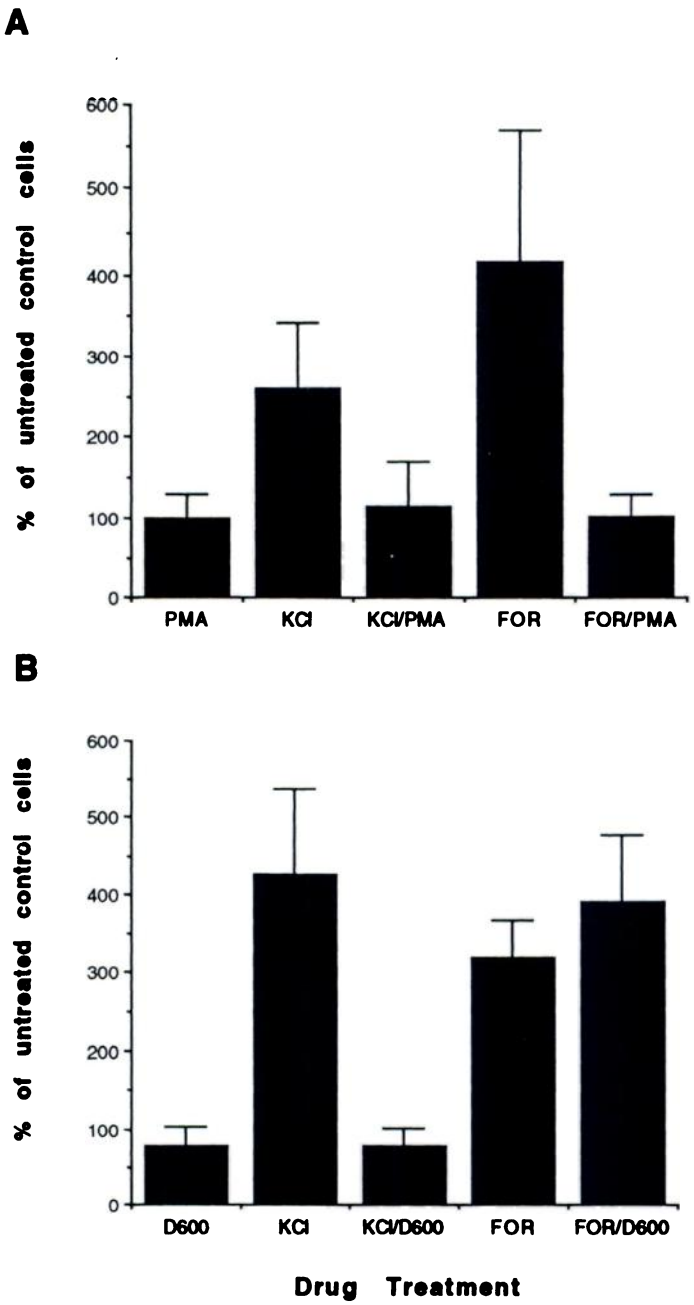


**Fig. 2.** Northern analysis of enkephalin expression in chromaffin cells. Cells were maintained in 24-well plates as described and RNA was purified 24 hr after the addition of drugs. Cells were treated with 40 mM KCl, 25 μM forskolin (*FOR*), 100 nM PMA, and/or 30 μM D600. A, Data represent proenkephalin A mRNA measured from three independent chromaffin cell dispersions. Autoradiographs were scanned and normalized to actin or rRNA. The percentage of induction of enkephalin mRNA by the drug treatments versus control was calculated for each experiment, and the mean and standard error are shown. B, Data represent proenkephalin A mRNA measured as in A except that cells were from a single dispersion (three wells/treatment group). The experiment was repeated multiple times. The standard error for the control cells varied up to 30% of the mean for the control cells.

increased enkephalin mRNA up to 7.2-fold. The effect of PMA addition to cultures varied from experiment to experiment. On average, treatment with PMA alone had no effect on mRNA levels (Fig. 2A). When cells incubated with either KCl or forskolin were also treated with PMA, a reduction in enkephalin mRNA to mean levels of 44% and 25%, respectively, of levels seen with KCl or forskolin alone was detected. Therefore, the reduction in Menk peptide in cells co-treated with PMA correlates with a decrease in enkephalin mRNA.

Because the depolarization of chromaffin cells with KCl results in an influx of calcium through voltage-gated ion channels, the effect of calcium on enkephalin mRNA levels was measured by pretreatment of cells with the calcium channel antagonist D600 before the addition of KCl or forskolin (Fig. 2B). As shown previously, pretreatment of cells with D600 blocked the KCl-induced induction of enkephalin mRNA but had no effect on the forskolin-stimulated increase in enkephalin mRNA, thereby confirming the KCl induction as calcium dependent and the forskolin pathway as calcium independent (35). Because PMA was equally effective at inhibiting the two pathways, either the mechanism of inhibition is calcium independent or two separate mechanisms of inhibition exist.

**Effects of depolarization and cAMP on enkephalin gene transcription.** A nuclear run-on assay was used to determine whether the increase in enkephalin mRNA in chromaffin cells after KCl or forskolin treatment occurred at the level of gene transcription. Chromaffin cells were treated with the agonists, nuclei were harvested, and radiolabeled run-on transcripts were synthesized and hybridized to membranes immobilized with DNA complementary to enkephalin or chromogranin A (which is not positively regulated by depolarization). As shown in Fig. 3A, enkephalin gene transcription increased 2-fold after depolarization with KCl (40 mM) and 5-fold after treatment with forskolin (25 μM), compared with plasmid sequences (pGem/7) used as a hybridization control. In Fig. 3B, the average and range of a series of run-on assays for KCl- and forskolin-stimulated cells are shown. Forskolin had an earlier and more robust effect on enkephalin gene

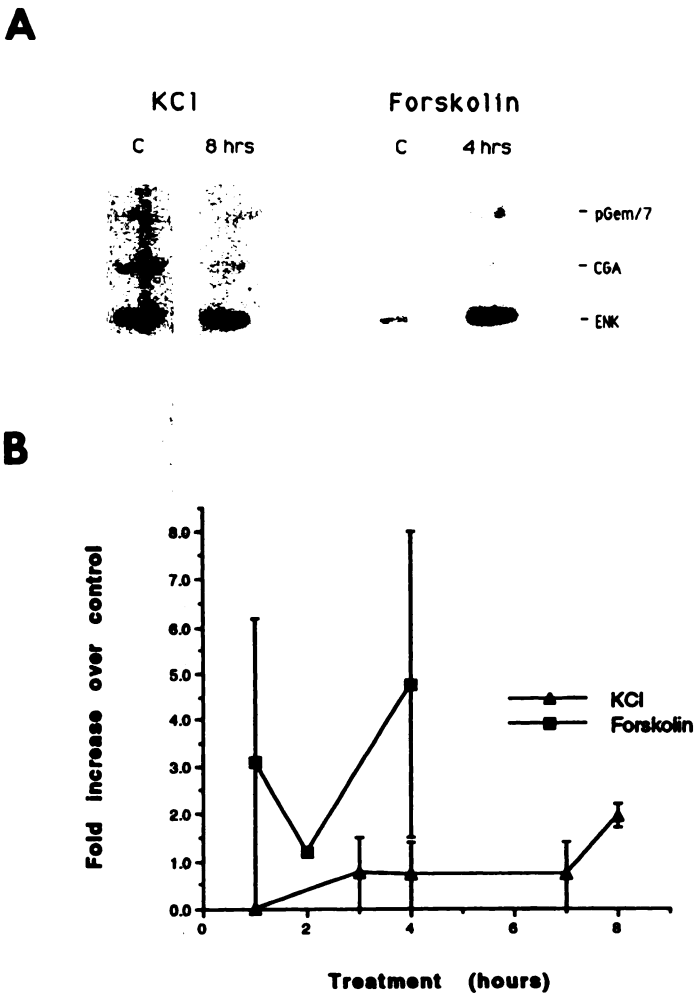


**Fig. 3.** A, Nuclear run-on analysis of enkephalin gene transcription in chromaffin cells. Cells were treated with 40 mM KCl for 8 hr or with 25 μM forskolin for 4 hr. The nuclei were prepared and transcripts were labeled as described in Materials and Methods. Equal counts of transcripts were hybridized to 2 μg of linearized plasmids containing bovine enkephalin (*ENK*) or chromogranin A (*CGA*) genes immobilized to Nytran membranes. The plasmid pGem/7 was used as a control for nonspecific background hybridization. After washing and RNase A treatment, the filters were exposed to Kodak XAR film for up to 2 weeks. Films were scanned with a Beckman spectrophotometer; plasmid hybridization (nonspecific background hybridization) was subtracted from specific hybridization. B, Summary of run-on transcription of cells treated with KCl or forskolin. Transcipts were hybridized to 1 μg of M13 DNA containing enkephalin or chromogranin A inserts or to M13 alone. Graph shows average and range of transcription induction (fold increase over control) at different time points.

transcription than did KCl; the increase in transcription was similar to the increase in mRNA seen by Northern analysis. Therefore, the increase in Menk peptide and enkephalin mRNA after activation of the calcium and protein kinase A pathways correlates with activation of enkephalin gene transcription.

**DNase I-hypersensitive sites in the enkephalin gene.** DNase I hypersensitivity mapping was used to identify regions on the enkephalin gene most likely to contain potential *cis*-acting regulatory elements important for gene regulation *in vivo*. We examined chromatin structure in the region surrounding transcription initiation. Nuclei were isolated from adrenal medulla, which expresses enkephalin mRNA, and adrenal cortex, which is negative for enkephalin expression. Cell nuclei were digested with increasing amounts of DNase I, and the

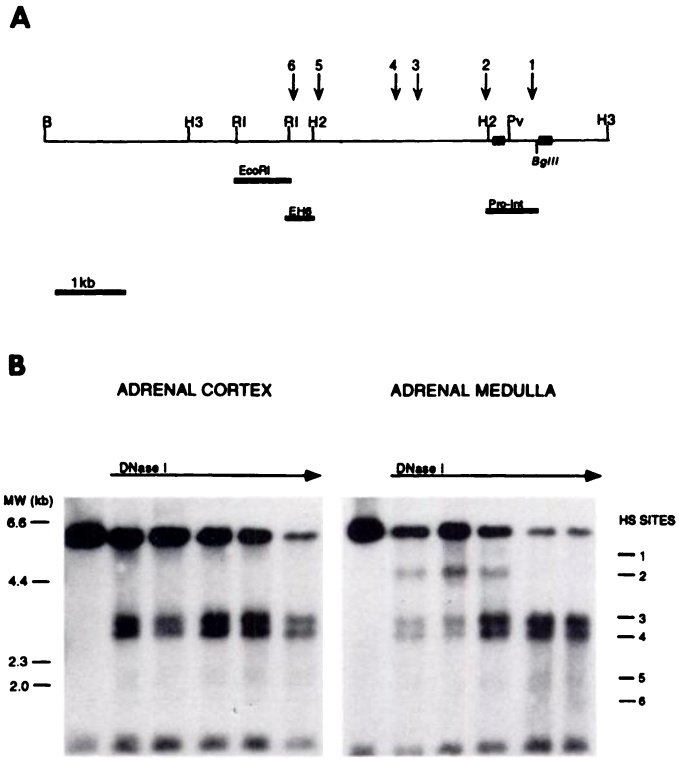


**Fig. 4.** A, DNase I-hypersensitive site map of the 5′ region of the bovine enkephalin gene. DNA was digested and probed as follows: (i) *Bgl*II/*Hind*III digest, *Eco*RI probe; (ii) *Hinc*II/*Hind*III digest, Pro-int probe; (iii) *Hind*III digest, *Eco*RI probe. Exons 1 and 2 (*black boxes*) and 5′ flanking sequences of the bovine enkephalin gene are shown. B, Southern blot analysis of *Hind*III-digested DNA (probed with the *Eco*RI probe) of DNase I-treated nuclei from bovine adrenal cortex and adrenal medulla. *Numbered lines*, positions of six hypersensitive (*HS*) sites identified in adrenal medulla, as shown in A. Four of these sites are present in the adrenal cortex. For the DNase I digestions, nuclei were purified, divided into six aliquots, and digested with 0, 10, 25, 50, 75, or 100 units/ml DNase I. After DNA purification and digestion with *Hind*III, 18 μg of DNA were electrophoresed in 0.8% agarose-TAE gels, transferred to Nytran membranes, and probed as described in Materials and Methods. *B*, *Bgl*II; *H3*, *Hind*III; *RI*, *Eco*RI; *H2*, *Hinc*II; *Pv*, *Pvu*II. The 3′ *Bgl*II site is present in the polylinker of the clone and not in the endogenous gene.

isolated DNA was cut with restriction endonucleases, size separated on agarose gels, blotted, and hybridized to the appropriate probes. Combinations of four different restriction digests and three probes were used to map the hypersensitive sites (Fig. 4A). The results for DNA digested with *Hind*III and probed with the *Eco*RI probe are shown in Fig. 4B. DNA digested with *Hind*III yielded a restriction fragment of aproximately 6.3 kb that spanned the region from −4.7 kb 5′ of transcription initiation to within intron 2. Sites 1 and 2, which correspond to regions of the enkephalin gene that were demonstrated to be hypersensitive to DNase I, mapped to intron 1 and the promoter region, respectively. Two strongly hypersensitive sites (sites 3 and 4) appeared at −1.85 kb and −1.5 kb, relative to the start of transcription initiation. Two weakly hypersensitive sites (sites 5 and 6) were present at −3.05 and −2.7 kb. However, only sites 1 and 2 were preferentially digested by DNase I in adrenal medulla and not adrenal cortex, thereby identifying hypersensitive sites that discriminate between the two tissues and correlate with enkephalin expression.

Nuclei from primary cultures of chromaffin cells were examined for the presence of DNase I-hypersensitive sites to determine whether the sites identified in adrenal medulla cor-

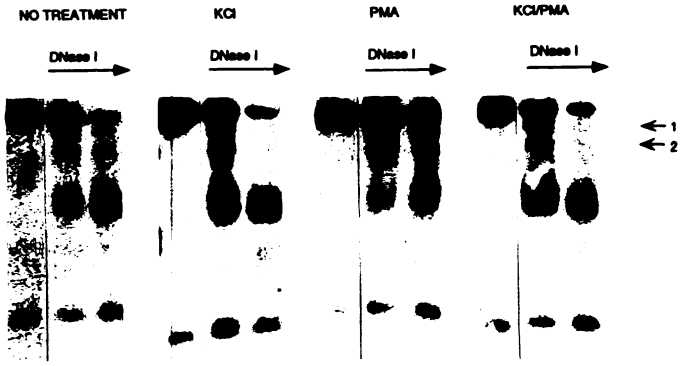


**Fig. 5.** DNase I-hypersensitive site map of enkephalin in primary cultures of bovine chromaffin cells. Primary chromaffin cells (80 × $10^6$ cells) were treated with serum-free medium containing 40 mM KCl, 100 nM PMA, both drugs together, or no drug treatment. Cells were harvested at 24 hr, and nuclei were purified, divided into four aliquots, and digested with 0, 10, 25, or 50 units/ml DNase I, as described for Fig. 4B.

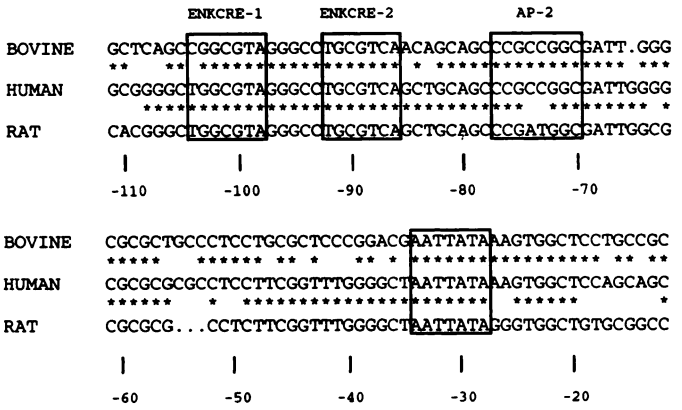


**Fig. 6.** Comparative analysis of the human, rat, and bovine enkephalin promoters. ★, Nucleotide identities between human and rat and between human and bovine sequences. *Numbers* start from the human transcription initiation site, designated as +1. The elements ENKCRE-1, ENKCRE-2, and AP-2, previously reported in the human gene, are shown in *boxes*.

related with changes in enkephalin gene transcription. In the absence of any drug treatment, hypersensitive sites 1–4 were seen in chromaffin cells (Fig. 5), although sites 1 and 2 were of equal hypersensitivity (compared with adrenal medulla, where site 2 was more prominent). Treatment of chromaffin cells with KCl, PMA, or both drugs together did not alter the hypersensitive site pattern, demonstrating that these sites are not dependent on the induction of enkephalin gene transcription but rather are constitutive sites maintained in chromaffin cells independently of the transcriptional state of the enkephalin gene.

**Sequence analysis of the bovine enkephalin promoter.** Previous analysis of the human enkephalin promoter defined several *cis*-acting DNA sequences or enhancer sequences required for its basal expression and regulation by calcium, cAMP, and phorbol esters *in vitro* (31). We therefore sequenced the bovine enkephalin promoter and compared it with the published sequences of the human and rat enkephalin promoters (Fig. 6). The enhancer sequences present in the human and rat genes were conserved in the bovine promoter in position as well as sequence, although there was a single base change in ENKCRE-1 and there were several differences in the nucleotide sequences surrounding ENKCRE-2.

## Discussion

Neuropeptide genes are typically expressed in postmitotic cells that are responsive to multiple signaling pathways. However, these genes have generally been studied with reporter gene constructs in mitotically active, transformed cell lines, with presumably quite different regulatory controls. Chromaffin cells represent well characterized, nondividing, neuroendocrine cells that respond to multiple signaling pathways and express neuropeptides in a physiologically relevent manner. In this report we examined the effects of activation of the calcium- and protein kinase A-dependent pathways on enkephalin peptide and mRNA abundance, enkephalin gene transcription, and chromatin structure surrounding the 5′ region of the enkephalin gene. The effects of protein kinase C activation on these two pathways were also examined.

As reported previously, the increase in Menk peptide after depolarization with KCl or activation of cAMP by forskolin correlated with an increase in enkephalin mRNA. We now show that this was preceded by an increase in transcriptional activity of the endogenous enkephalin gene, strongly suggesting that accumulation of enkephalin mRNA and peptides after stimulation of chromaffin cells with KCl or forskolin is due to direct stimulation of enkephalin gene transcription.

As shown here, activation of chromaffin cells with forskolin elicited a more rapid and greater fold stimulation of enkephalin transcription than did depolarization with KCl, where maximal induction was not observed until after 6 hr of treatment. One explanation for the difference in transcription kinetics between the two signaling pathways might be that depolarization by KCl requires *de novo* synthesis of transcription factors, whereas activation of transcription by cAMP does not. This is consistent with the work of Farin *et al.* (29), in which enkephalin mRNA induction in chromaffin cells stimulated with nicotine was blocked by the protein synthesis inhibitor cyclohexamide added up to 6 hr after nicotine stimulation. This contrasts with the effect of forskolin on enkephalin mRNA induction in C6 glioma cells (23), which does not require *de novo* protein synthesis. Therefore, two independent mechanisms of regulation by the depolarization and protein kinase A signaling pathways may exist at the level of enkephalin gene transcription.

Co-treatment of forskolin- and KCl-stimulated cells with PMA was associated with a decrease in Menk peptide and enkephalin mRNA. PMA alone did not significantly alter accumulation of Menk, enkephalin mRNA, or enkephalin gene transcription. Therefore, activation of protein kinase C via PMA treatment appears to antagonize the calcium- and cAMP-dependent pathways. In contrast to the reports of others (26), we did not consistently observe an increase in enkephalin mRNA after PMA treatment, although this may reflect differences in cell culture conditions.

To identify sequence elements that may be involved in the response of the enkephalin gene to the calcium and cAMP signal transduction pathways, we searched for DNase I-hypersensitive sites in the 5′ region of the enkephalin gene. DNase I hypersensitivity mapping has been used to map aproximate positions where transcription factors may interact with *cis*-acting DNA elements through a change in the positioning of nucleosomes along chromatin, thus revealing a site sensitive to DNase I (reviewed in Ref. 36). This method can identify regions of a gene that are active *in vivo*.

Limited DNase I digestion of nuclei purified from a tissue

that expresses enkephalin (adrenal medulla) and a tissue that does not (adrenal cortex) revealed two hypersensitive sites preferentially digested in adrenal medulla that were also present in cultured chromaffin cells. These sites were in the promoter and first intron. Additional sites (not tissue specific) were present upstream from the promoter site. The first intron was previously shown by others to be the site of a promoter used specifically in rat spermatogenic cells (37). The presence of an intron 1 DNase I-hypersensitive site in adrenal medulla and primary chromaffin cells strongly suggests that this region has an additional role in these tissues. A preliminary examination of the intron 1 sequence revealed an AP-2 consensus site (data not shown). Interestingly, an AP-2 consensus site is also present in the first intron of the rat enkephalin gene (37). This consensus sequence was previously reported to be a target for both the protein kinase C and protein kinase A signal transduction pathways (38).

Treatment of chromaffin cells with KCl, forskolin, or PMA did not alter the hypersensitivity pattern of the enkephalin gene at the promoter or intron sites, indicating that the sites are accessible constitutively before initiation or increase of transcription. Furthermore, PMA did not block enkephalin expression by altering the chromatin structure of the enkephalin gene at these sites. Constitutive DNase I-hypersensitive sites have been described for the cytochrome P-450IA1 gene. Transcription of this gene is dramatically induced through dioxin-responsive elements present at sites that are maintained hypersensitive to DNase I regardless of the transcriptional state of the gene (39). A recent report by Holloway and LaGamma (40) identified a tissue-specific hypersensitive site in the rat enkephalin promoter that appeared to be similar to the bovine gene, in that it was not affected by cholinergic induction of the enkephalin gene.

Sequence analysis of the bovine enkephalin promoter revealed conservation, in both sequence and position, of three previously identified enhancers in the human gene that are required for regulation by cAMP, calcium, and phorbol ester in *in vitro* reporter assays. The inhibitory effect of PMA on the bovine enkephalin gene in chromaffin cells contrasts with the effect of PMA on the human and rat enkephalin genes, which are both up-regulated by PMA *in vivo* and *in vitro* (16, 30, 41, 42). Several possible explanations for this difference in regulation include, firstly, the single base pair difference in ENK-CRE-1 or divergent sequences within the flanking sequences affecting the activity of the bovine enhancer or, secondly, the activity of the elements being dictated by *trans*-acting factors that are expressed in a cell- and tissue-specific manner (reviewed in Ref. 43). A third possibility is that additional regulatory sequences in the intron modulate the activity of the enhancers present in the promoter. The presence of hypersensitive sites in both the intron and promoter that correlate with enkephalin gene expression provides *in vivo* evidence for transcriptional regulation at these sites.

The mechanism by which phorbol esters antagonize the induction of enkephalin by the calcium and protein kinase A pathways in chromaffin cells is unclear. However, data presented here suggest that phorbol esters block by modulating transcription factor activity. Results obtained from transcription run-on assays show that induction of enkephalin by calcium and protein kinase A occurs at the level of transcription. PMA is equally effective at inhibiting a calcium-dependent pathway (depolarization) and a calcium-independent pathway (protein kinase A), suggesting that inhibition of calcium entry through voltage-dependent channels is not the primary mechanism of inhibition or that another mechanism exists for blocking the protein kinase A pathway. Finally, we have identified *in vivo cis*-acting DNA sequences in the bovine enkephalin promoter and intron that have previously been shown to respond to the three signaling pathways *in vitro*.

Interaction between the protein kinase C and protein kinase A signaling pathways has been demonstrated in a variety of cell types and can be synergistic or antagonistic (44). Regulation by these pathways converges at the gene for plasminogen activator inhibitor, for example, where transcription induced by PMA is blocked by treatment with forskolin (45). Similarly, stimulation of gene transcription by cAMP is blocked by phorbol ester (46). Both activation and repression of the phosphoenolpyruvate carboxykinase gene occur through the same 10-base pair element.

Results presented here demonstrate that regulation of the bovine enkephalin gene in primary cultures of chromaffin cells, by the calcium and protein kinase A signaling pathways, occurs at the level of transcription and may involve *cis*-acting sequences at several distinct loci. Chromaffin cells provide a well characterized, biologically relevent, model system for the future characterization of transcription factors that regulate neuropeptide genes after the stimulation of signal transduction pathways.

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