The regulation of proenkephalin expression in a distinct population of glial cells

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The expression of opioid genes was examined in isolated populations of glial cells in primary culture. Northern blot analysis of purified type I astrocytes, oligodendrocytes and mixed oligodendrocyte-type-2-astrocyte lineage cells derived from cerebral cortex demonstrated robust expression of proenkephalin mRNA exclusively in type I astrocytes. The expression of proenkephalin mRNA was stimulated by the β -adrenergic agonist isoproterenol, and 8-(4-chlorophenyl thio)adenosine 3'-5'-cyclic monophosphate (cpt-cAMP). Both of these compounds regulated a proenkephalin–chloramphenicol acetyltransferase fusion gene transiently transfected into type I astrocytes. HPLC and immunoassay of the cell culture media revealed significant levels of unprocessed proenkephalin secreted by the cell and this secretion was stimulated by isoproterenol and cpt-cAMP. The relatively high levels of proenkephalin expressed suggest that enhanced expression in astrocytes may be important during neural development, in trauma-induced gliosis and in neuroimmune interactions.

Key words: astrocytes/glia/gliosis/proenkephalin/opioid genes

Introduction

Glial cells execute essential functions in central nervous system (CNS) development and are also believed to play important roles during gliosis in response to trauma or disease (Bignami and Dahl, 1976; Varon and Somjen, 1979). These developmental and pathological states have also been associated with elevated CNS expression of opioid genes (Tsang *et al.*, 1982; Rataboul *et al.*, 1988). The anatomical distribution of the proopiomelanocortin, proenkephalin and prodynorphin derived peptides within the rat brain indicates regional variation between the individual peptides (reviewed in Khachaturian *et al.*, 1985). This discontinuous cellular expression suggests a neuronal rather than glial localization of opioid expression if glial cell populations are assumed to be homogeneous in function. However, immunohistochemical localization and *in situ* hybridization methodologies have not supplied incontrovertible evidence of a strict neuronal expression of the opioid peptides. In addition, regional differences in glial cell functions have been demonstrated (Cholewinski and Wilkin, 1988). To

investigate the possible expression and regulation of opioid genes within specific populations of glial cells, we utilized highly purified glial cultures isolated from the cerebrum of postnatal rat pups (1–2 day).

Methodologies have been developed to establish primary cultures of selected populations of glial cells. For example, McCarthy and de Vellis (1980) have demonstrated conditions for producing primary cultures of specific glial cell lineages from mixed glial cell cultures. When mixed oligodendrocyte-type-2-astrocyte lineage cells are cultured in the presence of a serum-free, chemically defined medium, virtually the entire cultured population of cells is induced to become oligodendrocytes (Saneto and de Vellis, 1985, 1987). However, when the same population of cells is cultured in the presence of fetal bovine serum (FBS), a subpopulation of cells expresses the astrocyte-specific protein glial fibrillary acidic protein (GFAP), indicating developmental commitment to type II astrocytes (Raff *et al.*, 1983). We have utilized these methodologies to prepare isolated cultures of different populations of glial cells to examine for proenkephalin expression.

At the onset of these studies, no information was available on the potential expression of opioid genes by normal glial cells. In an unexpected finding, the C6 rat glioma cell line was shown to express proenkephalin mRNA (Yoshikawa and Sabol, 1986a). More recently, cultured astrocytes were also shown to express proenkephalin mRNA (Vilijn *et al.*, 1988). Interestingly, regional differences in the levels of astrocyte proenkephalin expression were observed. The studies reported herein were conducted to determine the glial cell specificity of enkephalin expression, the regulation of proenkephalin gene expression at the mRNA and promoter levels and the expression of proenkephalin-derived peptides.

Results

Cell specificity of proenkephalin expression

Northern blot analysis of total RNA demonstrated a relatively high level of expression of proenkephalin mRNA in cultures of type I astrocytes, while expression was undetectable in cultures of oligodendrocytes and mixed oligodendrocyte-type-2-astrocyte lineage cells (Figure 1). Additionally, proenkephalin mRNA was undetectable by Northern blot analysis of poly(A)⁺ RNA from either oligodendrocytes or mixed oligodendrocyte-type-2-astrocyte lineage cells (data not shown). A subsequent wash and reprobing with the constitutively expressed cyclophilin cDNA (Danielson *et al.*, 1988) verified the quality and relative amounts of RNA loaded in each lane. These experiments are strongly indicative of distinct expression of proenkephalin mRNA within cultured type I astrocytes.

Type I astrocytes also expressed a large transcript of ~3.6 kb in addition to the previously described 1.4 kb proenkephalin mRNA (Yoshikawa *et al.*, 1984). Interestingly,

Fig. 1. Northern blot analysis of total RNA isolated from cultured type I astrocytes, mixed oligodendrocyte-type-2-astrocyte lineage cells and oligodendrocytes probed with rat proenkephalin and cyclophilin cDNAs. **Panel A** is a Northern blot of oligodendrocytes cultured in chemically defined medium (15 μ g RNA), **lane 1**; mixed oligodendrocyte-type-2-astrocyte lineage cells (15 μ g RNA), **lane 2**; and from another blot type I astrocytes (30 μ g RNA), **lane 3**. **Panel B** is the Northern blots after washing and reprobing with radiolabeled cyclophilin cDNA. Each lane contains RNA isolated from a separate experimental group.

a larger proenkephalin transcript of 1.7 kb has been observed in the rat testis (Kilpatrick *et al.*, 1987). These data suggest the presence of an alternately processed transcript in astrocyte total RNA.

The mixed oligodendrocyte-type-2-astrocyte lineage cells (cultured in the presence of FBS) used in this study are routinely 20–30% immunohistochemically GFAP positive (Saneto and de Vellis, 1985). While we have found a robust expression of proenkephalin mRNA in type I astrocytes, expression was undetectable in mixed oligodendrocyte-type-2-astrocyte lineage cultures (Figure 1). The lack of detectable proenkephalin mRNA in mixed oligodendrocyte-type-2-astrocyte lineage cells and oligodendrocytes suggests either the absence of proenkephalin expression in type II astrocytes or a greatly reduced expression in comparison to type I astrocytes.

Regulation of proenkephalin mRNA

To examine the possible regulation of proenkephalin mRNA by neurotransmitters in type I astrocytes, we utilized the β -adrenergic agonist, isoproterenol, and an analogue of its putative second messenger, cAMP. Previous studies have shown that the β -adrenergic agonists raise the intracellular levels of cAMP (Wu and de Vellis, 1983) and that cAMP can increase the expression of proenkephalin in the NG108-15 cell line (Yoshikawa and Sabol, 1986b). Both isoproterenol and the membrane-permeable cAMP analogue, 8-(4-chlorophenyl thio)adenosine 3'-5'-cyclic monophosphate (cpt-cAMP), were tested for their effects on proenkephalin mRNA levels. Cell cultures incubated for 20 h with either 10^{-6} M isoproterenol or 5×10^{-4} M cpt-cAMP exhibited increased proenkephalin mRNA levels compared to untreated cultures (Figure 2, Table I). Isoproterenol increased proenkephalin mRNA ~3-fold and cpt-cAMP 6-fold when compared to controls. In addition to the increased levels of proenkephalin mRNA, levels of GFAP mRNA were similarly increased ~3-fold by either treatment (Table I). These data suggest that proenkephalin and GFAP may be coordinately regulated.

Fig. 2. Northern blot analysis of cultured type I astrocytes treated with cpt-cAMP or isoproterenol. Type I astrocytes were cultured and isolated as described in Materials and methods. Cultures were either untreated (**lanes 1,2**) or treated with 500 μ M cpt-cAMP (**lanes 3,4**) or 10^{-6} M isoproterenol (**lanes 5,6**) for 20 h. 30 μ g of total RNA was isolated and subjected to Northern analysis using proenkephalin cDNA.

Table I.

Treatment	Proenkephalin/Cyclophilin	GFAP/Cyclophilin
Control	1.00 $\pm$ 0.10 ^a	1.00 $\pm$ 0.97 ^a
cpt-cAMP	6.02 $\pm$ 2.02	3.06 $\pm$ 0.88
Isoproterenol	2.76 $\pm$ 0.51	3.03 $\pm$ 0.04

^aData represent the mean $\pm$ SEM ($n = 4$) of either proenkephalin or GFAP mRNA normalized to the constitutively expressed cyclophilin mRNA. Autoradiograms were scanned at 633 nm and digitized using an LKB Ultrosan XL Laser Densitometer. Type I astrocyte cultures were treated with 500 μ M cpt-cAMP or 10^{-6} M isoproterenol for 20–24 h. Northern analyses and RNA isolations were performed as described in Materials and methods. In all treatments, the difference between control and treated was found to be significant ($P < 0.03$, Student's *t*-test).

Regulation of the proenkephalin promoter

Although the proenkephalin promoter has been shown to be regulated by cAMP in a transformed cell line, CV-1 (Comb *et al.*, 1986), little information is available concerning possible cAMP regulation of the promoter in non-transformed neural cells that normally express significant amounts of proenkephalin mRNA. To investigate possible transcriptional responses of the proenkephalin gene in a normal cell type that expresses proenkephalin mRNA, we transfected type I astrocytes (Figure 3) with the plasmid pENKAT-12 (Comb *et al.*, 1986). This construct contains 193 bp of the human proenkephalin 5' flanking sequence, exon I (70 bp), intron A (87 bp) and 53 of 56 bp of exon II fused to the bacterial chloramphenicol acetyl transferase (CAT) gene. cpt-cAMP was found to stimulate CAT activity ~6-fold. These data verified that increases in intracellular cAMP levels can regulate expression from the proenkephalin promoter in type I astrocytes. To examine the effect of a neurotransmitter agonist, cultures of type I astrocytes were exposed to various concentrations of isoproterenol. CAT activity was increased ~7-fold with 10^{-8} M, 17-fold in the presence of 10^{-7} M, and 9-fold with 10^{-6} M isoproterenol (Figure 3A and B). Together, these results demonstrate that both cAMP and a neurotransmitter agonist can regulate

Fig. 3. Regulation of the human proenkephalin gene promoter in cultured type I astrocytes. **Panel A** is an autoradiograph of a TLC separation of the extracted CAT enzyme reactions. Each lane is an analysis of a separate culture dish following 16 h of treatment post-transfection except lanes NE (no cytosolic extract control) and E (purified CAT enzyme control). Abbreviations: C, chloramphenicol; 1Ac, 1-acetylchloramphenicol; 3Ac, 3-acetylchloramphenicol. **Panel B** is the quantitation of non-acetylated and acetylated chloramphenicol by scraping and scintillation counting of corresponding bands from TLC plates. Data represent the mean $\pm$ SEM ($n = 3$). All treatments were found to be significantly greater than untreated cultures at $P < 0.02$.

expression from the proenkephalin promoter in a normal cell type.

Expression of proenkephalin peptide

To determine if high levels of proenkephalin mRNA were translated into high levels of protein end product, the medium was assayed for met-enkephalin-arg-phe immunoactivity. Astrocytes cultured for 20 h demonstrated relatively high levels of immunoactivity, 23.2 ± 3.6 pmol/flask (Figure 4). Further, treatment of the cells for 20 h with 10^{-7} M isoproterenol significantly increased secretion to 40 ± 1.4 pmol/flask while 10^{-6} M cpt-cAMP increased levels to 43.2 ± 3.3 pmol/flask. Interestingly, reversed-phase HPLC analysis of the secreted met-enkephalin-arg-phe related products under serum-free conditions indicated that a significant amount of the immunoactivity was in the form of unprocessed proenkephalin eluting at an identical position to unprocessed human vaccinia-derived proenkephalin (Thomas *et al.*, 1986) as shown in Figure 5. Another major peak of immunoactivity eluted at an identical position to synthetic met-enkephalin-arg-phe. The remaining peak of immunoactive peptide eluting at an intermediate position has not been characterized. It cannot be ruled out that leu- or met-enkephalin were also produced and secreted since the antisera to met-enkephalin-arg-phe do not recognize these peptides. This possibility is currently under investigation. These data indicate that astrocytes are secreting significant levels of unprocessed proenkephalin, met-enkephalin-arg-phe and an uncharacterized intermediate peptide.

Discussion

We have demonstrated the expression of proenkephalin mRNA in a specific population of cerebral glial cells, type I astrocytes. Other populations of glial cells in culture including mixed oligodendrocyte-type-2-astrocyte lineage cells and oligodendrocytes did not express detectable proenkephalin mRNA. Northern analysis has shown regulation of proenkephalin mRNA by a neurotransmitter agonist and its second messenger cAMP in normal, type I astrocytes. A similar regulation was shown for the proenkephalin promoter in transfection studies. These studies demonstrate the utility of a normal neural cell population for transfection studies.

This neurotransmitter-regulated expression of an opioid gene is suggestive of neuronal–glial cell communication which may be important during development, normal neural function, trauma and neuroimmune interactions. Recent data suggest that proenkephalin expression in addition to GFAP may be elevated in gliosis. Rataboul *et al.* (1988) demonstrated elevated proenkephalin mRNA levels in reactive gliosis using 6-hydroxydopamine induced lesions of the striatum. The authors interpreted this increase to indicate increased neuronal expression of proenkephalin following dopaminergic deafferentation. Our results suggest the possibility of an additional interpretation of these data. Proenkephalin expression may increase in astrocytes during reactive gliosis. It will be of interest to examine the

Fig. 4. Immunoreactive met-enkephalin-arg-phe in culture media from type I astrocytes. Cells were seeded at a density of 1×10^6 per 75 cm^2 flask and grown to confluency. Incubations were for 20 h in the absence (controls—CONT) or presence of 10^{-7} M isoproterenol (ISO) or $500 \mu\text{M}$ cpt-cAMP. Aliquots ($100 \mu\text{l}$ out of 10 ml) of the media from individual culture flasks were analysed for met-enkephalin-arg-phe by radioimmunoassay. Data are presented as the mean $\pm$ SEM. Asterisks denote significant differences in comparison with the controls at $P < 0.02$.

expression of proenkephalin mRNA in gliosis induced in regions of the brain devoid of enkephalinergic neurons.

The high levels of astrocyte proenkephalin expression raise many questions. The quantities of peptide secreted are equivalent to the quantities of ACTH secreted from primary cultures of anterior pituitary cells (Herbert *et al.*, 1980). This level of expression strongly implies an important functional role. Although the functions of astrocyte-derived enkephalin are unknown, the astrocyte population peaks at a time in development just prior to the major phase of neuronal degeneration (Cowan *et al.*, 1984). Additionally, administration of the opioid antagonist naltrexone from birth to day 10 rats has been shown to increase dendritic growth and neuronal maturation (Hauser *et al.*, 1987). It is therefore possible that enkephalin could play a role in this process, either trophic or inhibitory.

Another important question involves processing of the precursor peptide. We observed significant quantities of unprocessed proenkephalin in the astrocyte culture media along with met-enkephalin-arg-phe and an as yet uncharacterized intermediate peptide. It is possible that a uniquely processed peptide results from this process and that an extracellular pathway exists for processing the peptide. In this regard recent studies suggest that astrocytes secrete carboxypeptidase E (Vilijn *et al.*, 1989). In addition, astrocytes have been shown to express unprocessed angiotensinogen, presumably for extracellular processing (Stornetta *et al.*, 1988). It is of interest to determine if any unique processing of the proenkephalin precursor occurs and if the precursor or any non-opioid derivatives have any as yet uncharacterized functions.

Fig. 5. Reversed-phase HPLC analysis of met-enkephalin-arg-phe (MERF) immunoreactive products secreted from type I astrocytes. Cell culture medium was analysed by reversed-phase HPLC and immunoassay of individual fractions as described in Materials and methods. Peak 1 co-elutes with synthetic methionine enkephalin-arg-phe heptapeptide. Peak 2 co-elutes with unprocessed, vaccinia-derived human proenkephalin. The solid line indicates the gradient of CH_3CN used in the fractionation.

Gliosis induced by CNS trauma is characterized by both increases in GFAP content and pronounced morphological changes in astrocytes. *In vitro* models of gliosis have suggested that cAMP-induced changes in GFAP and morphology are valid models of several gliotic events (Fedoroff *et al.*, 1984). We observed the morphological change from flat and polygonal shape to fibrous and stellate observed in the presence of cpt-cAMP and isoproterenol although the effects of cpt-cAMP were more pronounced. Isoproterenol has previously been demonstrated to elevate intracellular cAMP levels (Wu and de Vellis, 1983). In addition, isoproterenol and cpt-cAMP treatments increased both proenkephalin and GFAP mRNA. Since gliosis has been correlated with type I astrocytes (Miller *et al.*, 1986) and GFAP levels are believed to be modulated by post-transcriptional events (Shafit-Zagardo *et al.*, 1988), the robust induction of proenkephalin mRNA transcription may be an early indicator of gliosis. It is tempting to speculate that elevated enkephalin expression in gliosis may contribute to the inhibition of central neuron regeneration following trauma. However, further information is needed on the expression of enkephalin in an *in vivo* model and in gliotic plaques as well as the functional roles of the peptides secreted.

Expression of enkephalin may be involved in neuro-immune interactions. Astrocytes have been shown to exhibit characteristics of immune cells such as the ability to present antigen (Fontana and Fierz, 1985) and the expression of cell surface antigens in common with immune cells. Enkephalins

have also been shown to have immunomodulatory effects (Chang, 1984; Jankovic and Maric, 1988) and to be expressed by specific populations of immune cells (Zurawski *et al.*, 1986). Localized trauma, inflammation or infection could activate a response involving astrocytes and enkephalin expression analogous to responses observed in the immune system.

In conclusion, these studies demonstrate a high level of proenkephalin expression at the promoter, mRNA and secreted peptide levels. This expression was regulated by β -adrenergic agonists coordinately with GFAP and may be an early marker for gliosis. The high levels of expression suggest important functions in development, neuron–glia communication, neuroimmune interactions, and gliosis following CNS damage.

Materials and methods

Glial cell cultures

Cells were isolated from neonatal rat cerebral cortices (1–2 day post-natal) and grown for 7–8 days in DMEM/Ham's F12 medium supplemented with 10% (v/v) FBS as previously described (Saneto and de Vellis, 1987). Overlying phase-dark bipotential glial precursor cells (termed mixed oligodendrocyte-type-2-astrocyte lineage cells) were subsequently shaken off at 200 cycles/min for 18–24 h and the remaining type I astrocyte cell population was reshaken for an additional 72–96 h to remove possible microglial contamination. The astrocytes were then reseeded into 75 cm² flasks and cultured until confluent. The supernatants containing mixed oligodendrocyte-type-2-astrocyte lineage cells were reseeded into 75 cm² flasks and cultured in the presence of 10% FBS for 24 h to allow attachment. At this time cultures were washed three times in basal medium (DMEM/F-12, 1:1 v/v) without serum and either medium containing 10% FBS or serum-free, chemically defined medium (Saneto and de Vellis, 1987) added for an additional 72 h. Representative populations of type I astrocytes prepared in this way were 98–99% homogeneous by positive immunohistochemical staining using antisera against GFAP and were negative for Mac-1. Representative populations of oligodendrocyte progenitor cells (termed oligodendrocytes) prepared in this way were routinely 90–98% homogeneous by immunohistochemical staining using antisera against myelin basic protein and glycerol phosphate dehydrogenase. Representative populations of mixed oligodendrocyte-type-2-astrocyte lineage cells prepared in this way were typically 20–30% GFAP positive.

Northern blots

Following culture, cells were rinsed in phosphate-buffered saline (PBS) and lysed in the presence of 4 M guanidine isothiocyanate solution (Chirgwin *et al.*, 1979) and RNA isolated by a modification of the method of Cathala *et al.* (1983). A proteinase K (150 μ g/ml) incubation step, 30 min at 43°C, was substituted for the 3 M LiCl reprecipitation step. RNA content was determined spectrophotometrically at 260 nm and samples of RNA were separated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH) as described previously (Low *et al.*, 1989). A rat proenkephalin cDNA fragment (Yoshikawa *et al.*, 1984) was labeled by the random priming method (Feinberg and Vogelstein, 1983, 1984). Blots were hybridized for 24–48 h at 45°C in 50% formamide, 7% SDS, 1% BSA and 5 $\times$ SSC (1 $\times$ SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Following hybridization, blots were washed under stringent conditions; one 30 min wash at 45°C with 5 $\times$ SSC and 1% SDS, two 30 min washes at 45°C with 2 $\times$ SSC and 0.1% SDS and a final 15 min wash at 60°C with 0.1 $\times$ SSC and 0.1% SDS. The blots were then air dried, wrapped with a single layer of Saran Wrap, and placed in X-ray cassettes with Kodak X-Omat AR film for autoradiography. For rehybridization, blots were washed for 2 h at 70°C in 5 mM Tris–HCl, pH 8.0, 0.2 M EDTA, 0.05% pyrophosphate, 0.1 $\times$ Denhardt's solution and 0.1% SDS to remove the hybridized probe and rehybridized to rat cyclophilin cDNA, a gene encoding peptidyl-prolyl *cis-trans* isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle (Danielson *et al.*, 1988; Takahashi *et al.*, 1989). Alternatively, the blots were rehybridized with a cDNA encoding murine GFAP (Lewis *et al.*, 1984).

Astrocyte transfections

Type I astrocytes were isolated, cultured and seeded into 60 mm culture dishes at a cell density of 10⁶ cells/dish. Cells were grown in the presence of FBS (10% v/v) until ~90% confluent. The plasmid pENKAT-12 (Comb *et al.*, 1986) containing 193 bp of the 5' flanking sequence, exon I (70 bp), intron A (87 bp) and 53 bp of exon II (56 bp) fused to the reporter gene CAT was transiently transfected in type I astrocyte cultures by calcium phosphate coprecipitation as described previously (Young *et al.*, 1989). These conditions were essentially a combination of those described by Parker and Page (1984) and Chepelinsky *et al.* (1985). Briefly, a CaPO₄-DNA co-precipitate was made by mixing 0.125 ml of each of two solutions: solution A, 700 μ M Na₂HPO₄, 700 μ M NaH₂PO₄, 0.01 M KCl, 2% glucose in HEPES-buffered saline and solution B, 45 μ g DNA in 0.24 M CaCl₂. First, 0.25 ml of precipitate was added to the wells and then after a 4 h exposure, cultures were glycerol-shocked for ~90 s and washed with Ca²⁺- and Mg²⁺-free PBS. At this time culture medium was added with and without treatments for 16 h. Cultures were treated with 10⁻⁸, 10⁻⁷ or 10⁻⁶ M isoproterenol or 500 μ M cpt-cAMP.

CAT assays and quantification

After treatment, cells were harvested by trypsinization, lysed and soluble cell extract collected by centrifugation. The cells were brought up in 100 μ l Tris buffer (0.25 M, pH 7.4) and lysed by three freeze–thaw cycles in a dry ice ethanol bath and a 37°C water bath. Soluble extract was separated from insoluble cell components by centrifugation at 15 000 g in a microcentrifuge for 5 min.

Cell extracts (85 μ l) were incubated with a solution of 1 M Tris buffer, pH 7.4, containing 2.5 μ l of [¹⁴C]chloramphenicol (New England Nuclear/DuPont, Boston, MA) and 53.4 mg/ml acetyl-coenzyme A (Sigma) at 37°C for 4 h. The chloramphenicol forms were then extracted from the solution with 1 ml ethyl acetate. The extract was dried in a speed-vac (Savant Instruments, Farmingdale, NY), solubilized in 15 μ l ethyl acetate and dispensed onto the loading zone of a TLC plate (type LK6, Whatman, Clifton, NJ). These conditions were adapted from those used in Young *et al.* (1989) and Gorman *et al.* (1982). Quantitation was achieved by scintillation counting of the spots on TLC plates detected by autoradiography and was expressed as per cent conversion of chloramphenicol to 3-acetylchloramphenicol (the product of enzymatic conversion).

Assay of proenkephalin peptides

Proenkephalin peptide levels were determined by radioimmunoassay using specific antisera recognizing met-enkephalin-arg⁶-phe⁷ essentially as described by Sonders and Weber (1987). We used met-enkephalin-arg⁶-phe⁷ (Peninsula Laboratories, Belmont, CA) as the tracer and standard. The assay was modified to use dextran-coated charcoal instead of second antibody to separate bound from free (Allen *et al.*, 1978). In this form the assay has a sensitivity of 20 pg and recognizes both free met-enkephalin-arg⁶-phe⁷ and intact proenkephalin. Media samples (100 μ l) from individual culture flasks were assayed either directly or following extraction and HPLC separation as described previously (Allen *et al.*, 1988; Thomas *et al.*, 1988). For reversed-phase HPLC analysis, cells were cultured in serum-free DMEM/F-12 containing 0.1% BSA, 3.3 μ g/l sodium selenite, 55 mg/l sodium pyruvate, 5 mg/l insulin and 2.75 mg/l transferrin. 2 ml of cell culture medium was acidified to 0.1% trifluoroacetic acid (TFA) and applied to a WP Butyl (C4) disposable extraction column (J.T. Baker, Phillipsburg, NJ). Preparation of the column has been previously described (Allen *et al.*, 1988). After washing the column with 5 ml of 0.1% TFA, peptides were eluted with 5 ml of 50% acetonitrile in 0.1% TFA. The eluate was diluted twice with water and lyophilized. The lyophilized extract was then resuspended in 1 ml of 13.6% acetonitrile/0.1% TFA, fractionated on a C4 reversed-phase column (Vydac 214TP54) and eluted with a linear gradient of acetonitrile from 12.8 to 39.2% over 80 min at a flow rate of 1 ml/min as described previously (Allen *et al.*, 1988). Subsequently, 50% of each fraction was reduced to dryness under vacuum at room temperature and assayed for met-enkephalin-arg-phe as described above. To characterize the reversed-phase HPLC system, vaccinia-derived proenkephalin (Thomas *et al.*, 1986) was kindly provided by Dr Gary Thomas, Vollum Institute of Advanced Biomedical Sciences and Oregon Health Sciences University, Portland, OR. Purified vaccinia-derived proenkephalin or synthetic met-enkephalin-arg-phe was mixed with 1 ml of unused cell culture medium and subjected to reversed-phase HPLC as described above. The elution positions of these standards are described in Figure 5. Recovery of peptides was estimated to be 60–70% by using 100 000 c.p.m. of ¹²⁵I-labeled met-enkephalin-arg-phe.

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