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INDUCTION OF DIFFERENTIATION INCREASES MET^5-ENKEPHALIN AND LEU^5-ENKEPHALIN CONTENT IN NG108-15 HYBRID CELLS: AN IMMUNOCYTOCHEMICAL AND BIOCHEMICAL ANALYSIS[1]

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Abstract

Regulation of cellular content of the endogenous opioid peptides Met^5-enkephalin and Leu^5-enkephalin was investigated in neuroblastoma × glioma hybrid cells NG108-15 grown in both serum-supplemented and serum-free defined media. Untreated cells and cells induced to differentiate were stained using anti-Met^5-enkephalin and anti-Leu^5-enkephalin with the peroxidase-antiperoxidase immunocytochemical technique at the light microscopic level. In untreated NG108-15 cells grown in serum-supplemented medium, intense enkephalin-like immunoreactivity was localized in cell bodies and short processes of a select population of cells. The volume fraction of stained untreated cells remained constant throughout the time period investigated. When cells were induced to differentiate with $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (dBcAMP) or 8-bromo cyclic adenosine monophosphate (1.0 mM) treatment for 5 days, staining was found throughout the cytoplasm of perikarya and the extensive processes which were expressed, and the volume fraction of stained cells increased over 2-fold. Receptor-mediated stimulation of adenylate cyclase by prostaglandin E_1 (10 μM) for 5 days produced results similar to those with dBcAMP. Pure cultures of differentiated cells with intense staining were obtained by further treatment of cultures, grown in the presence or absence of dBcAMP, with arabinosylcytosine (araC). Untreated, dBcAMP-treated and araC-treated NG108-15 cells grown in defined medium expressed staining patterns and volume fractions of stained cells similar to those grown in serum-supplemented medium; sodium butyrate (1.0 mM), however, increased the volume fraction of stained cells grown in defined medium over 3-fold, whereas it had little effect on staining of cells grown with serum. The presence of both Met^5- and Leu^5-enkephalin-like activities in NG108-15 cells was confirmed in acid extracts of cells by radioreceptor assay after separation by reverse phase high pressure liquid chromatography. Induction of differentiation in NG108-15 cells by dBcAMP treatment increased the cellular concentration of both enkephalins to over 2 times the levels found in untreated cells. The biochemical analysis for Met^5-enkephalin- and Leu^5-enkephalin-like activity compared well with the immunocytochemical data indicating that the enkephalin content is correlated with the state of differentiation of NG108-15 cells.

Subsequent to the discovery of stereospecific opiate receptors (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973), the endogenous opioid peptides Met^5-enkephalin and Leu^5-enkephalin (Hughes et al., 1975) have been found in a variety of cells and central and peripheral tissues. More recently, it has become clear through analysis of peptide precursors (Goldstein et al., 1979; Kanagawa et al., 1979; Gubler et al., 1982; Noda et al., 1982) that the enkephalins are synthesized via path-

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ways distinct from those for the larger endorphins (Mains et al., 1977). The existence of more than one functional opioid peptide system in brain tissue is complicated by the occurrence of several distinct opiate receptor populations with varying affinities for endogenous and synthetic opiate receptor agonists (Chang et al., 1980).

The neuroblastoma × glioma hybrid NG108-15 cell line has proven to be a very useful neuronal model. In contrast to the brain, these cells contain only δ-opiate (enkephalin-preferring) and ϵ-opiate (endorphin-preferring) receptors of high density (Klee and Nirenberg, 1974; Hammonds et al., 1981), while they express many neuronal characteristics (Hamprecht, 1977). NG108-15 cells have excitable membranes and form functional synapses with myotubes. They contain intracellular acetylcholine and choline acetyltransferase and acetylcholinesterase activities. In addition to δ-opiate receptors, they also possess α_2-adrenergic, muscarinic cholinergic, and prostaglandin E_1 (PGE_1) receptors among others. The enkephalin binding affinities to these cells approximate those for brain homogenates, and opiate agonists inhibit basal and PGE_1-stimulated adenylate cyclase activity (Sharma et al., 1975, 1977) in a sodium- and guanine nucleotide-dependent manner (Blume, 1978; Blume et al., 1979).

Recently, Glaser et al. (1980, 1982) showed that NG108-15 hybrid cells also contained Met^5-enkephalin and Leu^5-enkephalin, and that glucocorticoids elevated the intracellular concentrations of these peptides (Glaser et al., 1981). Similarly, the murine neuroblastoma clone N1E-115, which also possesses δ-opiate receptors, contains Met^5-enkephalin as demonstrated by immunocytochemical and biochemical methods (Knodel and Richelson, 1980; Gilbert et al., 1982).

NG108-15 cells can be induced to differentiate with numerous agents including N^6,$O^{2\prime}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (dBcAMP) (Daniels and Hamprecht, 1974). The differentiated cells are larger than control cells and express long, thick, neurite-like processes with many adhering cytoplasmic droplets. The processes contain increased amounts of mitochondria, endoplasmic reticulum, and electron transparent and large dense core vesicles. Ultrastructurally, differentiated cells appear similar to normal neurons.

We have utilized the neuroblastoma × glioma hybrid NG108-15 cell line to investigate the regulation of intracellular Met^5-enkephalin and Leu^5-enkephalin concentrations. We present in this report both immunocytochemical and biochemical evidence that the levels of the endogenous opioid pentapeptides can be correlated with the state of differentiation of NG108-15 cells.

Materials and Methods

Cell culture

Serum-supplemented medium. NG108-15 (108CC15) cells were obtained from Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD) and Dr. B. Hamprecht (University of Wurzburg, Wurzburg, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) without antibiotics, supplemented with 3.7 gm/liter of sodium bicarbonate, 10% newborn calf serum, 0.1 mM hypoxanthine, 1.0 μM aminopterin, and 16 μM thymidine as previously described (Daniels and Hamprecht, 1974). Cells of passages 15 to 25 were inoculated at a density of 4000 cells/cm^2 into 25-cm^2 plastic flasks (Corning) or Leighton tubes (Costar) for immunocytochemistry, or 175-cm^2 flasks (Falcon) for high pressure liquid chromotography (HPLC) analysis, and incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2. The medium was changed daily beginning 2 days after plating.

Serum-free defined medium. NG108-15 cells were also cultured in serum-free defined medium according to the method of Wolfe et al. (Wolfe et al., 1980; Wolfe and Sato, 1982). The cells were grown in a mixture of DMEM and Ham's F-12 (3:1) containing 3.7 gm/liter of sodium bicarbonate, 25 mM HEPES, trace elements (0.5 nM $MnCl_2 \cdot 4H_2O$, 0.5 nM $(NH_4)Mo_4O_{24} \cdot 4H_2O$, 0.25 nM $SnCl_2$, 25 nM $NaVO_4 \cdot 4H_2O$, 0.25 nM $NiSO_4 \cdot H_2O$, 15 nM H_2SeO_3, 25 nM $Na_2SiO_3 \cdot H_2O$, and 5 nM $CdSO_4$), 5 μg/ml of bovine insulin, 50 μg/ml of human transferrin, and linoleic or oleic acid (5 to 10 μg/ml) complexed to fatty acid-free bovine serum albumin (1 mg/ml). Culture conditions were identical to those described above for cells grown in serum-supplemented medium.

Treatment of cells

Serum-supplemented medium. To induce differentiation, cultures maintained in serum-supplemented medium were treated with 1.0 mM dBcAMP, 1.0 mM 8-bromo adenosine 3′,5′-cyclic monophosphate (8-BrcAMP), or 10 μM PGE_1 plus 100 μM 3-isobutyl-1-methylxanthine (IBMX) for up to 7 days, beginning 2 to 6 days after plating. Cultures were also treated with 2.0 mM sodium butyrate (NaB) or 100 μM IBMX under identical conditions. Additional flasks of cells grown in complete medium with or without dBcAMP were further treated for 4 days with 10 μM arabinosylcytosine (araC) to kill proliferating cells and obtain pure cultures of differentiated cells. PGE_1 was prepared in absolute ethanol and added to complete medium immediately before the daily treatment (1.0 μl of PGE_1/ml of complete medium). All remaining drugs were prepared fresh daily in complete medium.

Serum-free defined medium. Cultures of cells grown in serum-free defined medium were treated with 1.0 mM dBcAMP or 2.0 mM NaB for 5 days beginning 2 days after plating. Cultures of untreated or dBcAMP-treated cells were treated for an additional 4 days with araC.

Light microscopic immunocytochemical staining

Tissue preparation. Cultures were rinsed three times with DMEM and the cells were fixed to the culture flasks for 1.0 hr at room temperature with 1.0 or 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4, or with periodate/lysine/paraformaldehyde prepared according to the method of McLean and Nakane (1974). These fixatives were previously shown to best preserve the antigenicity of the enkephalins (Braas et al., 1980, 1981). Following fixation, the cultures were rinsed with

cold 0.1 M Sorensen's phosphate buffer, pH 7.4, before the immunocytochemical stain.

Immunocytochemical stain. The cultures were stained using a modification of the peroxidase-antiperoxidase (PAP) complex technique (Sternberger et al., 1970; Moriarty and Halmi, 1972). Antiserum to Leu^5-enkephalin, supplied by Dr. J. F. Wilber (Louisiana State University, New Orleans, LA), has been characterized using both radioimmunoassay (Kubek and Wilber, 1980) and immunocytochemical staining (Braas et al., 1980, 1981). Anti-Met^5-enkephalin was obtained from Immuno Nuclear Corp. (Stillwater, MN). The staining procedure was as follows: (*1*) *hydrogen peroxide reaction:* pretreatment with 3% H_2O_2 for 10 min to inhibit endogenous cellular peroxidase activity, followed by a water wash; (*2*) *blocking reaction:* incubation with 1:200 normal goat serum (NGS) for 10 min, then blotting off excess; (*3*) *primary antibody reaction:* incubation in 1:5,000 to 1:20,000 primary antiserum for 48 hr at 4°C, then washing with 0.1 M Sorensen's phosphate buffer, pH 7.4; (*4*) *second antibody reaction:* incubation in 1:100 goat anti-rabbit IgG in 1:200 NGS for 15 min, followed by a phosphate buffer wash; (*5*) *PAP complex:* incubation with 1:100 to 1:400 PAP complex in 1:200 NGS for 10 min, followed by a phosphate buffer wash; (*6*) *peroxidase reaction:* performed using diaminobenzidine and H_2O_2 as substrates (0.3 mg/ml of diaminobenzidine and 0.03% H_2O_2 in 0.05 M Tris buffer, pH 7.6) for 15 min with shaking, then washed with water; (*7*) *osmium tetroxide reaction:* exposure to osmium tetroxide vapors for 10 to 15 min to enhance the reaction product.

All antisera were diluted in sterile 0.05 M Sorensen's phosphate buffer, pH 7.4, containing 2.5 mg/ml of human serum albumin. All solutions were sterilized using 0.22-μm pore filters (Millipore Corp.), and all glassware was sterilized before use.

Staining controls. Solid phase immunoabsorption tests were performed using Met^5- or Leu^5-enkephalin (0.5 to 1.0 mg) coupled to cyanogen bromide-activated Sepharose 4B beads (1.0-ml bed volume). Primary antisera were absorbed with enkephalin-coupled Sepharose beads or with control ethanolamine-conjugated beads for 48 hr at 4°C prior to use in the immunocytochemical stain. Cultures were also stained using normal rabbit serum to replace the primary antibodies. Additional cultures were stained with omission of primary antibody, goat anti-rabbit IgG, or PAP complex.

Morphometrics

Morphometric analysis of the volume fraction of cells that stained was performed by a modification of the method of Weibel (1979), using light micrograph negatives. The cultures were photographed with Kodak Tri-X Pan film in a systematic sampling pattern at × 100 using a Zeiss Standard Research microscope with 35-mm camera attachments. The negatives were projected to a × 8 enlargement onto a test lattice and the volume fraction (V_v) was calculated as the number of test points in stained cells (P), divided by the total number of test points in all cells (P_t); or $V_v = P/P_t$. Two to eight thousand points per treatment were counted, and the volume fraction was calculated.

Enkephalin assay

Enkephalin extraction. Cells from 10 untreated and 10 dBcAMP-treated flasks were harvested in Hanks' balanced salt solution without phenol red, which interfered with subsequent HPLC analysis. After an aliquot was taken for cell counting and protein assay (Lowry et al., 1951), cells in suspension were pelleted and frozen at −70°C. For extraction, thawed cells were then homogenized in 5.0 ml of 0.2 N HCl with a Polytron homogenizer. After centrifugation at 48,000 × g for 10 min, the supernatants were frozen and lyophilized.

HPLC and radioreceptor assay of enkephalins. Lyophilized extracts were resuspended in 250 μl of 10 mM ammonium acetate, pH 4.15. After centrifugation to remove particulate matter, samples were subjected to HPLC separation on a Lichrosorb C-8 reverse phase column. Samples were eluted by a discontinuous gradient of 15% methanol in 10 mM ammonium acetate, pH 4.15, to 100% methanol, in a period of 50 min (1.0 ml/min). Elution of known quantities of Met^5- and Leu^5-enkephalin revealed that both peptides were eluted with greater than 95% recovery, with Met^5-enkephalin eluting at 33 min and Leu^5-enkephalin at 38 min. When standards were run, [^{3}H]Leu^5- and [^{3}H]Met^5-enkephalin were eluted in the presence of the same cellular extracts as those run for subsequent radioreceptor assay. One-milliliter fractions were collected, lyophilized, and resuspended in 250 μl of 50 mM Tris-HCl, pH 7.7. Aliquots (100 μl) were assayed in duplicate for inhibition of D-[^{3}H]Ala^2,D-Leu^5-enkephalin binding to rat brain membranes with 50 μg/ml of bacitracin added to retard hydrolysis of native enkephalin as previously described (Childers et al., 1979). Enkephalin levels were determined from separate standard competition curves of Met^5- and Leu^5-enkephalin; nonspecific binding was determined in the presence of 1 μM D-Ala^2,D-Leu^5-enkephalin.

Materials

Reagents were obtained from the following sources: Dulbecco's modified Eagle's medium, Ham's nutrient mixture F-12, newborn calf serum, and Hanks' balanced salt solution without phenol red, from Grand Island Biological Co. (Grand Island, NY); bovine insulin, human transferrin, linoleic acid, oleic acid, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate, 3-isobutyl-1-methlyxanthine, and 3,3′-diaminobenzidine tetrachloride, from Sigma Chemical Co. (St. Louis, MO); fatty acid-free bovine serum albumin, Miles Laboratories, Inc. (Elkhart, IN); arabinosylcytosine, The Upjohn Co. (Kalamazoo, MI); cyanogen bromide-activated Sepharose 4B beads, from Pharmacia Fine Chemicals (Piscataway, NJ); Met^5-enkephalin and Leu^5-enkephalin, from Peninsula Laboratories, Inc. (San Carlos, CA); methanol (HPLC grade) and hydrogen peroxide (30%), from Fisher Scientific Co. (Fair Lawn, NJ); D-[^{3}H]Ala^2,D-Leu^5-enkephalin, from New England Nuclear (Boston, MA). All other reagents were analytical grade.

Other immunochemicals were obtained from the following sources: goat anti-rabbit IgG and normal rabbit serum, from Kallestad (Chaska, MN); peroxidase-anti-peroxidase complex, from N. L. Cappel Laboratories (Cochranville, PA); normal goat serum, from Antibodies Inc. (Davis, CA).

Results

Morphology

Untreated neuroblastoma × glioma hybrid NG108-15 cells grown in serum-supplemented medium demonstrated many morphological neuronal characteristics in culture, as previously described by Daniels and Hamprecht (1974). The cells were polygonal, usually possessed short processes, and had a tendency to cluster (Fig. 1*A*). Cells grown in serum-free defined medium appeared similar, but generally had somewhat less angular perikarya (Fig. 1*B*). In both cases, there were morphological variabilities within each culture, possibly due to differences in the state of differentiation of the cells.

Induction of differentiation in NG108-15 cells by various treatments is shown in Figures 1, *C* to *F*, and 2, *A* and *B*. Treatment of NG108-15 cells grown in serum-supplemented medium with 1.0 mM dBcAMP reduced the rate of cell division and within 24 hr caused the cells to aggregate and emit long neurite-like processes which were strung between cell aggregates (Fig. 1*C*). By 5 days of treatment, cytoplasmic droplets appeared along both thickened processes and oval cell bodies. Similarly, cells grown in defined medium emitted long processes after dBcAMP treatment but did not form large aggregates. In addition, by 5 days of treatment, no thickening of processes or adhesion of cytoplasmic droplets was observed (Fig. 1*D*).

Since butyrate itself can induce a differentiation in some cultured cell types (Prasad and Vernadakis, 1972; Waymire et al., 1972; Richelson, 1973), cultures were also treated with 2.0 mM NaB for up to 5 days. In NG108-15 cells grown in serum-supplemented medium, NaB dramatically reduced cellular division. The cells aggregated into small groups and became very flattened and angular, and by 5 days of treatment a few cells had begun to emit short flattened processes (Fig. 1*E*). The effects of NaB on cells grown in defined medium was quite different. Again, cellular division was abruptly reduced; within 24 hr, however, the cell bodies became rounded and began to emit very long thick processes. Often, several cell bodies became associated together, with their long processes radiating outward (Fig. 1*F*). By 5 days of treatment, cytoplasmic droplets were occasionally present on the very extensive processes.

Exposure of cells grown in serum-supplemented medium to 8-BrcAMP, another nonhydrolyzable analogue of cAMP, did not affect cell division as markedly as did dBcAMP. These cells continued to proliferate at a rate slightly slower than that of untreated cells. Unlike dBcAMP, 8-BrcAMP-treated cells did not form large aggregates, and they emitted numerous short, branched processes with inclusive varicosities (Fig. 2*A*). In order to elevate intracellular cAMP content via a receptor-mediated stimulation of plasma membrane adenylate cyclase, NG108-15 cells grown in serum-supplemented medium were exposed to PGE_1 in the presence of the phosphodiesterase inhibitor IBMX. This treatment had effects similar to those of dBcAMP, although the rounded cell bodies were larger and varicosities were present along the processes (Fig. 2*B*).

Further treatment of cells grown in complete medium, with or without dBcAMP, with araC killed proliferating cells, resulting in pure cultures of differentiated cells. Previously untreated cells exposed to araC formed huge aggregates of very large rounded cells, and short processes could be found extending between the clusters of cells (Fig. 2*C*). araC treatment of cells previously grown with dBcAMP for 5 days unveiled the extensive network of thick processes with adhering cytoplasmic droplets found between cell aggregates (Fig. 2*D*).

Immunocytochemical staining

Untreated NG108-15 cells. Cells were fixed and stained beginning on day 3 through day 7 after plating. Under optimal conditions (2.5% glutaraldehyde fixation and the use of a 1:10,000 dilution of anti-Met^5-enkephalin), intense enkephalin-like immunoreactivity was found in select rounded or neurite-like cells, found either singly or in small clusters. The stain was localized to the cytoplasm and extended throughout the short processes (Fig. 3*A*). Staining was also found throughout the cell bodies and long axon-like processes of neurite-like cells (Fig. 3*B*). A similar staining pattern was observed with 1:10,000 anti-Leu^5-enkephalin (data not shown). Morphometric analysis, which provides an estimate of the extent but not the intensity of the stain, indicated that the volume fraction of stained cells remained constant at approximately 0.28 during this time period (Fig. 4), which was therefore chosen for the remaining experiments. The cell number rose to approximately 15 to 17 $\times 10^6$ cells/flask at the stationary phase, reached by days 9 to 10, and protein content (milligrams per flask) increased over 8-fold (Fig. 4).

Induction of differentiation in NG108-15 cells. The effects of various differentiation-inducing agents on the enkephalin staining pattern of NG108-15 cells were assessed at different treatment times. In cells grown in serum-supplemented medium, after 5 to 7 days of dBcAMP treatment, enkephalin-like immunoreactivity was localized throughout the cell body cytoplasm, with thickened processes extending between cell clusters and the cytoplasmic droplets appearing on both processes and perikarya (Fig. 3, *C* and *D*). The volume fraction of stained cells gradually rose from 0.29 in untreated cultures to approximately 0.68 after 5 days of dBcAMP treatment, for either anti-Met^5-enkephalin or anti-Leu^5-enkephalin staining (Fig. 5). Increases in the volume fraction of stained cells similar to those seen with dBcAMP treatment were observed after 5 days of 8-BrcAMP treatment ($V_v = 0.68$). Enkephalin-like immunoreactivity was found throughout the cell bodies, short branched processes, and varicosities (data not shown). On the other hand, 5 days of NaB treatment, which

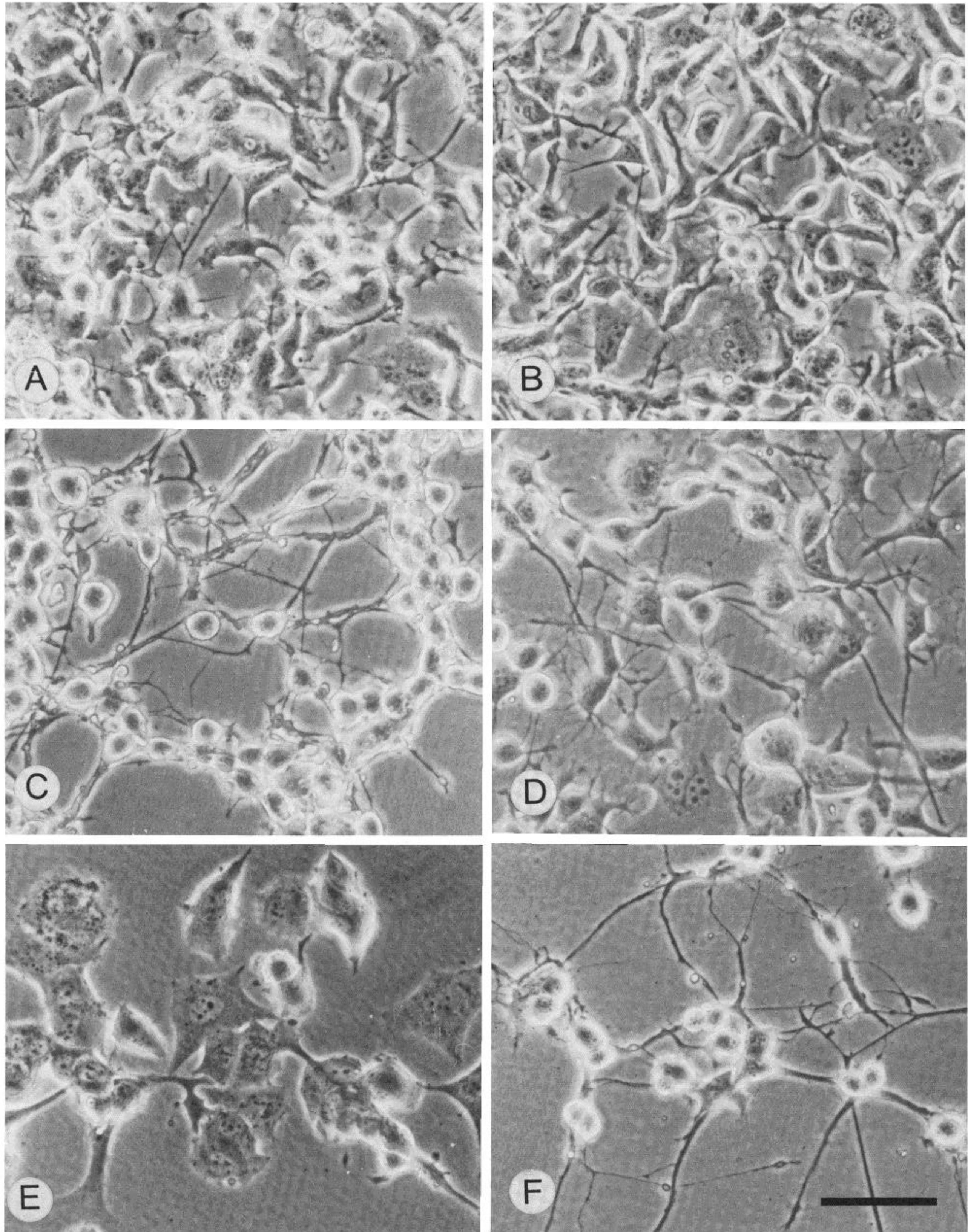

Figure 1. Morphological comparison of untreated, dBcAMP-treated, and NaB-treated NG108-15 cells. Phase contrast micrographs of NG108-15 cells grown in serum-supplemented (*A, C,* and *E*) or serum-free defined (*B, D,* and *F*) medium. Cells were grown as described under "Materials and Methods" with no drugs (*A* and *B*), 1.0 mM dBcAMP (*C* and *D*), or 2.0 mM NaB (*E* and *F*) for 5 days, beginning 2 days after plating. *Bar*, 100 μm.

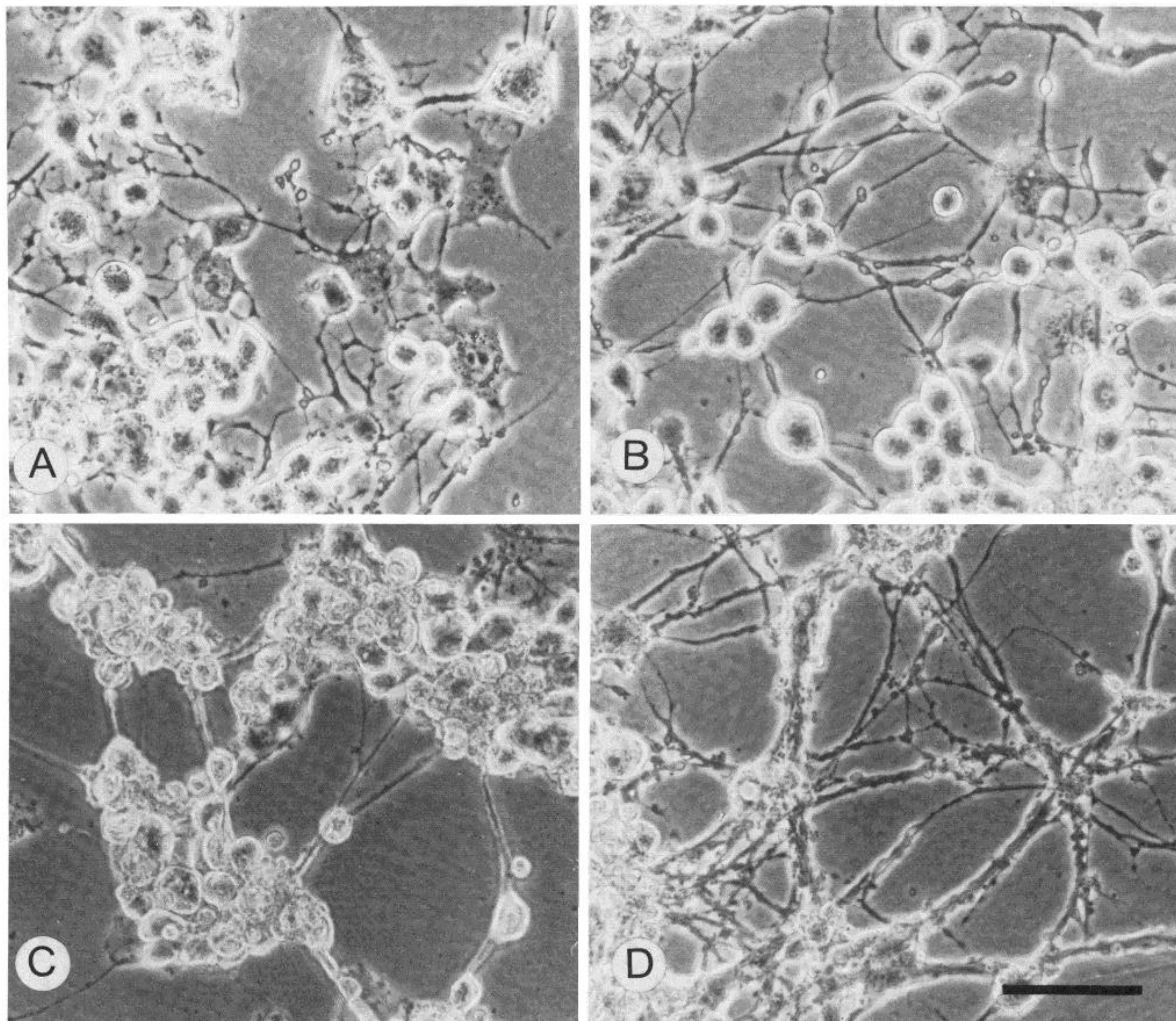

Figure 2. Morphological comparison of 8-BrcAMP-, PGE_1/IBMX-, and araC-treated NG108-15 cells. Phase contrast micrographs of NG108-15 cells grown in serum-supplemented medium as described under "Materials and Methods," which were treated with 1.0 mM 8-BrcAMP (*A*) or PGE_1 plus IBMX (*B*) for 5 days. Additional cultures, grown in the presence (*D*) or absence (*C*) of dBcAMP, were treated for 4 days with araC. *Bar,* 100 μm.

drastically reduced cellular division, only modestly increased the volume fraction of stained cells to 0.36. The stained cells were often isolated and had large indented nuclei (Fig. 6*A*).

After PGE_1 plus IBMX treatment for 5 days, the volume fraction of stained cells rose to levels slightly higher than those seen after similar dBcAMP and 8-BrcAMP treatments. Intense staining was localized in the large rounded cell bodies and throughout the long processes, varicosities, and cytoplasmic droplets (Fig. 7*A*).

Figure 8 compares the time course of increases in volume fraction of stained cells induced to differentiate with various treatments. This time course was identical for 8-BrcAMP and dBcAMP treatments. The time course for NaB treatment, however, was quite different. Within 24 hr of the initiation of the NaB treatment, the volume fraction of stained cells dropped to 0.17 from approximately 0.29 in untreated cells. After 3 days of treatment the volume fraction returned to normal levels and by day 5 rose to approximately 0.36. The time course of increase in the volume fraction following PGE_1 treatment was somewhat accelerated compared to dBcAMP and 8-BrcAMP treatments, reaching a slightly higher V_v value of 0.75 after 5 days.

When previously untreated cultures were exposed to araC and immunocytochemically stained using anti-Met^5-enkephalin, a very intense stain was localized in the cytoplasm of the large, rounded cell bodies and short processes. An identical, but less intense, staining pattern was seen when araC-treated cells were stained with anti-Leu^5-enkephalin (data not shown). In cultures first treated with dBcAMP for 5 days, then with araC, very intense enkephalin-like immunoreactivity was found in the cell bodies and throughout the exposed extensive network of processes with adhering cytoplasmic droplets

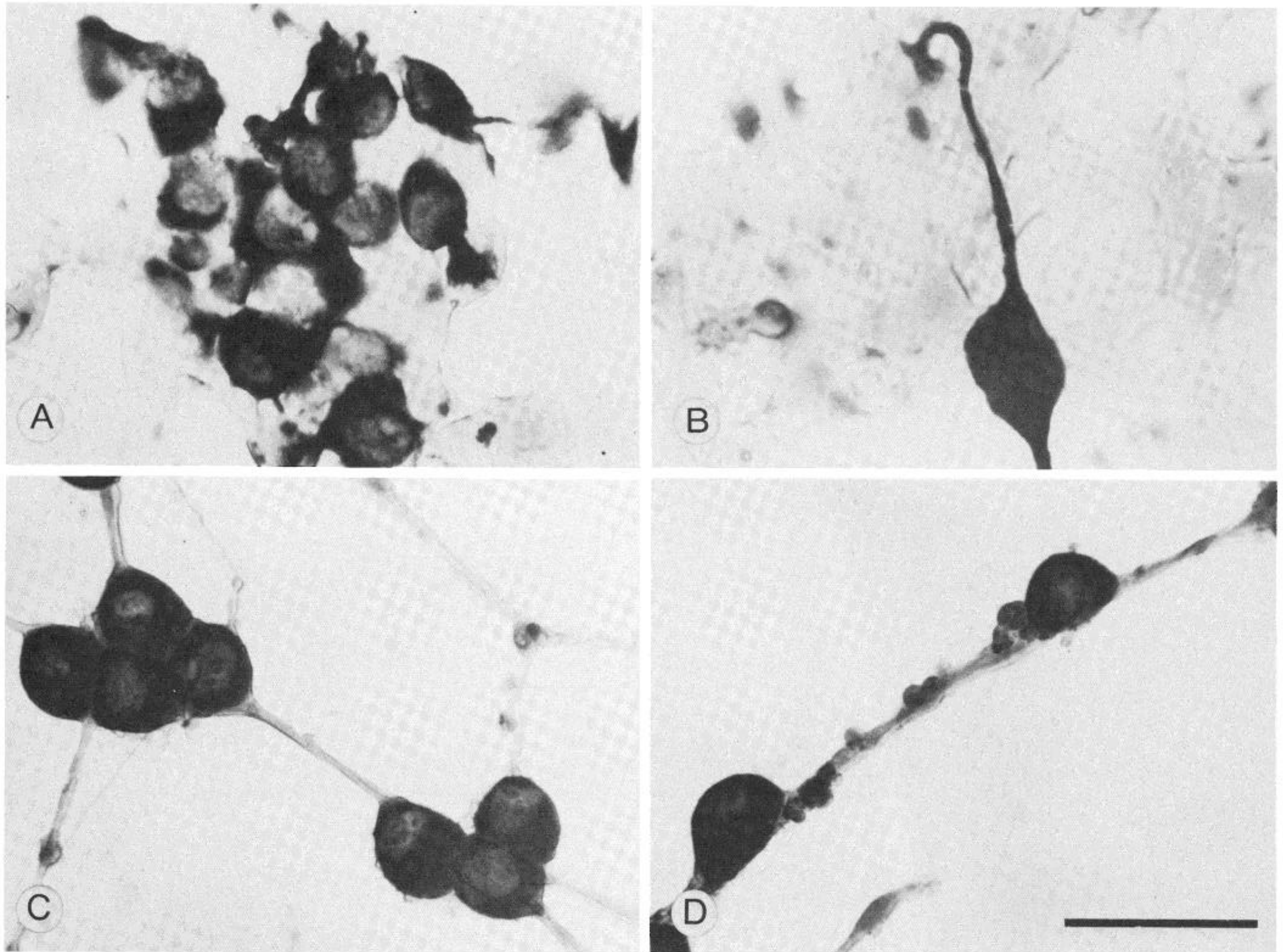

Figure 3. Immunocytochemical staining of NG108-15 cells for enkephalin in untreated and dBcAMP-treated cultures. NG108-15 cells grown in serum-supplemented medium were inoculated at an initial density of 4,000 cells/cm^2 and cultured in the presence of no drugs (*A* and *B*) or in 1.0 mM dBcAMP for 5 (*C*) or 7 (*D*) days beginning 2 days after plating. The cells were then rinsed, fixed with 2.5% glutaraldehyde, and stained using 1:10,000 anti-Met^5-enkephalin (*A, B, D*) or anti-Leu^5-enkephalin (*C*) with the peroxidase-antiperoxidase complex immunocytochemical technique as described under "Materials and Methods." Note the intense enkephalin-like immunoreactivity in the cell bodies and processes of a select population of cells and the absence of stain in the surrounding cells. *Bar,* 100 μm.

(Fig. 7*B*). In all cultures treated with araC, the volume fractions of stained cells were greater than 0.95.

NG108-15 cells grown in defined medium. NG108-15 cells were also cultured in serum-free defined medium in order to minimize the unknown effects of serum components and to decrease the possibility that some of the cellular stain was due to transport of enkephalins present in the serum. Untreated NG108-15 cells grown in defined medium expressed the same anti-Met^5-enkephalin and anti-Leu^5-enkephalin staining pattern as cells grown in serum-supplemented medium (data not shown).

Following 5 days of 1.0 mM dBcAMP treatment, enkephalin-like immunoreactivity was found in the cell bodies, often found grouped together, and throughout the long axon-like processes (Fig. 6*B*). The volume fraction of stained cells increased to 0.67 after 5 days of dBcAMP treatment, similar to cells grown in serum-supplemented medium. NaB treatment had very different effects on the morphology of NG108-15 cells grown in serum-supplemented and defined medium, such that nearly all cells cultured in defined medium emitted extremely long processes by 5 days of NaB treatment. Intense stain was localized to the cytoplasm of the rounded cell bodies, processes, and adhering cytoplasmic droplets with a volume fraction of stained cells of over 0.93 (Fig. 6*C*).

Pure cultures of differentiated NG108-15 cells were obtained from cells grown in defined medium with or without dBcAMP by further treatment with araC for 4 days. Although the cells did not form the tight, large aggregates found when cells grown in serum-supplemented medium were treated with araC, similar staining patterns were observed (data not shown).

Figure 9 compares the volume fractions of stained cells grown in serum-supplemented medium with cells grown in serum-free defined medium after various treatments. The volume fractions of cells stained using anti-Met^5-enkephalin or anti-Leu^5-enkephalin were all approximately 0.29 for cells cultured in both media, and they

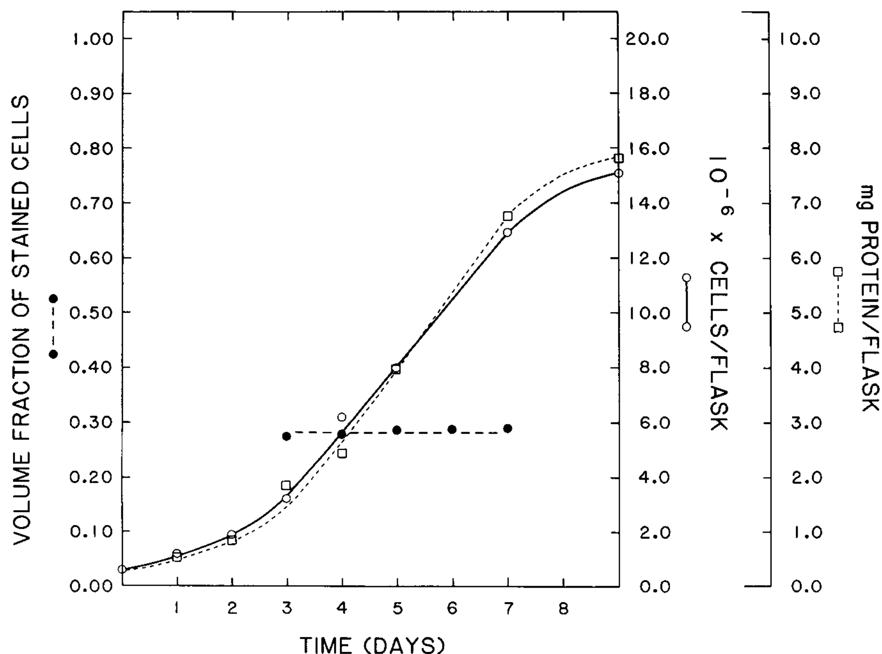

Figure 4. Comparison of the growth curve, protein content, and volume fraction of stained NG108-15 cells grown in serum-supplemented medium. NG108-15 cells were inoculated at an initial density of 4,000 cells/cm^2 into 75-cm^2 flasks (300,000 cells) for enumeration and protein assay, or into 25-cm^2 flasks (100,000 cells) for immunocytochemistry ($n = 3$). Cells were harvested daily for counting and for assay of protein content. In addition, cells in 25-cm^2 flasks were processed as described in Figure 3, using 1:10,000 anti-Met^5-enkephalin, and the volume fraction of stained cells was determined morphometrically.

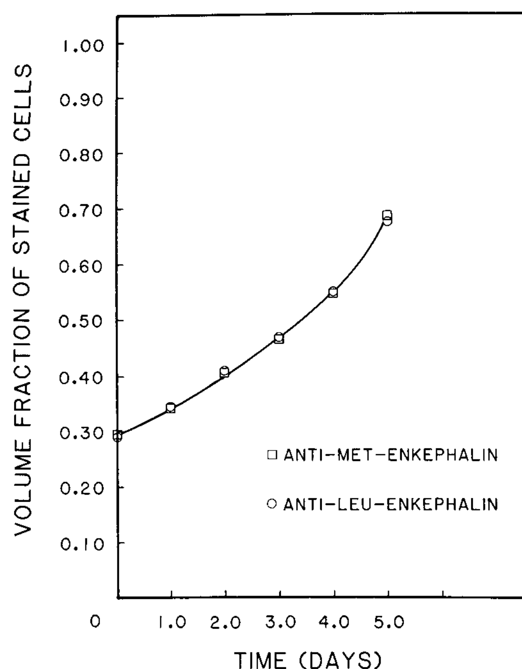

Figure 5. Time course of the volume fraction of stained cells treated with dBcAMP. NG108-15 cells grown in serum-supplemented medium were treated with 1.0 mM dBcAMP for 1 to 5 days beginning 2 to 6 days after plating. Following treatment, all cells, at day 7 after initial plating, were rinsed, fixed, and stained, using 1:10,000 anti-Met^5-enkephalin or anti-Leu^5-enkephalin, as described for Figure 3 ($n = 4$). The volume fraction of stained cells was then determined morphometrically.

increased to approximately 0.66 to 0.68 after 5 days of dBcAMP treatment. On the other hand, the volume fraction of stained cells after 5 days of NaB treatment was a modest 0.36 in cultures grown in serum-supplemented medium, whereas it increased to 0.93 for cells grown in defined medium. When previously untreated cells were exposed to araC, the volume fractions of stained cells were greater then 0.90 in all cases. Cultures treated with dBcAMP for 5 days, followed by araC treatment, all showed volume fractions of stained cells of greater than 0.95.

Staining controls. Solid phase immunoabsorption specificity tests were performed to remove the antibodies from the antiserum to avoid potential binding of enkephalin to cellular binding sites. Staining was reduced to background levels when cells were incubated with antiserum preabsorbed with enkephalin-conjugated Sepharose 4B beads (Fig. 10); however, staining remained intense when antiserum was preabsorbed with control ethanolamine-conjugated beads (data not shown). In addition, staining was absent when normal rabbit serum replaced the primary antibodies, or when cultures were stained with primary antibody, goat anti-rabbit IgG, or PAP complex omitted (data not shown).

Enkephalin levels in NG108-15 cells

In order to help confirm the presence of enkephalin-like activity in NG108-15 cells and its increase in treated cells, acid extracts of cells grown in serum-supplemented medium were analyzed after separation by reverse phase

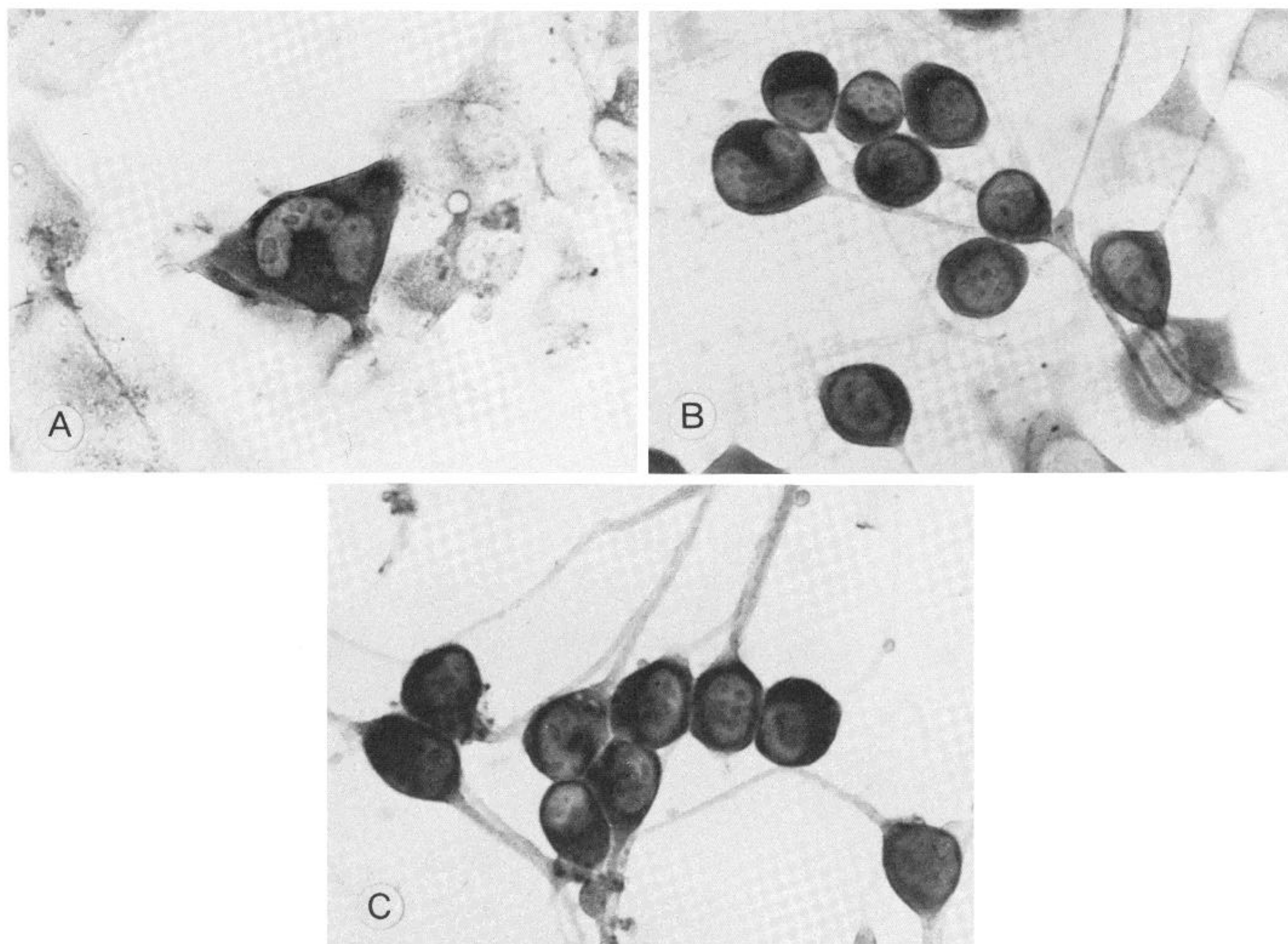

Figure 6. Immunocytochemical staining of NG108-15 cells for enkephalin in NaB- and dBcAMP-treated cultures. NG108-15 cells grown in serum-supplemented medium (*A*) or serum-free defined medium (*B* and *C*) were cultured in the presence of 2.0 mM NaB (*A* and *C*) or 1.0 mM dBcAMP (*B*) for 5 days beginning 2 days after plating. The cells were then stained as described in Figure 3 using 1:10,000 anti-Met^5-enkephalin.

HPLC. Fractions were assayed for enkephalin activity by radioreceptor assay, to provide independent (i.e., non-immunologic) measurements of biological activity. Analysis of HPLC elution patterns (Fig. 11) confirmed the presence of both Met^5-enkephalin- (at 33 min) and Leu^5-enkephalin-like (at 38 min) activities in all cell extracts. In many extracts a smaller peak of activity eluted near 24 min; this peak may represent oxidized Met^5-enkephalin since it also appeared during elution of authentic Met^5-enkephalin, but not of Leu^5-enkephalin (data not shown). Figure 11 also compares enkephalin peaks in control untreated and 5-day dBcAMP-treated cells. Despite the lower number of cells in the dBcAMP-treated sample (100×10^6 cells dBcAMP treated versus 205×10^6 cells untreated), both Met^5- and Leu^5-enkephalin peaks were clearly higher in the treated extract. Figure 12 shows the cellular concentrations of enkephalin equivalents present in these untreated versus 5-day dBcAMP-treated cells. The concentrations of both enkephalin equivalents in cells treated with dBcAMP increased to approximately 2 times the levels found in untreated cells. In addition, similar levels of enkephalin equivalents were found in untreated cells grown in either serum-supplemented or defined medium (data not shown). Immunocytochemical staining data at both the light and electron microscopic levels, as well as preliminary data from HPLC and radioreceptor assay analysis, indicated that treatment of NG108-15 cells with araC increased the cellular concentration of Met^5-enkephalin equivalents to over 5 times basal levels, while the level of Leu^5-enkephalin equivalents remained approximately the same (data not shown).

Discussion

NG108-15 cells can be induced by various agents to undergo morphological, biochemical, and physiological differentiation similar to many other neuronal cell lines. Biochemical differentiation in NG108-15 cells included increases in specific neurotransmitter enzyme activities such as acetylcholinesterase and choline acetyltransferase, while physiologically, the cells exhibit increased membrane excitability and stimulus-dependent release

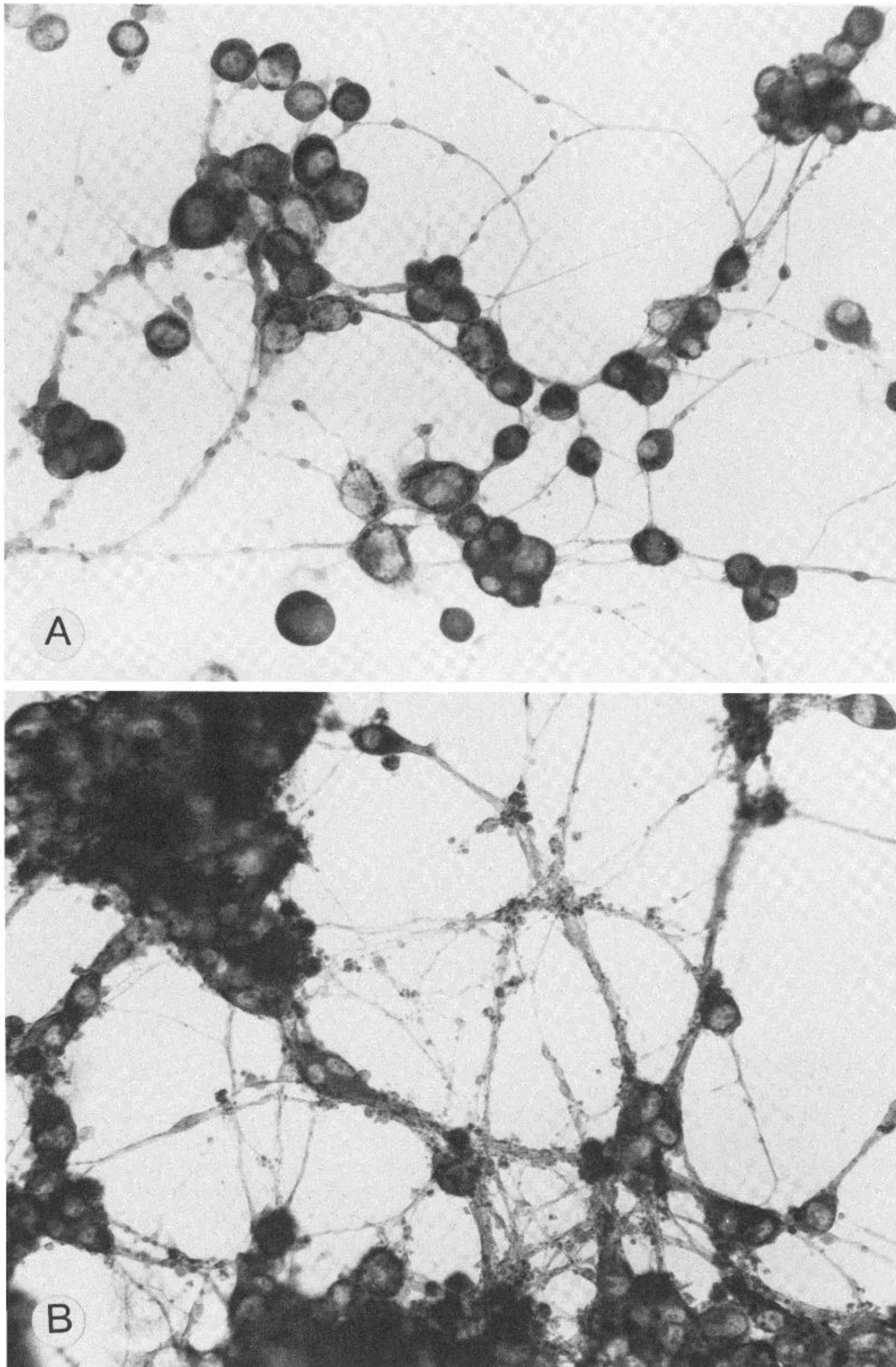

Figure 7. Immunocytochemical staining of NG108-15 cells for enkephalin in PGE_1/IBMX- and dBcAMP/araC-treated cultures. NG108-15 cells grown in serum-supplemented medium were cultured in the presence of 10 μM PGE_1 plus 100 μM IBMX for 5 days beginning 2 days after plating (*A*). Cultures grown in the presence of 1.0 mM dBcAMP for 5 days were subsequently treated for 4 days with araC (*B*). The cells were then processed as described in Figure 3, using 1:10,000 anti-Met^5-enkephalin.

of acetylcholine (Hamprecht, 1977; McGee et al., 1978). Using the peroxidase-antiperoxidase immunocytochemical technique, the results presented in this study confirm earlier biochemical reports of enkephalin-like peptides in NG108-15 cells (Glaser et al., 1980, 1982) and indicate that the levels of endogenous opioid peptides may be correlated to the state of differentiation of the cells.

In untreated NG108-15 cells grown in serum-supple-

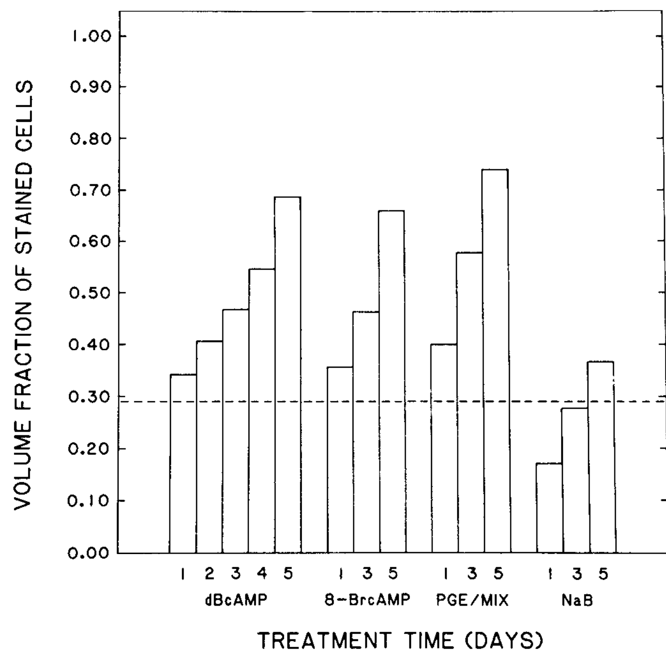

Figure 8. Comparison of the time courses of the volume fractions of stained cells induced to differentiate with various drugs. NG108-15 cells grown in serum-supplemented medium were treated with 1.0 mM dBcAMP ($n = 4$), 1.0 mM 8-BrcAMP ($n = 2$), 10 μM PGE_1 plus 100 μM IBMX ($n = 3$), or 2.0 mM NaB ($n = 3$) for 1 to 5 days beginning 2 to 6 days after plating. The cells, at day 7 after plating, were then rinsed, fixed, and stained using 1:10,000 anti-Met^5-enkephalin as described in Figure 3. The data demonstrate the volume fraction of stained cells determined morphometrically. – – –, volume fraction of stained cells in untreated cultures ($n = 5$).

mented medium, enkephalin-like immunoreactivity was found throughout the cytoplasm of the cell bodies and short processes of approximately 0.30 (30%) of the volume fraction of stained cells. The volume fraction of stained untreated cells remained constant from day 3 through day 7 of culture and was similar for both Met^5- and Leu^5-enkephalin antisera. In addition, biochemical determinations using radioreceptor assays of acid extracts after separation by reverse phase HPLC revealed approximately the same cellular concentrations of both peptides. This is in contrast to Glaser et al. (1982), who reported a Met:Leu concentration ratio in NG108-15 cells of 3 or 4:1, where peptide content was measured by radioimmunoassay and bioassay. A similar heterogeneous staining pattern was observed in the mouse neuroblastoma N1E-115 cell line, where approximately 10% of the stationary phase cells were labeled with anti-Met^5-enkephalin by indirect immunofluorescence (Knodel and Richelson, 1980). The heterogeneous staining patterns in the NG108-15 and N1E-115 cell lines appear to correlate with the cellular morphology heterogeneity, and may be due to inherent differences in the state of differentiation of untreated cells.

Treatment of NG108-15 cells grown in serum-supplemented medium with the cAMP analogues dBcAMP or 8-BrcAMP stimulated neurite extension in a manner characteristic of the differentiation process observed with other neuronal cell lines. The morphological differentiation produced by these agents was paralleled by a time-dependent increase in the immunocytochemical staining. The volume fractions of cells containing Met^5- or Leu^5-enkephalin-like immunoreactivity increased over 2-fold, to approximately 0.65 to 0.70 after 5 days of treatment. Intense staining was localized throughout the cell bodies, the extensive networks of processes, and the cytoplasmic droplets and varicosities which appeared along the processes and cell bodies. Similar staining patterns and slightly greater volume fractions of stained cells were observed with receptor-mediated stimulation of cell membrane adenylate cyclase by PGE_1 in the presence of IBMX. Treatment of NG108-15 cells for 5 days with IBMX alone resulted in only a modest increase in the stained cell volume fraction (data not shown). These light microscopic staining patterns for endogenous opioid peptides in NG108-15 cells induced to differentiate are similar to the cellular enkephalin staining patterns observed in mature neurons of numerous regions in the central nervous system (see review by Miller and Pickel, 1980) and in primary neuronal cell cultures (Neale et al., 1978; Knodel and Richelson, 1980; Wayhenmeyer et al., 1980).

Since sodium butyrate can be formed from the degradation of dBcAMP and may be a contaminant in commercially prepared dBcAMP (Kaukel and Hilz, 1972; Wright, 1973), it was used as a control treatment. Previous reports have demonstrated that many of the effects seen with dBcAMP treatment, including changes in neurotransmitter enzyme activities, can also be produced in neuroblastoma and other cell lines by sodium butyrate (Prasad and Vernadakis, 1972; Waymire et al., 1972; Richelson, 1973). Sodium butyrate treatment of NG108-15 cells cultured in serum-supplemented medium dramatically inhibited cellular division and did not induce morphological differentiation in a manner analogous to other neuronal cell lines (Prasad and Hsie, 1971; Waymire et al., 1972; Richelson, 1973). In addition, only a modest increase in the volume fraction of stained cells was observed in cells treated with sodium butyrate for up to 5 days.

The application of postembedding peroxidase-antiperoxidase immunocytochemical technique at the ultrastructural level demonstrated staining in the sparse, large dense core vesicles found throughout the cytoplasm and short processes of select untreated NG108-15 cells. Cells induced to differentiate with various agents for 5 to 21 days showed the ultrastructural characteristics of mature neurons and also increased the enkephalin-like immunoreactivity in the numerous large dense core vesicles throughout the cell bodies and neurite-like processes, providing additional evidence of increased levels of the endogenous opioid peptides in differentiated cells (K. M. Braas, S. R. Childers, and D. C. U'Prichard, manuscript in preparation). Similar vesicular enkephalin-like immunoreactivity has been demonstrated by pre-embedding electron microscopic staining in the rat midbrain and neostriatum (Pickel et al., 1979, 1980) and the primate basal ganglia (DiFiglia et al., 1982). Evidence from both pre- and postembedding electron microscopic immunocytochemical studies of NG108-15 cells also indicated staining in various other organelles, including the endoplasmic reticulum and Golgi complex, and in patches along the cell membranes.

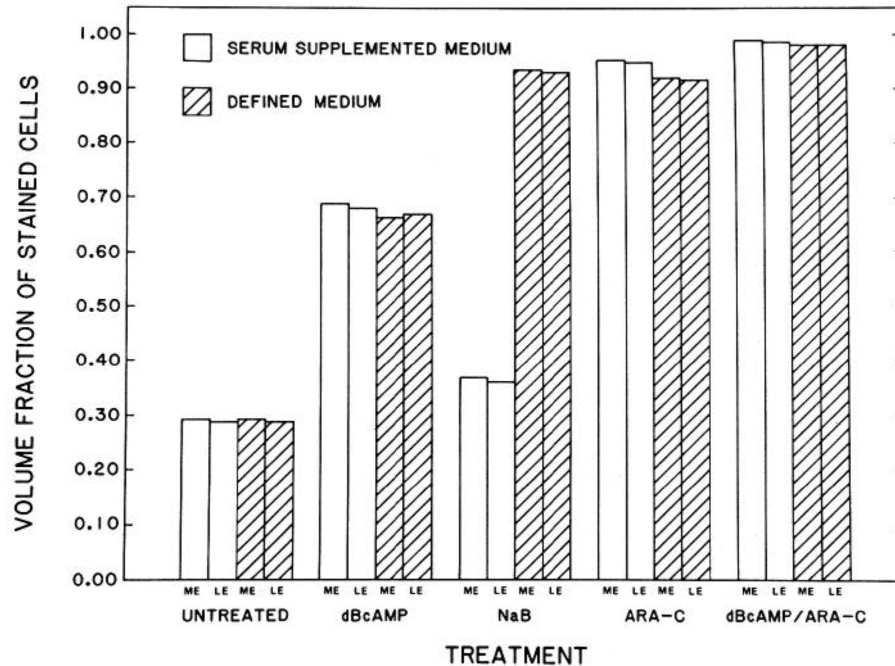

Figure 9. Comparison of the volume fractions of stained untreated and treated NG108-15 cells grown in serum-supplemented or serum-free defined medium. NG108-15 cells cultured in serum-supplemented or serum-free defined medium were treated with 1.0 mM dBcAMP ($n = 3$) or 2.0 mM NaB ($n = 3$) for 5 days beginning 2 days after plating. Untreated cells received no drug addition ($n = 5$). Cultures of untreated and 5-day 1.0 mM dBcAMP-treated cells were further treated for 4 days with araC ($n = 4$). The cells were then treated as for Figure 3, using 1:10,000 anti-Met^5-enkephalin (*ME*) or anti-Leu^5-enkephalin (*LE*). Data represent the volume fraction of stained cells determined morphometrically.

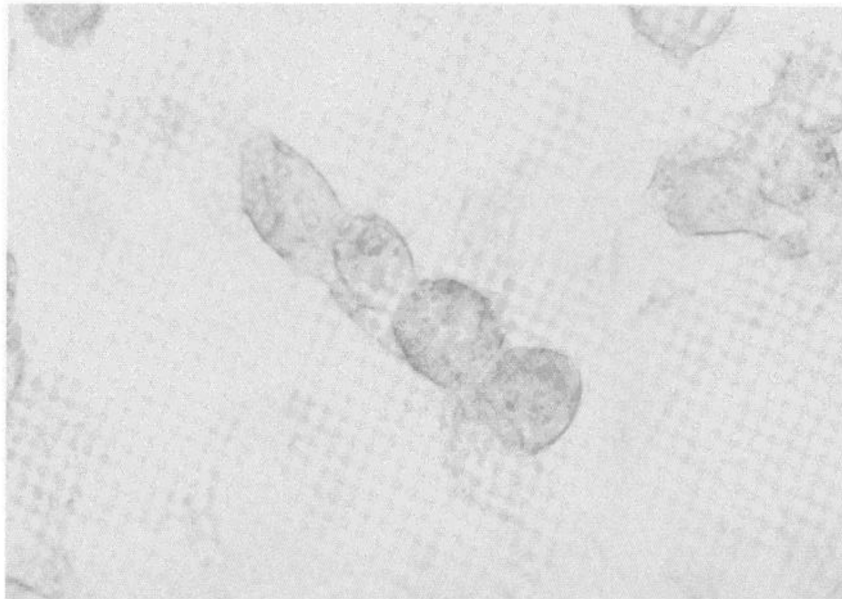

Figure 10. Solid phase immunoabsorption control. Untreated NG108-15 cells grown in serum-supplemented medium were stained as described in Figure 3, using anti-Leu^5-enkephalin preabsorbed with Leu^5-enkephalin-conjugated Sepharose 4B beads for 48 hr at 4°C.

Unlike cells in culture, neurons in the central nervous system of intact animals do not come in direct contact with serum. In addition, the newborn calf serum added to the cell culture medium contains many unknown components, possibly including opioid peptides, which vary from lot to lot. To reduce the unknown cellular environment, NG108-15 cells were also cultured in serum-free defined medium. Untreated and dBcAMP-

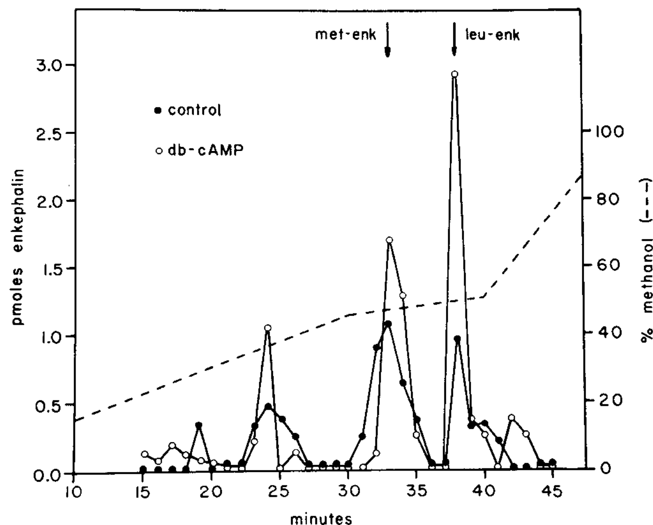

Figure 11. Separation of opioid peptides from NG108-15 cell extracts by HPLC. Lyophilized acid extracts of frozen untreated cells (205×10^6 cells; 160 mg of protein) or 5-day dBcAMP-treated cells (100×10^6 cells; 80 mg of protein) grown in serum-supplemented medium were resuspended in 250 μl of 10 mM ammonium acetate, pH 4.15, and eluted on a Lichrosorb C-8 reverse phase column by a discontinuous gradient of methanol in 10 mM ammonium acetate, pH 4.15 (flow rate: 1.0 ml/min). One-milliliter fractions were lyophilized, resuspended to 250 μl, and assayed for enkephalin activity by displacement of D-[^{3}H]Ala^2, D-Leu^5-enkephalin binding to rat brain membranes as described under "Materials and Methods" (0.2 pmol limit of detection). Data represent picomoles of Met^5-enkephalin equivalents for fractions 15 to 36 and 41 to 50, and picomoles of Leu^5-enkephalin equivalents for fractions 37 to 40, for duplicate 100-μl aliquots (n = 6, untreated cells; n = 3, dBcAMP-treated cells).

treated cells grown in defined medium expressed the same staining patterns and volume fractions of stained cells for both enkephalin antisera as their serum-supplemented counterparts. However, NaB treatment of cells grown in defined medium caused extensive process formation and increased the volume fraction of stained cells over 3-fold. This disparity between defined and serum-supplemented medium NaB effects could be due to differences in the lipid content of the cell membranes or differences in the interactions of medium components with NaB, altering the concentration and/or form of the NaB exposed to the cells. Glazer and Schnieder (1975) reported morphological and enzyme activity changes with lower NaB concentrations which were absent at higher concentrations. Therefore, a change in the effective dose of NaB may have produced the vastly different effects seen in the NG108-15 cells.

Cultures of pure differentiated cells were obtained by further treatment of untreated or 5-day dBcAMP-treated cells with araC, which is cytotoxic to proliferating cells. In cells cultured in either serum-supplemented or defined medium, very intense Met^5-enkephalin-like immunoreactivity was localized to a stained cell volume fraction of greater than 0.90. Although similar volume fractions were obtained with the anti-Leu^5-enkephalin, the staining intensity was observed to be much lower. Likewise, preliminary biochemical assays indicated a selective increase in cellular Met^5-enkephalin-like activity content.

The same variation in staining intensities between the two antisera was also observed on serial ultrathin sections of NG108-15 cells at the electron microscopic level (K. M. Braas, S. R. Childers, and D. C. U'Prichard, manuscript in preparation).

Staining was abolished when solid phase immunoabsorption specificity tests were performed, by preabsorption of antiserum with enkephalin-conjugated Sepharose 4B beads. In addition, absorption with β-endorphin, β-lipotropin, or ACTH failed to reduce staining at the electron microscopic level. Radioimmunoassay characterization of the Leu^5-enkephalin antiserum showed less than 4.0 and 0.01% cross-reactivity with Met^5-enkephalin and β-endorphin, respectively, and essentially no cross-reactivity with β-lipotropin or ACTH (Kubek and Wilber, 1980; J. F. Wilber, personal communication). The Met^5-enkephalin antiserum (Immuno Nuclear Corp.) has also been reported to exhibit very little cross-reactivity. Although cross-reactivity of the antisera with other related peptides not yet identified in NG108-15 cells cannot be eliminated, the presence of enkephalins has been demonstrated in these cells, using biochemical methods, by us and others (Glaser et al., 1982). Immunocytochemical staining of NG108-15 cells with anti-β-endorphin or anti-β-lipotropin resulted in weak to no staining (data not shown).

The presence of enkephalin-like activities in untreated NG108-15 cells, and their increase in treated cells, was confirmed by radioreceptor assay of acid extracts after separation by reverse phase HPLC. These activities had retention times that were identical to those of authentic Met^5- and Leu^5-enkephalin. The levels for both Met^5- and Leu^5-enkephalin equivalents from cells cultured in

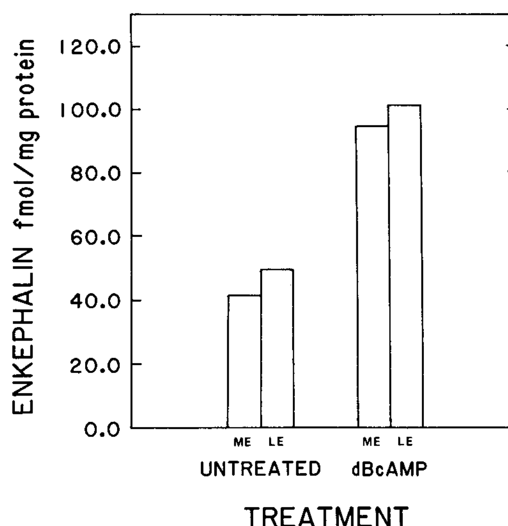

Figure 12. Enkephalin levels in untreated and dBcAMP-treated NG108-15 cells. Lyophilized extracts of untreated (n = 6) or 5-day dBcAMP-treated (n = 3) cells grown in serum-supplemented medium were separated by reverse phase HPLC and the fractions were assayed for enkephalin activity by radioreceptor assay (see Fig. 11). Data shown are from a typical experiment (same extracts as in Fig. 11) and represent femtomoles of Met^5-enkephalin equivalents or Leu^5-enkephalin equivalents per milligram of protein, summated from fractions 33 to 35 (Met^5-enkephalin) and 38 to 40 (Leu^5-enkephalin) indicated in Figure 11.

serum-supplemented medium of approximately 40 to 50 fmol/mg of protein were comparable to levels found in cells cultured in serum-free defined medium. Induction of differentiation with dBcAMP increased the levels of both enkephalin equivalents 2-fold, to approximately 95 to 100 fmol/mg of protein by 5 days of treatment, complementing the results obtained by immunocytochemistry. The figures for Met^5-enkephalin equivalents, however, represent minimum levels since they do not include possible contributions of oxidized Met^5-enkephalin. Preliminary results indicate that sodium butyrate-induced differentiation of cells cultured in serum-free defined medium, which increased the volume fraction of stained cells over 3-fold, resulted in even greater increases in the intracellular enkephalin equivalent concentrations. Glaser et al. (1982) recently reported that partially purified extracts of untreated NG108-15 cells contained between 30 and 100 fmol Leu^5-enkephalin equivalents/mg of protein as determined by radioimmunoassay. In addition, glucocorticoid treatment for 4 days specifically increased the opioid peptide levels 3- to 5-fold (Glaser et al., 1981).

The enkephalin precursor and the presence of additional peptides with opioid activities in the NG108-15 cells remain to be examined in future studies. Whereas preproenkephalin contains a Met^5-enkephalin:Leu^5-enkephalin ratio of 4:1 (Noda et al., 1982), the similar levels of Met^5- and Leu^5-enkephalin-like activities we observed in the NG108-15 cells could result from partial processing of this precursor, if present. Changes in the ratios of the enkephalin-like activities, as seen with araC treatment, could be due to alterations in peptide synthesis, degradation, processing, or modification. Alternately, the opioid peptides could be the products of a precursor other than preproenkephalin, or more than one precursor.

Gilbert et al. (1982) recently reported that stationary phase cells of the mouse neuroblastoma N1E-115 line, which show heterogeneous enkephalin staining patterns, contained approximately 135 fmol immunoreactive Met^5-enkephalin/mg of protein. We have extended these studies in N1E-115 cells using radioreceptor assays (Braas et al., 1982) and observed the same Met^5-enkephalin equivalent levels and similar levels of Leu^5-enkephalin equivalents, which are comparable to levels found in NG108-15 cells. Immunocytochemical staining also revealed that, as with the NG108-15 hybrid cell line, dBcAMP treatment of N1E-115 cells increased the volume fraction of cells staining for the endogenous opioid peptides. This same phenomenon was also observed in other neuronal cell lines including the mouse neuroblastomas N4TG-1 and N18TG-2 (parent of NG108-15) and the mouse neuroblastoma × hamster brain NCB-20 hybrid (Braas et al., 1982). Surprisingly, untreated C6BU-1 rat glioma cells (parent of NG108-15) exhibited low immunocytochemical staining, which greatly increased with dBcAMP treatment. A detailed comparison of enkephalin staining patterns, and the effects of differentiation status, in different neural cell lines, will be the subject of a separate communication. We are currently investigating the modulation of differentiation status and intracellular enkephalin content in neural cells in culture, by activation of cell surface neurotransmitter receptors.

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