Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2259–2263, June 1977
Biochemistry

Synthesis of opiate peptides by a clonal pituitary tumor cell line*

(adenylate cyclase/endorphin/enkephalin/β-lipotropin)

GABRIELLA GIAGNONI†, STEVEN L. SABOL, AND MARSHALL NIRENBERG

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014

Contributed by Marshall Nirenberg, March 10, 1977

ABSTRACT **Clonal mouse pituitary tumor cells, AtT-20, synthesize at least four species of peptides with opiate activity. The endorphin concentration of AtT-20 cells was estimated to be 300–600 pmol/mg of protein. The two most abundant endorphins with apparent molecular weights of 1800 and 2400 were purified 300- and 24-fold, respectively; additional minor components were found with apparent molecular weights of >3000 and <750.**

A recently discovered class of peptides, the endorphins, found in mammalian pituitary gland and in certain neurons, possess properties which resemble those of morphine and other opiates (1, 2). For example, endorphins act like opiates in tissue bioassays (1), bind to opiate receptors with high affinity (3), and are potent inhibitors of adenylate cyclase (4). The endorphins thus may regulate the responses of neurons with opiate receptors to various transmitters and hormones which activate adenylate cyclase.

A pituitary "prohormone," β-lipotropin (5), is thought to be a precursor of endorphins as well as β-melanotropin [(β-lipotropin-(41–58)]. The smallest peptide fragment that retains opiate activity is the pentapeptide methionine-enkephalin (1) with the sequence Tyr-Gly-Gly-Phe-Met, which corresponds to β-lipotropin-(61–65). A similar pentapeptide with a carboxy-terminal leucyl rather than methionyl residue, leucine-enkephalin, is present in brain and possesses opiate activity. Peptides of longer chain length, such as α-, γ- and β-endorphin which correspond to β-lipotropin-(61–76), β-lipotropin-(61–77), and β-lipotropin-(61–91), respectively, also possess high opiate activity (6–9).

In this study, various cell lines were examined for their ability to synthesize endorphins. A clonal line of mouse pituitary cells, AtT-20, known to synthesize corticotropin (ACTH) (10) and peptides with melanotropin activity (11), was shown to synthesize at least four species of endorphins.

MATERIALS AND METHODS

The following chemicals were kind donations: normorphine, Everett May; Met-enkephalin (synthetic), Werner Klee; Leu-enkephalin, S. Snyder; prostaglandin E_1 (PGE_1), John Pike (Upjohn Co.); naloxone, Endo Laboratories; RO-20-1724, Hoffmann-La Roche Inc.; and ACTH-(1–24), CIBA-Geigy Corporation. Synthetic α-endorphin and camel β-endorphin were obtained from Peninsula Labs.

Cell Growth. AtT-20 cells, subcultures 41 and 47, obtained from the American Type Culture Collection (no. CCL89), Rockville, MD, were grown in a humidified atmosphere of 10% CO_2/90% air in the Dulbecco-Vogt modification of Eagle's minimal essential medium supplemented with either 10% fetal bovine serum and 5% heat-inactivated horse serum or 2.5% fetal bovine serum and 15% heat-inactivated horse serum, in stationary Falcon flasks or stirred at 96 rpm in 1-liter spinner bottles. In both types of vessels the cells form aggregates that do not adhere to the vessel surface. Pleuropneumonia-like organisms were not detected in cells or medium.

NG108-15 hybrid cells were grown as described (12). For best results, the pH of the growth medium was kept within 7.2–7.4, especially 48 hr before harvest of cells.

Endorphin Extraction. AtT-20 cells were harvested by centrifugation at 160 × *g* for 5 min. The pellets were washed three times by suspension in 25 volumes of DI solution (152 mM NaCl/5.4 mM KCl/0.17 mM Na_2HPO_4/0.22 mM KH_2PO_4/24 mM glucose, pH 6.7) and centrifugation. The washed pellets, 9×10^8 cells per g wet weight, were suspended in 10 volumes of water and immersed in a boiling-water bath for 15 min. The suspension was cooled in ice and centrifuged at 100,000 × *g* for 1 hr at 4°; the supernatant fraction, termed "boiled extract," was utilized as a source of endorphins. Protein was determined by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

Adenylate Cyclase Assay for Opiate Activity. PGE_1-stimulated adenylate cyclase activity was determined as described (14). The assay mixture (100 μl) was 30 mM Tris·HCl, pH 7.8/5 mM magnesium acetate/1 mM $MnCl_2$/50 mM sucrose/10 μM PGE_1/1 mM [α-^{32}P]ATP, sodium salt (3×10^6 cpm)/1 mM adenosine 3′:5′-cyclic monophosphate (cAMP), sodium salt/0.25 mM RO-20-1724/20 mM creatine phosphate, sodium salt/10 units of creatine phosphokinase (65 μg of protein)/75–100 μg of NG108-15 homogenate protein. Incubation was for 5 min at 37°. Duplicate values generally differed by <5%. For assay of opiate activity in cell extracts, the extract was added to the reaction mixture and the difference in adenylate cyclase activity due to naloxone (100 μM) was divided by the activity in the presence of naloxone, and this percentage was compared to a standard concentration curve of percentage inhibition of adenylate cyclase activity by Met-enkephalin. One Met-enkephalin unit is defined as the naloxone-reversed inhibition of PGE_1-stimulated adenylate cyclase activity due to 1 pmol of Met-enkephalin (10 nM, final concentration).

Bioassay of Opiate Activity. Inhibition, by opiates, of electrically induced contractions of the guinea pig ileum–myenteric plexus preparation was measured as described by Paton (15). Each ileum strip was suspended in 3 ml of modified Krebs solution (16) at 37° and gassed with 95% O_2/5% CO_2. Rectilinear electric pulses (60 V, 0.4 msec) were applied every 10 sec by a Grass stimulator, and contractions were measured by a Statham isometric transducer coupled to a polygraph.

RESULTS

Extracts from 33 cell lines were prepared and assayed for opiate activity by determining their effects on PGE_1-stimulated

Abbreviations: ACTH, corticotropin; PGE_1, prostaglandin E_1; cAMP, adenosine 3′:5′-cyclic monophosphate.

* Preliminary results were presented at the American Society for Neurochemistry meeting, Denver, CO, March 14–17, 1977.

† Guest worker, Laboratory of Biochemical Genetics, from the Institute of Pharmacology, University of Milan, Milan, Italy.

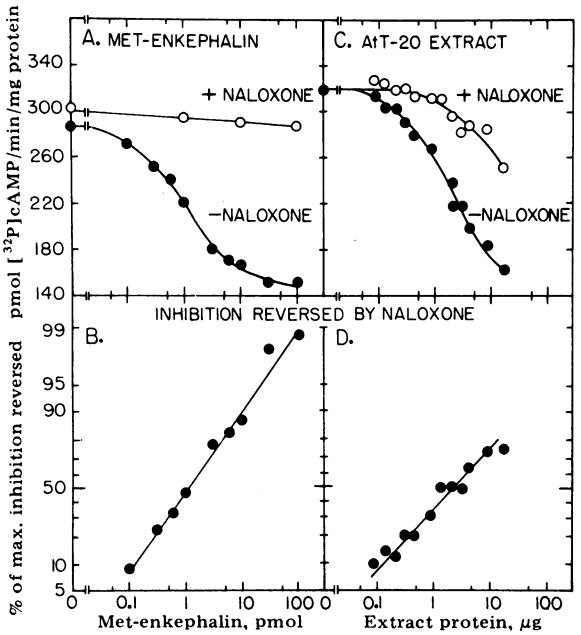

FIG. 1. The inhibition of PGE_1-stimulated NG108-15 adenylate cyclase activity by Met-enkephalin (*A* and *B*) and by boiled AtT-20 extract (*C* and *D*). (*Upper*). Specific activity of PGE_1-stimulated adenylate cyclase with and without 100 μM naloxone·HCl. The best fit for maximum inhibition of adenylate cyclase activity in *A* was found by computer to be 48%. (*Lower*) Logit-log plots of same data with opiate activity expressed as the percentage of maximum naloxone-reversed inhibition of adenylate cyclase activity, which was assumed to be 48%.

adenylate cyclase activity in NG108-15 homogenates (12, 14) in the presence and absence of 100 μM naloxone, which competitively inhibits opiate binding to the opiate receptor. Opiate receptor-mediated inhibition is reversed by naloxone and thus can be distinguished from nonspecific inhibition. Opiate activity was found in extracts from clonal mouse pituitary tumor cells (AtT-20) but not in extracts from 32 other cell lines.‡ The inhibition of PGE_1-stimulated adenylate cyclase activity by different concentrations of Met-enkephalin or boiled AtT-20 extract is shown in Fig. 1. Half-maximal inhibition of adenylate cyclase activity was obtained with 1.1 pmol (11 nM) of Met-enkephalin, and the inhibition was completely reversed by 100 μM naloxone (Fig. 1*A*). Half-maximal inhibition also was obtained with 8.9 pmol (89 nM) of α-endorphin or 6.3 pmol (63 nM) of β-endorphin (not shown). These values confirm those previously found by Werner Klee (personal communication) under slightly different assay conditions. In Fig. 1*B*, the logit of the percentage of maximum inhibition of adenylate cyclase activity reversed by naloxone was shown to be a linear function of the logarithm of the Met-enkephalin concentration over the range 0.1–100 pmol. A boiled extract from AtT-20 cells also inhibited adenylate cyclase activity (Fig. 1*C*) and part of the inhibition was reversed by naloxone; thus, both opiate-specific and nonspecific inhibitions were seen. The opiate-specific inhibition was a linear function of AtT-20 extract protein concentration on a logit-log plot (Fig. 1*D*), but the slope of the line was slightly lower than that observed with Met-enkephalin. Two micrograms of boiled AtT-20 extract protein was equivalent in activity to 1 pmol of Met-enkephalin (i.e. 1 Met-enkephalin unit). The concentration of endorphins in different preparations of AtT-20 cells was 40–100 Met-enkephalin units/mg of cell protein. Opiate activity was not detected in unused growth medium or serum.

‡ Opiate activity was not detected (<0.1–0.5 Met-enkephalin unit/mg of cell protein) in the following cell or hybrid lines: rat pituitary clone GH3; mouse neuroblastoma clones N10, NS20, NS20Y, N1E115, and N18TG2; rat glioma clone C6BU1; NG108-15; NX-31T; NCB-7, -10, -13, -15, -17, -20, -21, -22; NCFL-1, -2, -3, -4, -6, -7, -7A, -8, -10, -13, -13A, -15, -18, and -22; and NSC-12 (24).

Table 1. AtT-20 endorphin purification

Fraction	Units* × 10^{-3} Total	Units* × 10^{-3} Pooled	Protein, mg	Units* × 10^{-3} per mg protein	Endorphin, nmol per mg protein†
Experiment A					
Cells, 4.2 g	(39)	—	416.0	(0.09)	(0.6)
Boiled extract	39	—	25.8	1.50	10.2
Lyophilized	22	—	25.8	0.85	5.8
Bio-Gel P-4, Tris·HCl					
I	0.8	—	—	—	—
II (36–40)	5.1	2.4	0.94	2.5	14.3
III (46–50)	8.5	7.0	0.30	23.4	189.0
IV (79–81)	0.8	0.6	<0.09	>6.7	>6.7
Experiment B					
Cells, 11.4 g	(51)	—	1270	(0.04)	(0.3)
Boiled extract	51	—	85	0.6	4.0
2 M acetic acid					
Solution	30	—	18	1.7	11.0
Precipitate	6	—	67	0.09	0.6
Bio-Gel P-4, acetic acid					
II (34–37)	5.6	2.2	2.22	1.0	5.7
III (50–53)	13.2	3.6	0.71	5.0	40.8
IV (94–96)	2.8	0.5	1.27	0.4	0.4

* Met-enkephalin units.

† Met-enkephalin units were converted to picomoles of endorphin by assuming that the activities of endorphins II, III, and IV are similar to those of β-endorphin, α-endorphin, and Met-enkephalin, respectively, for inhibition of PGE_1-stimulated adenylate cyclase activity. One picomole of Met-enkephalin (1 unit) is thus equivalent to 8.1 pmol of α-endorphin or 5.7 pmol of β-endorphin. The recoveries were assumed to be the same for each species of endorphin.

AtT-20 cells have been shown to synthesize ACTH (10, 11). Although ACTH binds to opiate receptors with relatively low affinity (17), ACTH-(1–24) (0.3–30 μM) did not inhibit adenylate cyclase activity or block the inhibition of adenylate cyclase activity by opiates.

AtT-20 boiled extracts were fractionated on a Bio-Gel P-4 column equilibrated and eluted with 0.1 M Tris·HCl, pH 7.5 (Fig. 2*A*). Four species of endorphins (termed I, II, III, IV) were found. A portion of a boiled AtT-20 extract soluble in 2 M acetic acid also was fractionated on a Bio-Gel P-4 column with 2 M acetic acid (Fig. 2*B*) rather than Tris·HCl. Three peaks of endorphin activity were found. Endorphin I was not detected and thus may be insoluble in 2 M acetic acid. The mobility of endorphin IV was retarded compared to that shown in Fig. 2*A*, and the elution of Met-enkephalin was also retarded (not shown).

Comparison of elution volumes of AtT-20 endorphins in Tris·HCl buffer with those of standard peptides, assuming no adsorption effects, yielded tentative molecular weight estimates of >3000, 2400, 1800, and <750 for endorphins I–IV, respectively (not shown). Synthetic β-endorphin (apparent molecular weight 2300) eluted later than expected based on its known molecular weight, 3439, possibly because the molecules had a net positive charge and differed slightly in chromatographic mobility from endorphin II (Fig. 2*A*). Endorphin III had the

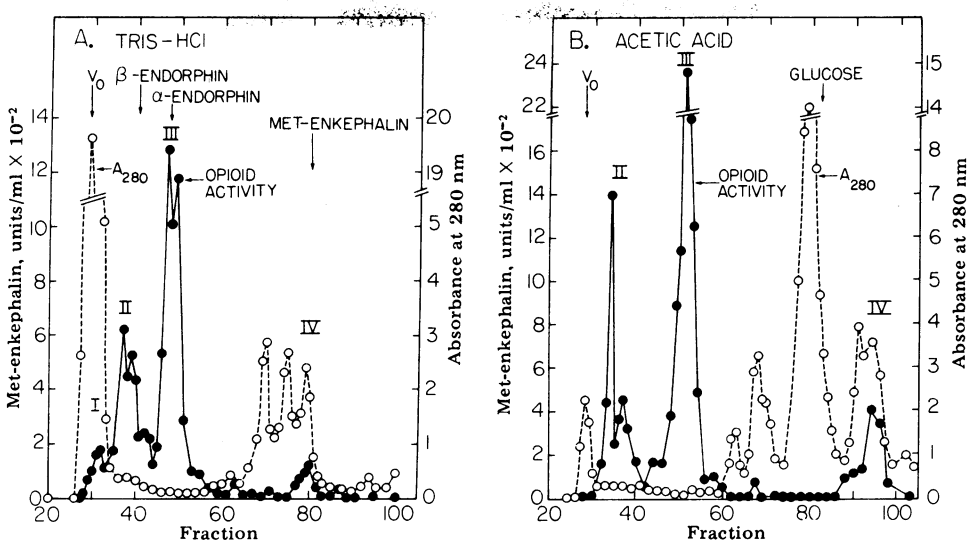

FIG. 2. Boiled AtT-20 extracts were fractionated on a Bio-Gel P-4 column (1.5 × 84 cm, 200–400 mesh) equilibrated and eluted with 0.1 M Tris·HCl, pH 7.5 (*A*) or 2 M acetic acid (*B*) at 4°. In *A*, 25.8 mg (39,000 Met-enkephalin units) of lyophilized boiled extract protein in 2 ml H_2O was applied to the column, and 1.5-ml fractions were collected in silicon-coated tubes and assayed for (●) opiate activity and (○) absorbance at 280 nm. The flow rate was 6 ml/hr. In *B*, 85 mg of lyophilized boiled extract was suspended in 2 ml of 2 M acetic acid and the 12,000 × *g* supernatant fraction containing 18 mg of protein and 30,000 Met-enkephalin units was applied to the column. Each 1.42-ml fraction was lyophilized, dissolved in 0.2 ml of 0.05 M Tris·HCl at pH 7, and assayed for opiate activity (●) and absorbance at 280 nm (○).

chromatographic mobility of synthetic α-endorphin. Thus, AtT-20 cells possess multiple species of molecules with opiate activity, and the most abundant forms resemble the larger endorphin peptides such as α-, β-, or γ-endorphin, while a minor fraction of the opioid peptides resembles the enkephalins.

Endorphin partial purifications are summarized in Table 1. The recovery of activity after Bio-Gel P-4 column chromatography was 39 and 42% for Experiments A and B, respectively. However, in Experiment B, 37–70% of endorphin activity was lost after column fractions were pooled. In Experiment A, after column chromatography, endorphins I–IV represented approximately 5, 34, 56, and 5%, respectively, of the total Met-enkephalin units. However, if one assumes that the activities of endorphins I + II and endorphin III are similar to those of β-endorphin and α-endorphin, respectively, endorphins I, II, III, and IV account for 5, 28, 66, and <1% of the total endorphins, respectively. Assuming that all endorphin species were recovered to the same extent during purification, one may calculate that the endorphin content of AtT-20 cells is about 0.6 and 0.3 nmol/mg of protein for Experiments A and B, respectively. Endorphins thus account for as much as 0.15% of the total cell protein.

The effects of proteolytic enzymes on partially purified AtT-20 endorphins are shown in Fig. 3. Peak II opiate activity was relatively resistant to leucine aminopeptidase, α-chymotrypsin, and carboxypeptidase A and was increased 4-fold by incubation with trypsin. Peak III was inactivated by leucine aminopeptidase and, to a limited extent, by chymotrypsin and its activity was increased by carboxypeptidase A and trypsin. Peak IV was completely inactivated by leucine aminopeptidase or chymotrypsin and was partially inactivated by carboxypeptidase A; trypsin decreased activity slightly. Thus, the AtT-20 opioid molecules are peptides that have different sensitivities to inactivation or activation by proteases. The sensitivity of peaks III and IV and Met-enkephalin to leucine aminopeptidase is consistent with a requirement for an amino-terminal tyrosine for opiate activity (18). However, peak II activity resembles camel β-endorphin in which the tyrosyl[1] residue is protected from aminopeptidase action (19). Treatment of peaks II and III with trypsin and peak II with carboxypeptidase A resulted in reaction products more active than the parent molecules. Chymotrypsin is known to inactivate β-endorphin (19); therefore, the resistance of peak II activity to chymotrypsin and the limited sensitivity of peak III activity are unexplained. Peak IV activity was more sensitive than Met-enkephalin to inactivation by chymotrypsin, but less sensitive to carboxypeptidase A; these results suggest that peak IV may contain one or more endorphins similar but not identical to Met-enkephalin.

Cyanogen bromide treatment of the boiled AtT-20 extract soluble in 2 M acetic acid resulted in the loss of 94% of the opiate activity (Table 2). Cyanogen bromide also inactivated Bio-Gel P-4 fractions corresponding to endorphins II–III (unresolved) and IV (data not shown). Thus, most or all of the AtT-20 opioid peptides possess a methionine residue that is essential for activity and thus resemble endorphins thought to be derived from β-lipotropin.

As shown in Fig. 4, purified AtT-20 opioid fractions inhibited the twitch amplitude of electrically stimulated guinea pig ileum contractions (15). Naloxone reversed the inhibitions 97, 95, and 68% for peak II, III, and IV activities, respectively. Thus, AtT-20

Table 2. Effect of cyanogen bromide on AtT-20 endorphin activity

Endorphin	Met-enkephalin units: Minus cyanogen bromide	Met-enkephalin units: Plus cyanogen bromide	%
Met-enkephalin	1150	38	3
Leu-enkephalin	460	600	130
AtT-20 extract	224	14	6

Met-enkephalin (1000 pmol), Leu-enkephalin (1000 pmol), or boiled AtT-20 extract protein (370 μg) soluble in 2 M acetic acid was incubated in 1 ml of 70% formic acid with or without 470 μmol of cyanogen bromide for 24 hr at 21°. Samples then were diluted with Tris·HCl, pH 8.5 (25 mM, final concentration), lyophilized three times, dissolved in water, and assayed for opioid activity.

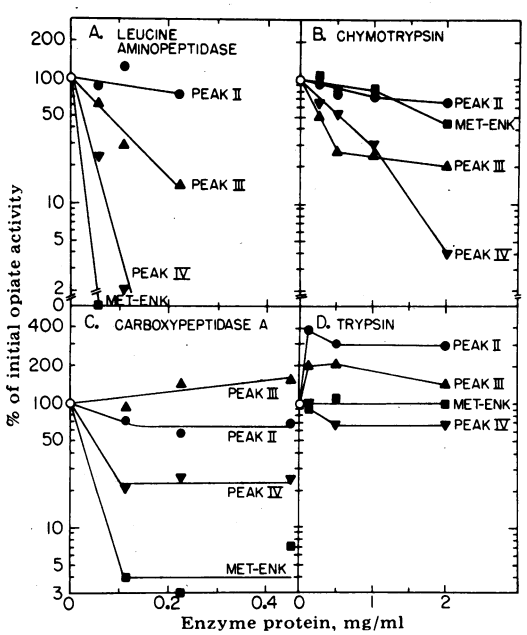

FIG. 3. The effects of proteolytic enzymes on the opiate activities of endorphin fractions eluted from the Bio-Gel P-4 column shown in Fig. 2*B* and Table 1, Experiment B. Symbols correspond to the following: ●, endorphin II (56 μg of protein); ▲, endorphin III (18 μg of protein); ▼, endorphin IV (43 μg of protein); and ■, Met-enkephalin (50 pmol). Reaction mixtures (25 μl) also contained 0.05 M Tris·HCl, pH 7.7, and were incubated for 2 hr at 37° and then for 15 min at 100°. Specific activities of proteases (Worthington) in units/mg of protein were as follows: leucine aminopeptidase, 227; α-chymotrypsin, 61; carboxypeptidase A (treated with phenylmethylsulfonyl fluoride), 47; and trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone), 203. Reaction mixtures with leucine aminopeptidase contained 2.5 mM $MgCl_2$, and those with carboxypeptidase A contained 0.24 M LiCl. One hundred percent corresponds to the Met-enkephalin units found after incubation without protease; averages of triplicate determinations were as follows: 56 units for peak II, 91 for III, 19 for IV, and 28 for Met-enkephalin.

extracts contain substances that fulfill pharmacological criteria of opiate activity.

DISCUSSION

The results show that clonal cells of the mouse pituitary tumor line AtT-20 synthesize at least four species of opioid peptides. Endorphin I has a molecular weight of at least 3000 and may be a precursor of a more active species of endorphin. Endorphin II, with an apparent molecular weight of 2400 by gel filtration analysis, resembles β-endorphin (molecular weight 3439) in elution from the gel and resistance to inactivation by leucine aminopeptidase and carboxypeptidase A. Endorphin III (apparent molecular weight 1800) resembles α- or γ-endorphin (molecular weights 1747 and 1878) in gel filtration analysis. Endorphin IV is a small peptide (molecular weight <750) similar, but possibly not identical to Met-enkephalin.

After adjustment for differences in the potencies of the various endorphins as inhibitors of NG108-15 adenylate cyclase, one may calculate (Table 1) that endorphins comprise as much as 0.15% of the total cell protein. Furthermore, the content of AtT-20 endorphins, expressed in normorphine equivalents in the guinea pig ileum assay, is similar to the endorphin content of bovine pituitary posterior lobe (20).

Preliminary experiments (unpublished) indicate that most of the AtT-20 endorphin activity is associated with the particulate (27,000 × *g*) fraction. This suggests that secretory vesicles that contain endorphins may be present in these cells.

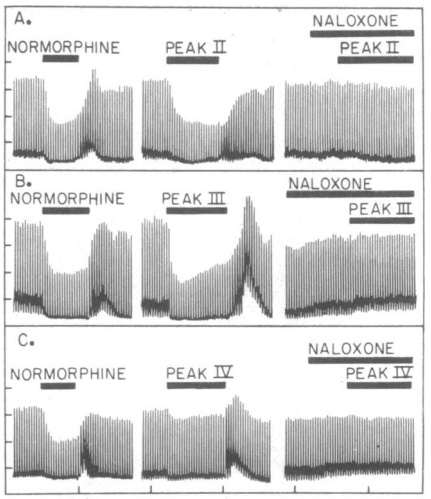

FIG. 4. Inhibition of electrically induced contractions of guinea pig ileum by AtT-20 endorphin fractions purified by Bio-Gel P-4 column chromatography (Fig. 2*B* and Table 1, Experiment B), and the reversal of inhibition by the narcotic antagonist naloxone (0.9 μM). Normorphine concentrations were 0.33 μM in *A* and *C* and 0.13 μM in *B*. Endorphin fractions added to the 3-ml bath were as follows: 167 μg of peak II protein (168 Met-enkephalin units); 44 μg of peak III protein (222 units); and 105 μg of peak IV protein (45 units). Different normorphine concentrations were tested; peak II and peak III endorphins had 5290 and 22,000 pmol of normorphine equivalents per mg of protein, respectively. Each interval marked on the ordinate represents a pen displacement resulting from 1 g of tension; each interval on the abscissa represents 5 min.

AtT-20 cells originated from an adenocarcinoma of the anterior pituitary of an irradiated LAF_1 mouse (21). The tumor cells later were adapted to culture conditions (10) and cloned (22). The clonal cells synthesize and secrete ACTH as well as biologically active melanotropin not identical to either α- or β-melanotropin (11). The presence of melanotropins and endorphins in AtT-20 cells strongly suggests that the cells synthesize β-lipotropin and contain specific proteases that catalyze the conversion of β-lipotropin to endorphin and melanotropin peptides. AtT-20 endorphins are probably related to Met- rather than Leu-enkephalin because they are inactivated by cyanogen bromide (19, 20). This result suggests that the gene for the Met-enkephalin class of opioid peptides can be expressed independently of the gene for the Leu-enkephalin class of peptides.

The syntheses of β-lipotropin and ACTH may be coupled to one another. For example, all cells in the human pituitary gland that contain ACTH also contain molecules reacting with an antibody to β-melanotropin (23). The presence of endorphins in AtT-20 cells suggests that endorphin and ACTH synthesis may be coupled in normal pituitary cells, as well as in AtT-20 and other neoplastic cells. Studies of clonal AtT-20 cells provide opportunities to explore possible coupling between endorphin and ACTH synthesis and perhaps to determine whether pituitary endorphins and ACTH are products of separate genes which are coordinately regulated or are products of the same gene. The cells also can be used to define steps in the biosynthesis and activation of opioid peptides from a prohormone precursor and to study factors which may regulate endorphin synthesis, activity, and secretion.

We are most grateful to Dr. Werner Klee for advice and for materials used in this study.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the

1. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. & Morris, H. R. (1975) *Nature* **258,** 577–579.
2. Kosterlitz, H. W., ed. (1976) *Opiates and Endogenous Opioid Peptides* (North Holland, Amsterdam), 456 pp.
3. Simantov, R. & Snyder, S. H. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2515–2519.
4. Klee, W. A. & Nirenberg, M. (1976) *Nature* **263,** 609–612.
5. Li, C. H., Barnafi, L., Chrétien, M. & Chung, D. (1965) *Nature* **208,** 1093–1094.
6. Li, C. H. & Chung, D. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1145–1148.
7. Ling, N., Burgus, R. & Guillemin, R. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3942–3946.
8. Cox, B. M., Gentleman, S., Su, T.-P. & Goldstein, A. (1976) *Brain Res.* **115,** 285–296.
9. Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. & Hulme, C. E. (1976) *Nature* **260,** 793–795.
10. Buonassisi, V., Sato, G. & Cohen, A. I. (1962) *Proc. Natl. Acad. Sci. USA* **48,** 1184–1190.
11. Orth, D. N., Nicholson, W. E., Mitchell, W. M., Island, D. O., Shapiro, M. & Byyny, R. L. (1973) *Endocrinology* **92,** 385–393.
12. Klee, W. A. & Nirenberg, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3474–3477.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
14. Sharma, S. K., Nirenberg, M. & Klee, W. A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 590–594.
15. Paton, W. D. M. (1957) *Br. J. Pharmacol.* **11,** 119–127.
16. Hughes, J. (1975) *Brain Res.* **88,** 295–308.
17. Terenius, L., Gispen, W. H. & de Wied, D. (1975) *Eur. J. Pharmacol.* **33,** 395–399.
18. Morgan, B. A., Smith, C. F. C., Waterfield, A. A., Hughes, J. & Kosterlitz, H. W. (1976) *J. Pharm. Pharmacol.* **28,** 660–661.
19. Doneen, B. A., Chung, D., Yamashiro, D., Law, P. Y., Loh, H. H. & Li, C. H. (1977) *Biochem. Biophys. Res. Commun.* **74,** 656–662.
20. Gentleman, S., Ross, M., Lowney, L. I., Cox, B. M. & Goldstein, A. (1976) in *Opiates and Endogenous Opioid Peptides,* ed. Kosterlitz, H. W. (North Holland, Amsterdam), pp. 27–34.
21. Cohen, A. I. & Furth, J. (1959) *Cancer Res.* **19,** 72–78.
22. Yasumura, Y. (1968) *Am. Zool.* **8,** 285–305.
23. Phifer, R. F., Orth, D. N. & Spicer, J. S. (1974) *J. Clin. Endocrinol. Metab.* **39,** 684–692.
24. Minna, J. D., Yavelow, J. & Coon, H. G. (1975) *Genetics* **79** (Suppl.), 373–382.