thepsin B also transforms a procollagenase found in bone to its active form (*17*). Liotta *et al.* (*18*) demonstrated that the activity of a collagenase specific for basement membrane collagen is twice as great in $B16F_{10}$ melanoma cells as it is in $B16F_1$ cells. Since maximal collagenase activity in vitro required trypsin treatment, the elevated cathepsin B activity in $B16F_{10}$ cells (Table 1) might activate the basement membrane collagenase in vivo, thus correlating it with the higher metastatic potential of $B16F_{10}$.

Cathepsin B activity, previously shown to be associated with human breast carcinoma (*1*) and vaginal adenocarcinoma (*4*), is a specific property of the tumor cell. In the mouse model, increased cathepsin B activity is correlated with increased metastatic behavior. These results warrant an investigation of lysosomal cathepsin B activity in human tumors with high metastatic potential. Cathepsin B may be a locus for therapeutic intervention in the metastatic cascade.

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20. Subcutaneous tumors were removed, diced, and placed in sterile Eagle's minimal essential medium (MEM) buffered with sodium bicarbonate (15 m*M*) and Hepes (25 m*M*). The MEM used for tumor cell dispersion contained collagenase type III (Worthington, 1 mg/ml; 174 U/mg), deoxyribonuclease I (Sigma, 50 μg/ml), soybean trypsin inhibitor (Worthington, 100 μg/ml), and fatty acid–free human serum albumin (Sigma, 10 mg/ml). Cells were dispersed (for 30 minutes and then for 60 minutes at 37°C) under air in a Dubnoff metabolic shaker (90 oscillations per minute). Supernatants were collected through cheesecloth and centrifuged (100*g* for 10 minutes); the pellets were resuspended in MEM and fractionated by centrifugal elutriation (*5*) into an α and a β fraction. Elutriation was accomplished with a Beckman JE6 elutriator rotor fitted with a Sanderson separation cell and operated (1300 rev/min) in a Beckman J2-21 centrifuge at 25°C. The separation medium (MEM) was pumped through the system with a Cole Palmer Master Flex pump (model 7014). The pump control was modified with a ten-turn potentiometer [W. D. Grant and M. Morrison, *Anal. Biochem.* **98**, 112 (1979)]. Monodispersed (> 99 percent) cells were introduced into the chamber and washed (3.3 ml/min) for 15 minutes to remove cellular debris and red blood cells. Fifty-milliliter fractions were then elutriated at flow rates of 3.7, 7.8, 9.5, 11.8, 30.0, and 43.5 ml/min. Each fraction was examined microscopically for composition. The first three fractions were designated α and the last three fractions were designated β. The viability (trypan blue dye exclusion) of β fraction cells was generally > 97 percent, with homogeneity > 99 percent. Whole tumors, dispersed cells, α fractions and β fractions were homogenized in 250 m*M* sucrose with 5 m*M* EDTA, *p*H 7.3, at 4°C with two 5-second bursts of a Tekmar homogenizer at full speed.
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Selective Protection of Methionine Enkephalin Released from Brain Slices by Enkephalinase Inhibition

Abstract. *Methionine enkephalin release was evoked by depolarization of slices from rat striatum with potassium. In the presence of 0.1 μM thiorphan [(N(R,S)-3-mercapto-2-benzylpropionyl)glycine], a potent inhibitor of enkephalin dipeptidyl carboxypeptidase (enkephalinase), the recovery of the pentapeptide in the incubation medium was increased by about 100 percent. A similar effect was observed with the dipeptide phenylalanylalanine, a selective although less potent enkephalinase inhibitor. Inhibition of other known enkephalin-hydrolyzing enzymes—aminopeptidase by 0.1 mM puromycin or angiotensin-converting enzyme by 1 μM captopril—did not significantly enhance the recovery of released methionine enkephalin. These data indicate that enkephalinase is critically involved in the inactivation of the endogenous opioid peptide released from striatal neurons.*

Whether specific inactivation mechanisms similar to those found in the well-known neurotransmitter synapses are responsible for turning off the signals conveyed by neuropeptides has not been established. Regarding the enkephalins Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu (Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Met, methionine; Leu, leucine), at least three well-characterized peptidases are candidates for this role in the central nervous system: an aminopeptidase (*1*), a peptidyl dipeptide hydrolase, angiotensin-converting enzyme (ACE) (*2*), and the recently characterized enkephalin dipeptidyl carboxypeptidase, enkephalinase (*3–8*). All three enzymes cleave the enkephalins into biologically inactive peptides—the first by releasing the NH_2-terminal Tyr residue, the others by hydrolyzing the Gly^3-Phe^4 amide bond. Enkephalinase may be more strategically located than the others, that is, in the vicinity of putative enkephalinergic synapses (*7, 9, 10*).

One approach to this kind of study consists in ascertaining whether selective inhibitors of the various enzymes protect the endogenous enkephalins released from their neuronal stores. Brain slices constitute a preparation in which the morphological organization is preserved and on which release of endogenous enkephalins by depolarizing agents has been demonstrated (*11, 12*). Inhibition of enkephalinase by the dipeptide Phe-Ala (Ala, alanine) or by thiorphan (*13*) enhances recovery of Met-enkephalin when its release from rat striatal slices is evoked by K^+, whereas selective inhibition of the two other peptidases does not provide such protection.

Selective inhibition of the Tyr-releasing enzyme activity can be obtained with puromycin, a compound that does not significantly affect either enkephalinase or ACE (*7*). Captopril (SQ14,225), a potent ACE inhibitor with an inhibition

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constant (K_i) in the nanomolar range (*14*) is a rather weak enkephalinase inhibitor (K_i = 10 μM) (*15*) and does not significantly affect aminopeptidase activity (K_i > 10 μM) (*7*). Selective inhibition of enkephalinase can be obtained either with Phe-Ala, a dipeptide selected among a large series or by thiorphan, [(*R,S*)-3-mercapto-2-benzylpropionyl) glycine] (*13*). Thiorphan displays both a high inhibitory potency toward enkephalinase activity from mouse striatum (K_i = 4.7 ± 1.2 nM) and a good selectivity as shown by the much higher K_i values for ACE (150 nM) and aminopeptidases (> 10 μM) (*13*). The dipeptide Phe-Ala, although much less potent than thiorphan as an enkephalinase inhibitor (K_i = 1 μM) is still a selective agent (K_i > 0.5 mM on ACE and K_i = 0.3 mM on aminopeptidase).

Potassium-evoked release of radioimmunoassayable Met-enkephalin was studied on slices from rat striatum suspended in an oxygenated Krebs medium (Fig. 1A). Removing the tissue, incubating portions of the Krebs medium with 20 nM ^{3}H-labeled Leu^5-enkephalin, and analyzing the products by thin-layer or Porapak column chromatography (*3*) showed that considerable aminopeptidase activity leached out of the tissue, as observed on the guinea pig ileum (*1*). Leaching mainly took place during the initial incubation period, but after a 30-minute incubation with renewal of the medium, enkephalin-hydrolyzing activity was hardly detectable in the medium. Hence the preincubation pattern described in the legend of Fig. 1A was adopted in order to minimize the influence of leaching out enzymes in the degradation of Met-enkephalin after its K^+-evoked release.

The release of Met-enkephalin evoked by 50 mM K^+resulted in a 10- to 20-fold increase of the pentapeptide level in the medium, which was completely abolished in a Ca^{2+}-free medium (*11, 12*). The effect of peptidase inhibitors, which were added 20 minutes before K^+-evoked release and maintained until the end of the experiments, was assessed at concentrations ensuring selective inhibition. In the presence of 0.1 μM thiorphan, the K^+-evoked release of Met-enkephalin was increased by more than 100 percent (Fig. 1B). The spontaneous efflux, measured during the incubation periods preceding depolarization, was not significantly modified, and increasing the thiorphan concentration to 1 μM did not further enhance the recovery after either spontaneous or evoked release (not shown). An effect almost identical to that of thiorphan was observed with the dipeptide Phe-Ala, although at a concentration 1000 times higher.

In contrast with the two enkephalinase inhibitors, neither puromycin nor captopril, in concentrations at which they inhibit aminopeptidase and ACE activities, respectively, enhanced the recovery of Met-enkephalin.

Under all conditions, the K^+-evoked increase of the Met-enkephalin concentration in the medium was accompanied by a sharp decrease of the concentration in the tissues (compared to levels in control slices transferred into normal Krebs medium for the last 5 minutes of incubation). In fact, even in the presence of thiorphan, the amount of endogenous peptide released into the medium (1.5 ± 0.1 nmole per gram of protein) was much less than that disappearing from the tissue (9.9 nmole/g representing the difference between 22.2 ± 1.6 nmole/g in control slices and 12.3 ± 0.6 nmole/g in depolarized slices; values are means ± standard error for nine independent measurements).

Our major finding is that enkephalinase inhibition results in enhanced recovery of Met-enkephalin released from its endogenous stores by depolarization and that such an effect is not observed after inhibition of other known enkephalin-hydrolyzing enzymes. This might appear surprising, because inactivation of exogenous enkephalins by brain homogenates mainly occurs by cleavage of the Tyr^1-Gly^2 bond (*1*) and because a high ACE activity is found in striatum (*16*). This suggests that, in contrast with these other enzymes, enkephalinase plays a key role in the inactivation of Met-enkephalin, probably because of its strategic location close to enkephalinergic nerve terminals. Other properties of enkephalinase, unshared by the other enzymes, also suggest its presence in the vicinity of putative enkephalinergic synapses. Thus its distribution between cerebral regions parallels that of opiate receptors and, furthermore, lesions induced by 6-hydroxydopamine or kainate within the striatum affect both types of enkephalin recognition sites to approximately the same extent (*6, 7, 9*). Also, the subcellular distribution of enkephalinase is strikingly parallel to that of opiate receptors,

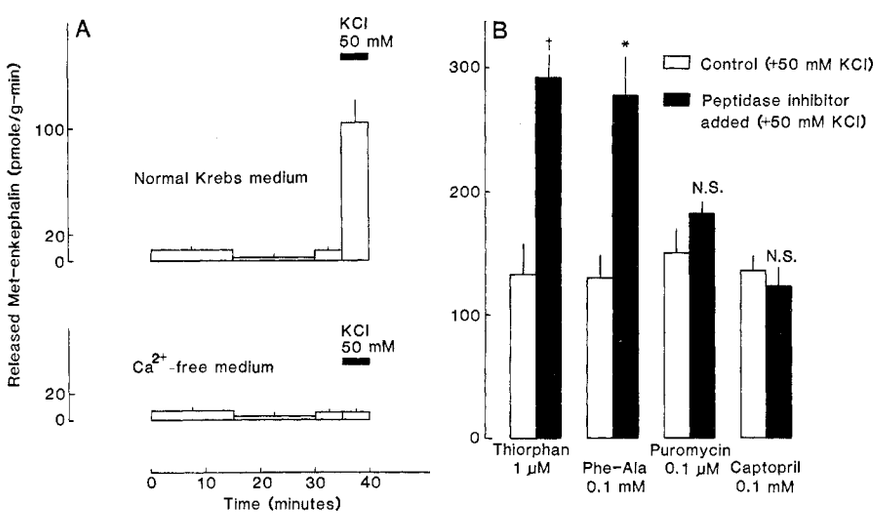

Fig. 1. Potassium-evoked release of Met-enkephalin from slices of rat striatum and the effects of various peptidase inhibitors. Slices of rat striatum obtained with a McIlwain tissue chopper (350 μm thickness) were suspended (about 10 mg of tissue per milliliter) in Krebs-Ringer medium (glucose, 5.9 mM; $NaHCO_3$, 27.5 mM; NaCl, 120 mM; KCl, 5 mM; $CaCl_2$, 2.6 mM; $MgSO_4$, 0.67 mM; KH_2PO_4, 1.2 mM). After two washes with fresh medium, 5 ml of the suspension was transferred in an open cylinder with a nylon mesh fitted to the bottom as a small basket (*18*). The whole system was placed in a 10-ml beaker, and incubations were performed at 37°C under a constant flow of 95 percent O_2 and 5 percent CO_2. The slices were transferred after 15 and 30 minutes into beakers containing fresh standard Krebs medium. After 35 minutes they were transferred for 5 minutes into Krebs medium in which 50 mM NaCl was replaced by 50 mM KCl. Immediately after the slices were removed from the successive incubation media, the media were acidified with HCl (0.1N, final concentration). At the end of experiments, the slices were sonicated in 2 ml of 0.1N HCl. (A) Met-enkephalin levels in tissue and media measured by radioimmunoassay (*19*) and expressed per gram of tissue protein; lower graph shows data obtained when calcium is omitted. (B) Effects of peptidase inhibitors. Experiments were conducted as in (A) in the presence of calcium and various compounds added at 15 minutes. The values represent Met-enkephalin levels in medium at the end of the 5-minute incubation in the presence of 50 mM K^+. Met-enkephalin levels in media before addition of 50 mM K^+ were not significantly modified in the presence of any peptidase inhibitor. Values shown are means ± standard error for six to nine experiments. N.S., nonsignificant. *P < .005. †P < .0005 in comparison with respective controls.

namely with identical enrichment of fractions containing synaptic membranes (*8, 17*). Finally the selective increase in cerebral enkephalinase activity after long-term administration of morphine (other enkephalin-hydrolyzing enzymes are not affected) could reflect the participation of the enzyme in the long-term control of enkephalinergic transmission (*3, 7*).

Enkephalinase inhibition in vivo should result in a facilitated enkephalinergic transmission reminiscent, for instance, of the enhanced cholinergic activity that follows administration of acetylcholinesterase inhibitors. In support of this hypothesis, it has recently been observed that thiorphan displays naloxone-sensitive antinociceptive activity in rats and mice (*13*).

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Calcium Spike Electrogenesis and Other Electrical Activity in Continuously Cultured Small Cell Carcinoma of the Lung

Abstract. *Spike electrogenesis, local depolarizing and hyperpolarizing responses, spontaneous rhythmic firing, and alternating resting potentials were measured in cells from a continuously cultured small cell carcinoma of the lung. Spike generation was blocked by $MnCl_2$. In view of this evidence for calcium-spike electrogenesis and previous evidence of secretory activity in these cells, this cell line (DMS 53) can provide a model for the study of excitation-secretion behavior in human neoplastic cells.*

Calcium-spike electrogenesis appears to be a widely occurring membrane-mediated process that participates in the initiation and regulation of excitation-secretion coupling. Endocrine and other secretory cells in both invertebrates and mammals generate Ca^{2+} spikes to signal the release of hormones and other chemical substances (*1–8*). We have studied cells of the highly malignant tumor known as small cell carcinoma of the lung (SCCL) because of their functional and growth characteristics (*9, 10*). These include the synthesis and secretion of estrogens and peptide hormones and the presence of cytoplasmic dense core vesicles, which have been associated with the synthesis and storage of peptide hormones. Although spike electrogenesis has been reported in cells isolated from a primary tumor SCCL (*11*), neither the ionic mechanism nor other electrical potentials were described. We have now determined the types of electrogenic activity that are present in long-term continuous cultures and report that extracellular calcium ions carry a major portion of depolarizing transmembrane current.

Table 1. Switching between two levels of membrane resting potential. Data were obtained during a prolonged impalement of DMS 53 cell 5/16/80D. The 12 observed epochs of decreased resting potential occurred as four discrete periods interspersed with periods of normal resting potential. *N* is the number of 150-msec observation epochs.

Resting potential (mV)	Class of resting potential measurement	
	Normal (N = 15)	Spontaneously decreased (N = 12)
Average	− 32.8	− 21.3
Median	− 32.0	− 19.2
S.D.	± 3.5	± 4.6

Cells of a continuous SCCL line (DMS 53) were trypsinized for subculture with 0.25 percent trypsin in calcium- and magnesium-free Hanks balanced salt solution (*9*). The cells (2.5×10^6) were plated in 35-mm Falcon plastic petri dishes in 5 ml of Waymouth's MB 752/1 medium containing 20 percent fetal calf serum and 25 m*M* Hepes buffer. Cultures were examined 4 days after plating; at this time the monolayer was approximately half confluent. The culture dish was mounted on a temperature-controlled (35 ± 1°C) stage of a Zeiss inverted phase-contrast microscope. Microelectrodes, filled with 3*M* KCl and having resistances of 45 to 55 megohms, were coupled to a bridge circuit to allow intracellular injection of constant-current pulses. Recordings of transmembrane potential and injected current were obtained from a cathode-ray oscilloscope with a Polaroid camera or were directly led into an Able-40 (New England Digital) computer for analysis. Software for real-time data acquisition and analysis were developed specifically for this project.

Resting potentials recorded in these cells displayed two significant characteristics: (i) relatively low values and (ii) a switching back and forth between two values (Table 1). There is a tendency to dismiss low-voltage membrane poten-

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