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Characterization of Aminopeptidases Responsible for Inactivating Endogenous (Met[5])Enkephalin in Brain Slices Using Peptidase Inhibitors and Anti-Aminopeptidase M Antibodies

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SUMMARY

In addition to "enkephalinase" (EC 3.4.24.11), two enkephalin-hydrolyzing aminopeptidases recently identified in cerebral membranes—aminopeptidase M (EC 3.4.11.2) and a "puromycin-sensitive" aminopeptidase (also designated "MII" or "aminoenkephalinase")—are potentially involved in endogenous enkephalin inactivation. Their participation in the hydrolysis of the endogenous (Met[5])enkephalin released by depolarization of slices from rat globus pallidus was assessed, using three inhibitory agents: bestatin, puromycin, and anti-aminopeptidase M antibodies. The selectivity and potency of these agents were first determined by evaluating their IC_{50} values for inhibition of [^{3}H](Met[5])enkephalin hydrolysis by increasingly complex preparations comprising semipurified aminopeptidases, pallidal membranes, and pallidal slices. Bestatin was a fairly potent inhibitor but lacked selectivity, as there was only a 3-fold difference between its IC_{50} values for the two aminopeptidases, and it displayed restricted diffusion and degradation in the slice preparation. Puromycin discriminated well between the two aminopeptidases (30-fold difference in IC_{50} values) and did not show any apparent restricted diffusion in the slice preparation. Anti-aminopeptidase M antibodies were highly discriminant (>300-fold difference in IC_{50} values for the two aminopeptidases) but displayed restricted diffusion. Analysis of the concentration-protection curves of the three agents for recovery of the (Met[5])enkephalin released from pallidal slices in the presence of the "enkephalinase" inhibitor, thiorphan, indicated that both aminopeptidases participated in enkephalin degradation but that the role of aminopeptidase M was largely predominant, in contrast with its low relative activity in the preparation.

Like other neurotransmitters, enkephalins are rapidly inactivated after their release from cerebral neurons, and much work was recently devoted to identifying the peptidases responsible (1–5). Since enkephalins are substrates for a number of peptidases, some of which may not be involved in their physiological inactivation, assessment of the effects of selective inhibitors using biological assays constitutes an important means of identifying physiologically relevant neuropeptidases.

Selective inhibition of "enkephalinase" (EC 3.4.24.11), a membrane-bound peptidase that hydrolyzes the Gly[3]-Phe[4] amide bond and is found in brain (6) and peripheral organs like the kidney (7–11), partially protects endogenous enkephalins from complete inactivation (12–14) and, *in vivo*, induces several opioid-like responses like analgesia, which are reversible by naloxone (15, 16). In addition, aminopeptidase involvement in enkephalin inactivation was suggested by the opioid-like, naloxone-reversible neurochemical (17), behavioral (18), or analgesic (16, 19, 20) effects of the inhibitor, bestatin, and by the complete protection of the endogenous enkephalins released from brain slices in the presence of bestatin and of thiorphan, an "enkephalinase" inhibitor (14, 21).

Three membrane-bound aminopeptidases from rat brain, all sensitive to inhibition by bestatin, recently have been characterized. Whereas the first, designated MI aminopeptidase, displayed an extremely low affinity for enkephalins, thus making its participation in enkephalin inactivation unlikely (22, 23), this was not the case for the two others, whose affinity for enkephalins was higher, with K_M values in the micromolar range (23–25). One of the two, identified as aminopeptidase M (EC 3.4.11.2) (2, 25), is characterized by its low sensitivity to puromycin, an aminopeptidase inhibitor (26, 27). In contrast, the other, designated as MII aminopeptidase (22, 23), "membrane-aminoenkephalinase" (24, 28) or "puromycin-sensitive" aminopeptidase (2, 25), is highly sensitive to both puromycin and bestatin.

In the present work, three inhibitory agents were used to establish whether one or both of these two aminopeptidases are responsible for the inactivation of endogenous (Met[5])-enkephalin: bestatin, puromycin, and anti-aminopeptidase M antibodies, raised against a highly purified enzyme preparation (25). Their influence was evaluated from the recovery of the (Met[5])enkephalin released by depolarization of slices from rat

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.

globus pallidus, a region in which it seems almost exclusively stored in the axon terminals (29). Although this relatively simple preparation has the distinct advantage of maintaining the topographic relationships between the terminals releasing neuropeptides and the peptidases truly responsible for their hydrolysis, diffusion and inactivation problems are liable to obscure the interpretation of the concentration-response curves showing the effects of the inhibitors. Hence we began by evaluating the potency and selectivity of these three agents in relation to [^{3}H]enkephalin hydrolysis by increasingly complex preparations: semipurified aminopeptidases, membranes, and brain slices.

Materials and Methods

Chemicals. [^{3}H](Met^5)Enkephalin (30–50 Ci/mmol) was purchased from Amersham International plc (Amersham, U.K.), Porapak Q was from Waters Associates (Milford, MA), and bestatin was a generous gift from Laboratoire R. Bellon (Neuilly s/Seine, France). Thiorphan (DL-3-mercapto, 2-benzylpropanoyl-glycine) was from Laboratoire Bioprojet (Paris, France), and puromycin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical grade reagents were from Prolabo (Paris, France).

Rat globus pallidus membranes. Male Wistar rats (Iffa Credo, France) weighing 180–240 g were allowed free access to food and water. After decapitation, the globus pallidus was rapidly dissected out and homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7.5, using a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min and the resulting supernatant was centrifuged again at 100,000 × *g* for 20 min. To eliminate any soluble enzyme activity, the resulting pellet was washed three times by suspension in 50 mM Tris-HCl buffer, pH 7.5, and subsequent centrifugation. The final pellet was resuspended in a slightly modified 5 mM K^+ Krebs-Ringer medium comprising 5.9 mM glucose, 27.5 mM Na-HEPES, 120 mM NaCl, 3.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, and 1.2 mM KH_2PO_4, pH 7.4, and was kept at 4° until use.

Rat globus pallidus slices. Rats were decapitated and the globus pallidus was rapidly dissected out and sliced using a McIlwain tissue chopper. Slices (generally 0.35 mm thick) were pooled and washed by suspension in the modified Krebs-Ringer medium previously gassed with pure O_2. In order to obtain about 1 mg of tissue/incubation, 10 animals were generally needed for 24 incubations. After four successive washes with fresh medium, slices were transferred to a 35-ml plastic test tube, gassed with pure O_2, and maintained at 37°. They were immediately used either for [^{3}H](Met^5)enkephalin hydrolysis or endogenous (Met^5)enkephalin release studies.

Semipurified aminopeptidases from rat brain membranes. Two semipurified aminopeptidase preparations from rat brain membranes were obtained as previously described (25). Briefly, a whole brain pellet was extensively washed to eliminate any soluble aminopeptidase activity and submitted to 1% Triton X-100 solubilization. Aminopeptidase M (EC 3.4.11.2, also designated aminopeptidase N) was purified about 500-fold from the solubilized preparation by chromatography on concanavalin A-Sepharose and DEAE cellulose columns followed by chromatofocusing (pI = 5.0–4.5). Its specific activity, as measured by hydrolysis of 40 nM [^{3}H](Met^5)enkephalin, was about 200 pmol/min/mg of protein, and the preparation was devoid of any enkephalin-hydrolyzing activity other than aminopeptidase M, as shown by the complete inhibition of this preparation in the presence of antibodies raised against highly purified aminopeptidase M from rat kidney (25).

A second aminopeptidase activity, designated "puromycin-sensitive aminopeptidase" (2, 25), "MII aminopeptidase" (22, 23), or "membrane aminoenkephalinase" (24), was separated from aminopeptidase M at the lectin affinity column step and isolated by DEAE cellulose column chromatography. The specific activity of this preparation was 20 pmol/min/mg of protein.

With both preparations, [^{3}H](Met^5)enkephalin hydrolysis only occurred at the level of the Tyr^1-Gly^2 amide bond as shown by thin layer chromatography (14), indicating the absence of any enkephalin-hydrolyzing activity other than aminopeptidases.

Antibodies against aminopeptidase M. Active site-directed antibodies were obtained in rabbits against a highly purified preparation of aminopeptidase M from rat kidney as described previously (25). Several bleedings from the same rabbit were selected on the basis of their aminopeptidase M-inhibiting activity. A purified immunoglobulin gamma fraction was prepared by Na_2SO_4 precipitation and DEAE Sephadex A-50 chromatography (30). The inhibitory activity of this fraction was compared to that of a corresponding fraction prepared from the preimmune serum. Until use, the antibodies were kept at 4° in 0.01% sodium azide.

Assay of [^{3}H](Met^5)enkephalin-hydrolyzing activities. The three preparations, i.e., semipurified aminopeptidases, pallidal membranes, and pallidal slices, were preincubated for 10 min at 37° in the absence or presence of the various peptidase inhibitors, or for 20 min whenever anti-aminopeptidase M antibodies were included. To allow for comparisons, all incubations lasted for 10 min at 37° in the modified Krebs-Ringer medium described for the incubation of pallidal membranes. Incubations of membranes and purified enzymes (in a final volume of 200 μl), were stopped by adding 50 μl of 0.2 N HCl. In the case of pallidal slices, the incubations (in a final volume of 800 μl) were stopped by adding $HClO_4$ (final concentration, 0.4 N); then, slices were sonicated in the incubation medium, proteins were allowed to precipitate overnight at 4°, and the mixture was centrifuged. Tritiated hydrolysis products were quantitatively separated from the intact substrate by polystyrene bead column chromatography (31) as described by De la Baume *et al.* (14).

Endogenous (Met^5)enkephalin release by depolarization of rat globus pallidus slices. About 25 ml of fresh medium were added to washed rat globus pallidus slices from 10 rats, and the medium was then changed twice every 15 min, for 45 min, for complete removal of soluble peptidase activity. Its elimination was checked in the last supernatant (see Results). Slices were then suspended in 25 ml of fresh medium, and 360-μl aliquots of the suspension (corresponding to 0.5–2 mg of protein) were poured into Eppendorf plastic tubes containing 40 μl of the various agents to be tested, dissolved in 5 mM K^+ control medium. After a 10-min preincubation at 37°, 400 μl of fresh medium (with or without drugs), containing amounts of KCl calculated to give final concentrations of 5 or 50 mM K^+, were added. In the presence of 50 mM K^+, the NaCl concentration was reduced in order to maintain isoosmolarity. After 5 min, incubations were stopped by rapid centrifugation, and the supernatants were transferred to tubes containing 90 μl of 4 N $HClO_4$. Pellets were sonicated in 500 μl of 0.4 N $HClO_4$ and kept at 4° overnight for complete precipitation of proteins.

Protein pellets were separated from tissue extracts by centrifugation and dissolved in 0.5 ml of 0.4 N NaOH for protein determination. Perchlorate in tissue extracts and medium supernatants was precipitated by addition of potassium hydroxide and potassium phosphate, pH 6.4. The clear supernatants obtained after centifugation were passed through polystyrene bead columns in order to isolate (Met^5)enkephalin. The columns were washed three times with 2 ml of water, and the (Met^5)enkephalin eluted in 1 ml of ethanol was radioimmunoassayed using antibodies directed toward the C-terminal part of the molecule and displaying negligible cross-reactivity toward its various fragments (32). In several experiments, the immunoreactive material eluted was identified as authentic (Met^5)enkephalin by HPLC on a C_{18} μBondapak column using a linear gradient (5–25% acetonitrile in 1.4 mM triethylamine and 1.7 mM orthophosphoric acid; running time of 30 min), at a flow rate of 1 ml/min (retention time of 25.5 min).

Data from triplicate assays were averaged, and results were expressed in pmol of (Met^5)enkephalin/mg of tissue protein.

The amount of (Met^5)enkephalin released from slices by depolarization was calculated as the difference between the mean levels in slices incubated with 5 and 50 mM K^+ and expressed per mg of protein.

The amount recovered in the medium was calculated as the difference between the mean levels in the 5 and 50 mM K^+ media and expressed per mg of protein. Recovery was taken as the ratio of (Met^5)enkephalin recovered in the medium to the (Met^5)enkephalin released from the slices, and was expressed as a percentage. In studies in which antibodies were used, slight modifications were introduced in the experimental protocol: slices were 0.25 mm thick (instead of 0.35 mm) and the preincubation time was increased to 20 min (instead of 10 min).

Protein determination. Protein content was determined in purified enzyme fractions, using bovine serum albumin as standard, with a Bio-Rad protein assay kit. and in membranes or slices by the method of Lowry *et al.* (33).

Data analysis and statistics. Inhibition curves were analyzed according to a nonlinear least squares curve-fitting procedure using a one- or two-component model derived from Parker and Waud (34) as described by Martres *et al.* (35). This method provided an estimation of IC_{50} values and, in the case of a two-component model, the relative proportions of high and low affinity components. An F test (F = ratio of the deviation mean squares obtained in the one site model to that obtained in the two-site model) was performed to select the best fitting model. Other statistical comparisons were performed using the Student's t test.

Results

Effects of peptidase inhibitors and anti-aminopeptidase M antibodies on [^{3}H](Met^5)enkephalin hydrolysis by semipurified aminopeptidase M and "puromycin-sensitive" aminopeptidase and in membranes or slices of rat globus pallidus. To allow for comparisons, [^{3}H](Met^5)-enkephalin hydrolysis by the various preparations was always evaluated under the same conditions, i.e., in modified Krebs-Ringer incubation medium and with a 40 nM concentration of [^{3}H](Met^5)enkephalin. To allow for a predominant hydrolysis of the enkephalin by aminopeptidases (14, 20), thiorphan (0.2 μM), an enkephalinase inhibitor, was present in most experiments with membranes or slices. The hydrolysis rates, measured in the presence of 40 nM [^{3}H](Met^5)enkephalin, were: 0.26 ± 0.01, 0.23 ± 0.01, 200 ± 5, and 20 ± 1 pmol/min/mg of protein for slices, membranes, semipurified aminopeptidase M, and "puromycin-sensitive" aminopeptidase, respectively. In contrast, for the medium in which slices had been incubated for 15 min and then removed by centrifugation, the hydrolysis rate was <0.01 pmol/min/mg of tissue protein, indicating that none of the peptidases leaching out from the slice preparation participated significantly in enkephalin degradation.

Puromycin completely inhibited [^{3}H](Met^5)enkephalin hydrolysis by the two semipurified aminopeptidases in a monophasic manner, with a 30-fold difference in IC_{50} values [presumably corresponding to K_i values since the substrate concentration was by 3 orders of magnitude below its K_M values for the two enzymes (25)] (Fig. 1, Table 1). The ^{3}H-peptide hydrolysis by pallidal membranes, evaluated in the presence of 0.2 μM thiorphan, was maximally inhibited by 70–75% (at a puromycin concentration of 1 mM), in an apparently monophasic manner, with an IC_{50} value not significantly different from that found for the "puromycin-sensitive" aminopeptidase (Table 1). In the presence of thiorphan, puromycin inhibited [^{3}H](Met^5)-enkephalin hydrolysis by pallidal slices in an incomplete manner (80% maximal inhibition) that was clearly biphasic (Fig. 1). Analysis of these data by a computerized curve-fitting procedure indicated that they were best fitted ($p < 0.05$) to a two-site model with IC_{50} values not significantly different from those obtained with the two semipurified aminopeptidases. The

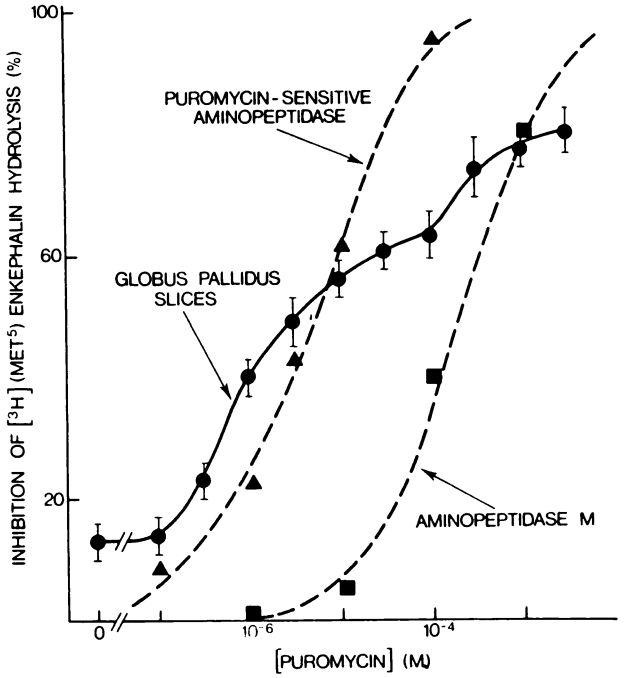

Fig. 1. Effect of puromycin on [^{3}H](Met^5)enkephalin hydrolysis by slices of rat globus pallidus and by purified aminopeptidase preparations. Pallidal slices were preincubated for 10 min in the presence of 0.2 μM thiorphan and increasing concentrations of puromycin. The purified enzyme preparations were directly incubated in the absence of thiorphan. Ten-min incubations were started by the addition of 5 mM K^+ medium containing the above drugs and 40 nM [^{3}H](Met^5)enkephalin. Hydrolysis products were isolated by polystyrene bead column chromatography. In the absence of puromycin hydrolysis rates (pmol/mg of protein/min) were 0.26 ± 0.01, 200 ± 5, and 20 ± 1 for slices, aminopeptidase M, and "puromycin-sensitive" aminopeptidase, respectively. Values shown represent the means ± standard error from 8–12 experiments for slices and the means from 6 experiments for the purified aminopeptidase preparations.

relative contributions of the two components distinguished by puromycin were 75% and 25% for the high and low affinity components, respectively (Table 1). When slices were preincubated in the presence of 20 μM puromycin and 0.2 μM thiorphan for periods of 0–30 min, inhibition of [^{3}H](Met^5)enkephalin hydrolysis was similar (50 ± 3%), indicating that the diffusion of puromycin within the tissue occurred at least as rapidly as that of the enkephalin, and that equilibrium had been reached by the end of the 10-min preincubation time selected for most experiments.

Bestatin completely inhibited [^{3}H](Met^5)enkephalin hydrolysis by the two purified aminopeptidases in a monophasic manner, with only a 3-fold difference in their IC_{50} values (Table 1). Inhibition of hydrolysis by pallidal membranes was also monophasic, with an IC_{50} value close to that found for the "puromycin-sensitive" aminopeptidase (Table 1). With pallidal slices, the maximal inhibition with 0.1 mM bestatin and 0.2 μM thiorphan was about 80% (Table 2), and analysis of the concentration-inhibition curve (not shown) was not improved by using a two-site model instead of a one-site model; the mean IC_{50} value for bestatin was 7 times higher than the value for [^{3}H](Met^5)enkephalin hydrolysis by pallidal membranes (Table 1). The lower potency of bestatin in the slice model was apparently attributable to restricted diffusion or degradation of this inhibitor since, when slices were preincubated with 1 μM bestatin and 0.2 μM thiorphan for 0, 5, 10, or 30 min before

TABLE 1

Comparative potencies of peptidase inhibitors and anti-aminopeptidase M antibodies in various experimental models

[^{3}H](Met^5)enkephalin was incubated at a concentration of 40 nM. Values for peptidase inhibitors are derived from experiments with pallidal membranes or slices incubated in the presence of 0.2 μM thiorphan.

	IC_{50} Values (μM)			Antibodies (50% inhibitory dilutions)
	Bestatin		Puromycin	
A. Hydrolysis of exogenous [^{3}H] (Met^5)enkephalin				
Purified aminopeptidase M	0.7 ± 0.2		112 ± 30	0.0003
Purified "puromycin-sensitive" aminopeptidase	0.2 ± 0.1		3.5 ± 1.5	>0.1
Pallidal membranes	0.3 ± 0.1		1.2 ± 0.4	0.0004[a]
Pallidal slices	2.1 ± 0.7	2 ± 0.2	{ 1.3 ± 0.4 (75%)[b] 155 ± 70 (25%)	0.0070[a]
B. Hydrolysis of endogenous (Met^5)enkephalin released from pallidal slices	12 ± 3	35 ± 14	{ 1.2 ± 0.7 (30%)[b] 170 ± 66 (70%)	0.0150[a]

[a] Data were obtained from experiments in the presence of 0.2 μM thiorphan and 20 μM puromycin to inhibit "enkephalinase" and "puromycin-sensitive" aminopeptidase, respectively.

[b] Values bracketed together indicate the IC_{50} values and relative participation of the two components distinguished by puromycin (two-site model analysis of inhibition data by an iterative program).

TABLE 2

Effects of peptidase inhibitors and anti-aminopeptidase M antibodies on [^{3}H](Met^5)enkephalin hydrolysis by rat globus pallidus slices

Slices (0.5–1.5 mg of protein) were preincubated for 20 min in the presence of inhibitory agents and incubated for a further 10 min in the presence of 40 nM [^{3}H](Met^5)enkephalin; then, the ^{3}H-metabolites formed were isolated from the intact ^{3}H-peptide by polystyrene bead chromatography. Values are means ± standard error from four to eight experiments.

Inhibitory agents	[^{3}H] Metabolites Formed		Inhibition (%)
	(pmol/mg protein)	(%)	
None	2.55 ± 0.12	12.1 ± 0.5	
Thiorphan (0.2 μM)	2.22 ± 0.06	10.6 ± 0.3	13 ± 2
Antibodies (1/50)	2.16 ± 0.07	10.2 ± 0.3	15 ± 3
Puromycin (20 μM)	1.57 ± 0.11	7.4 ± 0.5	38 ± 4
Bestatin (0.1 mM)	1.04 ± 0.04	4.9 ± 0.2	59 ± 4
Thiorphan (0.2 μM) + puromycin (20 μM)	1.23 ± 0.04	5.9 ± 0.2	51 ± 2[a,b]
Thiorphan (0.2 μM) + bestatin (0.1 mM)	0.63 ± 0.03	3.0 ± 0.2	76 ± 2[a,c]
Thiorphan (0.2 μM) + puromycin (20 μM) + antibodies (1/500)	1.07 ± 0.04	5.1 ± 0.2	58 ± 2[a,d]
Thiorphan (0.2 μM) + puromycin (20 μM) + antibodies (1/100)	0.90 ± 0.03	4.3 ± 0.2	65 ± 4[a,e]
Thiorphan (0.2 μM) + puromycin (20 μM) + antibodies (1/50)	0.75 ± 0.04	3.6 ± 0.1	70 ± 1[a,f]

[a] $p < 0.001$ compared to thiorphan alone.
[b] $p < 0.05$ compared to puromycin alone.
[c] $p < 0.01$ compared to bestatin alone.
[d] Not significant compared to thiorphan + puromycin.
[e] $p < 0.05$ compared to thiorphan + puromycin.
[f] $p < 0.001$ compared to thiorphan + puromycin.

the addition of [^{3}H](Met^5)enkephalin, the hydrolysis inhibitions were 25 ± 7, 42 ± 6, 68 ± 4, and 55 ± 3%, respectively (means ± standard error of four to eight experiments).

In addition, HPLC analysis (solvent system: 25.5% acetonitrile in 1.4 mM triethanolamine and 1.7 mM orthophosphoric acid) of the medium in which 50 μM bestatin had been incubated for 30 min in the presence of slices indicated that the concentration of this inhibitor (retention time of 6 min) had decreased by about 20%. Inhibitor degradation within the slice itself could not be evaluated by HPLC analysis, due to interference by endogenous tissue components (not shown).

The anti-aminopeptidase M antibodies progressively and completely inhibited [^{3}H](Met^5)enkephalin hydrolysis by the purified aminopeptidase M preparation with 50% inhibition occurring at a 1/3000 dilution; in contrast, the preimmune serum had no significant effect, even at a 1/100 dilution (Fig. 2). At a 1/10 dilution, the anti-aminopeptidase M antibodies only inhibited the "puromycin-sensitive" aminopeptidase by about 20%. Inhibition of the hydrolyzing activity of pallidal membranes by antibodies was hardly detectable when evaluated in the presence of thiorphan but in the absence of puromycin (not shown). Hence, this inhibition was investigated in the presence of 0.2 μM thiorphan and 20 μM puromycin, the concentration of the latter being chosen to obtain selective inhi-

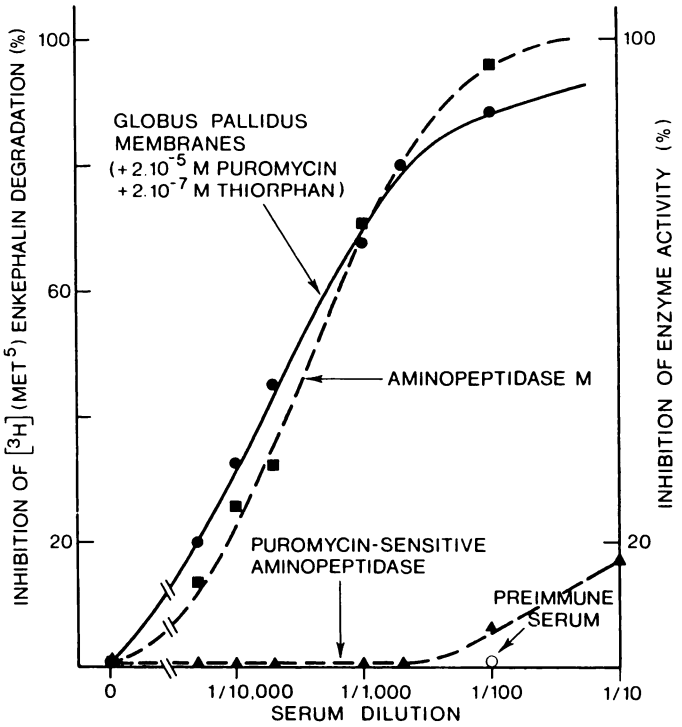

Fig. 2. Effects of anti-aminopeptidase M antibodies on [^{3}H](Met^5)enkephalin hydrolysis by membranes of rat globus pallidus or purified aminopeptidase preparations. After 20 min preincubation at 37° in the presence of antibodies, membranes or purified enzyme preparations were incubated for 10 min in the presence of 40 nM[^{3}H](Met^5)enkephalin and the indicated agents. Hydrolysis products were isolated by polystyrene bead column chromatography. Values are means from six experiments.

bition of the "puromycin-sensitive" aminopeptidase activity from the membranes, without affecting aminopeptidase M activity (Fig. 1). Under these conditions, the remaining enkephalin-hydrolyzing activity was progressively inhibited by increasing concentratins of antibodies with a half-maximal effect occurring at a 1/4000 dilution, whereas the antibodies from the preimmune serum were ineffective, even at a 1/100 dilution (Fig. 2). In rat globus pallidus slices, 0.25 mm thick, significant inhibition of [^{3}H](Met^5)enkephalin hydrolysis by the anti-aminopeptidase M antibodies (tested after 20 min preincubation) was also observed, but approximately 10-fold higher concentrations were required, and even at a 1/50 dilution, the inhibition was slightly less marked than in the presence of 0.1 mM bestatin (table 2). This shift in the IC_{50} values might be due to slow diffusion of the antibodies; thus, at a 1/100 dilution, and for preincubation times of 0, 10, 20, and 40 min, inhibition increased steadily, the values being 37 ± 3, 58 ± 2, 65 ± 4, and 73 ± 2%, respectively (means ± standard error of four determinations). In contrast, no significant inhibition could be detected with the preimmune serum or when the anti-aminopeptidase M antibodies were used with slices 0.35 mm thick (not shown).

Effects of peptidase inhibitors and anti-aminopeptidase M antibodies on the recovery of endogenous (Met^5)enkephalin released from pallidal slices. As shown in Table 3, in slices 0.25 mm thick incubated in the absence of any peptidase inhibitor, only 8 ± 1% of the endogenous (Met^5)enkephalin released by depolarization was recovered in intact immunoreactive form in the medium. When evaluated in the presence of 0.2 μM thiorphan, recovery increased significantly to 13 ± 1% ($p < 0.01$) and further increased to 35 ± 3% after the addition of 20 μM puromycin (Table 3). When antibodies at a 1/50 dilution were added with thiorphan, recovery also increased significantly. When 20 μM puromycin was added to these two inhibitory agents, the recovery elicited by 20 μM puromycin alone was potentiated, although complete protection of endogenous enkephalin was not achieved; in contrast, thiorphan and bestatin ensured almost complete protection.

Complete concentration-response curves for the protective effects of aminopeptidase inhibitors and antibodies were established on the same preparation (Figs. 3 and 4). With puromycin, a shallow protection curve was obtained, with almost total protection observed at 1 mM and half-maximal inhibition at 35 μM (Fig. 3). However, computer analysis of this curve indicated that it was best fitted ($p < 0.05$) to a two-component model, one component corresponding to 30% protection and an IC_{50} value of 1.2 ± 0.6 μM for puromycin, and the other corresponding to 70% protection and an IC_{50} value of 170 ± 70 μM. With bestatin, a steeper concentration-inhibition curve was obtained (best fitted to a single component model), with an IC_{50} value of 12 ± 3 μM and total protection occurring at 0.1 mM. In the

TABLE 3

Effects of peptidase inhibitors and anti-aminopeptidase M antibodies on the recovery of (Met^5)enkephalin released by K$^+$-induced depolarization of Rat Globus Pallidus Slices

Slices were preincubated for 20 min in the presence of peptidase inhibitors and/or antibodies and incubated for a further 5 min after the addition of KCl. (Met^5)Enkephalin levels were evaluated in slices and medium. The release of (Met^5)enkephalin evoked by the K$^+$-stimulus under the various conditions was evaluated as the difference between its tissue levels in slices incubated in the presence of 5 mM K$^+$ (21.3 ± 1.8 pmol/mg of protein) and 50 mM K$^+$, respectively. (Met^5)Enkephalin levels in slices incubated with 5 mM K$^+$ and the various inhibitory agents did not vary significantly. Values are means ± standard error of 12 experiments.

Conditions	(Met^5)Enkephalin		(Met^5)Enkephalin recovery (% of released)
	Recovered in medium	Released from slices	
	pmol/mg protein		
50 mM K$^+$	0.56 ± 0.03	6.4 ± 1.2	8.5 ± 1
50 mM K$^+$ + 0.2 μM thiorphan	0.86 ± 0.07	6.8 ± 1.1	13 ± 1
50 mM K$^+$ + 20 μM puromycin	1.51 ± 0.16	7.5 ± 0.9	20 ± 2
50 mM K$^+$ + 0.1 mM bestatin	2.1 ± 0.2	6.2 ± 0.5	34 ± 3
50 mM K$^+$ + 0.2 μM thiorphan + 20 μM puromycin	2.6 ± 0.2[a]	7.4 ± 1.4	35 ± 3[a]
50 mM K$^+$ + 0.2 μM thiorphan + anti-bodies (1/50)	1.7 ± 0.3[a]	6.9 ± 1.0	24 ± 2[a]
50 mM K$^+$ + 0.2 μM thiorphan + 20 μM puromycin + anti-bodies (1/50)	4.1 ± 0.3[a,b]	6.8 ± 1.0	61 ± 5[a,b]
50 mM K$^+$ + 0.2 μM thiorphan + 0.1 mM bestatin	4.7 ± 0.5[a]	5.1 ± 1.2	91 ± 6[a]

[a] $p < 0.001$ compared to 50 mM K$^+$ + thiorphan.
[b] $p < 0.001$ compared to 50 mM K$^+$ + thiorphan + puromycin.

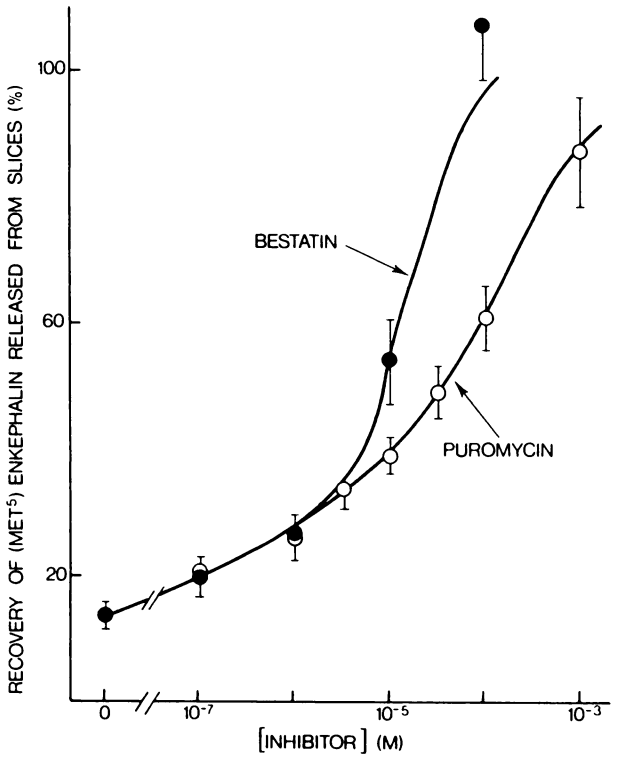

Fig. 3. Effects of puromycin or bestatin on the recovery of endogenous (Met^5)enkephalin released from K$^+$-depolarized slices of rat globus pallidus. Pallidal slices were preincubated for 10 min in the presence of 0.2 μM thiorphan and increasing concentrations of the inhibitors, and were exposed for 5 min to 50 mM K$^+$. The amount of (Met^5)enkephalin released from slices was evaluated as the difference between the mean tissue peptide contents at 5 mM K$^+$ (17.9 ± 1.3 pmol/mg of protein) and at 50 mM K$^+$ (11.5 ± 0.96 pmol/mg of protein). The amount of (Met^5)enkephalin recovered in the medium was 0.30 ± 0.11 pmol/mg of protein and 1.26 ± 0.13 pmol/mg of protein at 5 mM K$^+$ and 50 mM K$^+$, respectively (in the absence of inhibitor). The recovery represents the percentage ratio of the (Met^5)enkephalin recovered in the medium to the (Met^5)enkephalin released from slices. Values are means ± standard error from 12 experiments.

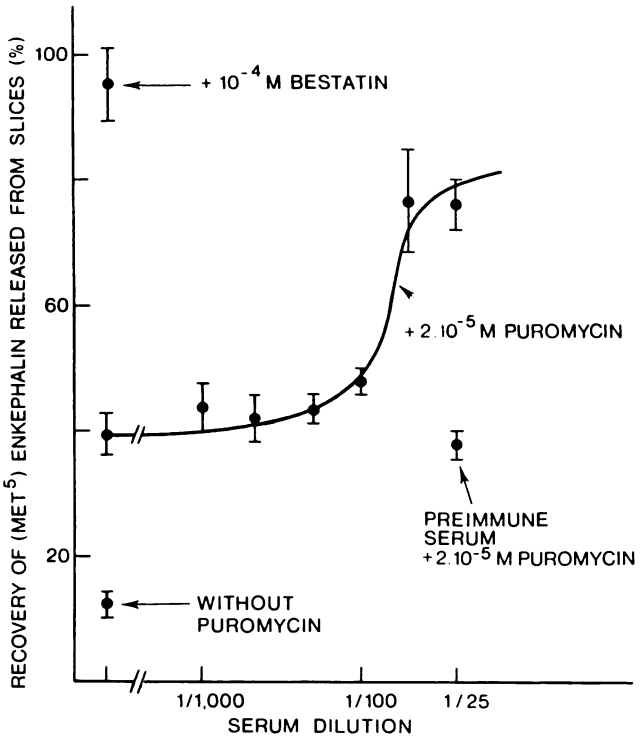

Fig. 4. Effects of anti-aminopeptidase M antibodies on the recovery of endogenous (Met^5)enkephalin released from slices of rat globus pallidus. Experiments were performed in the presence of 0.2 μM thiorphan, as described in the legend to Fig. 3 (except that the preincubation time was 20 min). Values are means ± standard error of 12–18 experiments.

presence of 20 μM puromycin, a concentration chosen to obtain selective inhibition of the "puromycin-sensitive" aminopeptidase, inhibition by anti-aminopeptidase M antibodies was half-maximal at a 1/70 dilution, the maximal recovery being 80%, compared to 95% in the presence of bestatin (Fig. 4, Table 1). Addition of the preimmune serum (1/25 dilution) in the presence of 20 μM puromycin had no effect on recovery.

Discussion

The present attempt to identify the aminopeptidase(s) that inactivate(s) the endogenous (Met^5)enkephalin released by depolarization of pallidal slices was based upon the use of three aminopeptidase-inhibiting agents in addition to the "enkephalinase" inhibitor, thiorphan. The first, bestatin, is a rather potent aminopeptidase inhibitor (14, 20, 36, 37) but displays limited specificity, with only a 3-fold difference in K_i values for "puromycin-sensitive" aminopeptidase (23, 25, 38) versus purified aminopeptidase M (25) (Table 1). The effect of bestatin was first studied on pallidal membranes incubated in the presence of thiorphan and [^{3}H](Met^5)enkephalin as a substrate, the latter at a 40 nM concentration, in the range of those at which opiate receptor stimulation occurs (39) but which is 3 orders of magnitude lower than K_M values for the two membrane-bound aminopeptidases (25). Under these conditions, the IC_{50} value was close to the K_i value for the "puromycin-sensitive" aminopeptidase, which presumably reflected the great predominance of the latter in cerebral membranes (25). In contrast, the potency of bestatin in inhibiting exogenous enkephalin hydrolysis by the pallidal slice preparation was 7-fold lower, a difference attributable to restricted diffusion and/or degradation of this peptide-like inhibitor inside the tissue. Complete protection of [^{3}H](Met^5)enkephalin was not achieved in either membranes or slices, probably because of the inability of thiorphan and bestatin to inhibit enkephalin-hydrolyzing peptidases such as angiotensin-converting enzyme or dipeptidylaminopeptidases (14). Conversely, when these agents were used together, they provided almost complete protection of the endogenous (Met^5)enkephalin released from pallidal slices, as previously shown with striatal slices (14). In the pallidal slice model, the IC_{50} value for bestatin was significantly higher than that for the inhibition of exogenous enkephalin hydrolysis: conceivably, the effect of the diffusion and degradation of the inhibitor was greater on the endogenous peptide emanating from the inner layers of the slice than on the exogenous peptide, probably hydrolyzed in the outer layers.

The use of puromycin provided information that was easier to interpret because this aminopeptidase inhibitor (26, 27) is more discriminant, displaying a 30-fold difference between its K_i values for each of the two purified aminopeptidases. Furthermore, in the pallidal slices, there was apparently no interference by diffusion or degradation processes. Puromycin inhibited [^{3}H](Met^5)enkephalin hydrolysis by this preparation in a biphasic manner, and the two components displayed IC_{50} values fairly close to those for the purified aminopeptidases. A predominant participation of the "puromycin-sensitive" aminopeptidase (corresponding to 75% of the exogenous enkephalin hydrolysis) was also shown by analysis of the concentration-inhibition curve for puromycin. In the presence of thiorphan, puromycin provided complete protection of endogenous (Met^5)enkephalin, and analysis of the shallow concentration-protection curve clearly suggested the participation of both aminopeptidases, with two IC_{50} values for puromycin close to its K_i values for the purified enzymes (Table 1). This implies that, after the release of (Met^5)enkephalin into the extracellular space of the slices, its concentration did not reach a value significantly above its K_M for the two aminopeptidases, i.e., 18 and 45 μM (25). Interestingly, the contribution of aminopeptidase M to endogenous enkephalin metabolism seemed clearly predominant, corresponding to about 70% of total metabolism. The predominant participation in this process by this enzyme which is poorly sensitive to inhibition by puromycin, probably accounts for the slight nonsignificant protection previously observed with striatal slices when a low concentration of this inhibitor was used in the absence of thiorphan (12).

The anti-aminopeptidase M antibodies are certainly the most selective tool of the three used in the present study, with a more than 300-fold difference between inhibitory potencies for the two purified aminopeptidases. However, restricted diffusion of this high molecular weight agent inside the slices was indicated by the fact that the potency of anti-aminopeptidase M antibodies in inhibiting exogenous enkephalin hydrolysis in pallidal slices was 20 times lower than in membranes or purified aminopeptidase M, both on account of their slow equilibration and of their nonsignificant effect on thicker slices. Whereas the preimmune serum had no inhibitory effect, these antibodies provided a concentration-dependent protection of endogenous enkephalin, and their IC_{50} value was fully consistent with selective inhibition of aminopeptidase M. However, the maximal effect was less marked than with bestatin, presumably because the antibodies did not reach sufficiently high concentrations inside the tissue.

Hence, the effects of all three agents indicate that, in addition

to "enkephalinase," both aminopeptidases contribute to the inactivation of the endogenous (Met^5)enkephalin released from pallidal slices. The larger contribution of aminopeptidase M to this process, mainly suggested by the effects of puromycin, contrasts with the much greater contribution of the "puromycin-sensitive" aminopeptidase toward exogenous enkephalin degradation in cerebral membranes (25). This apparent discrepancy suggests that a close topographic relationship between aminopeptidase M and enkephalin-releasing axon terminals might be responsible for its predominant participation in the inactivation of endogenous enkephalins, a hypothesis that could be checked by immunohistochemical and/or subcellular distribution studies.

Finally, it should be stressed that the above conclusions were mainly derived from the use of brain slices. This *in vitro* model combines two advantages: it preserves the topographic relationships between the sites of endogenous peptide release and inactivation that exist *in vivo* (a crucial factor, as shown in the present study) and allows precise definition of the potencies of available aminopeptidase inhibitors which display limited specificities, or, like the antibodies, are not easy to use *in vivo*. In this model it clearly appears that aminopeptidase M is the most important enzyme for endogenous enkephalin inactivation. However, this model of pallidal slices also has inherent limitations which preclude direct extrapolation to events involved in the physiological inactivation of enkephalins *in vivo*. Highly selective inhibitors that easily enter the brain are therefore required to confirm that aminopeptidase M is also the major enkephalin-inactivating aminopeptidase *in vivo*.

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