Identification and characterization of aminopeptidases from *Aplysia californica*

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Aminopeptidase activities were identified in extracts of kidney, ovotestis, head ganglia, heart and haemolymph of *Aplysia californica*. These enzyme preparations hydrolysed [³H][Leu]enkephalin at the Try-1–Gly-2 bond as determined by h.p.l.c. analysis of cleavage products. In all these tissues, enkephalin-degrading aminopeptidase activities were present both in membrane-bound and cytosolic fractions. The bivalent-cation-chelating agent, 1,10-phenanthroline, inhibited kidney membrane aminopeptidase activity with an IC₅₀ of 30 μM, suggesting that this enzyme is a metalloproteinase. The aminopeptidase inhibitor amastatin was the most potent inhibitor of [Leu]enkephalin degradation (IC₅₀ 25 nM) by membrane-bound aminopeptidase, and bacitracin, bestatin and puromycin were about 100–1000 times less potent. In contrast with membrane-bound aminopeptidase, the cytosolic form is sensitive to puromycin. Angiotensin-converting enzyme inhibitor had no effect on [Leu]enkephalin degradation by kidney membranes, while the neutral endopeptidase inhibitors were poor inhibitors of the enzymes in this preparation. The K_m values of the aminopeptidase in the kidney membranes and cytosolic fractions for the [Leu]enkephalin substrate were 2.4 and 7.4 μM respectively. The aminopeptidase present in the kidney membranes also hydrolysed endogenous Phe-Met-Arg-Phe-amide peptide at the Phe-1–Met-2 bond as well as synthetic alanine *p*-nitroanilide and leucine *p*-nitroanilide. When used in a competition assay, these substrates inhibited hydrolysis of [³H][Leu]enkephalin, suggesting that the same enzyme degraded all these substrates. Taken together, these results suggest that *Aplysia* tissues contain both a membrane-bound aminopeptidase related to the mammalian aminopeptidase N and a cytosolic puromycin-sensitive aminopeptidase.

INTRODUCTION

The marine mollusc *Aplysia californica* is a useful model to study the central nervous system because of the relatively small number and large size of its neurons (Kandel, 1979). By using molecular-biology techniques, a large number of neuropeptides has been identified from *Aplysia* neurons [for reviews see DesGroseillers & Scheller (1988)] and some of the putative peptides derived from the precursor proteins show remarkable sequence similarities to mammalian neuropeptides (Taussig & Scheller, 1986; Wickham & DesGroseillers, 1991).

Secreted neuropeptides can act over short distances within the constrained space of the synapse or travel some distance from their site of release to mediate their physiological functions (Bousfield, 1985). In contrast with chemical neurotransmitters, which can be degraded and/or recaptured at the synapses, recapture does not seem to be a predominant mechanism for terminating the action of neuropeptides. Peptide neurotransmitters rather appear to be mostly degraded by a broad range of relatively non-specific peptidases (Kenny *et al.*, 1987). In the mammalian brain, several neuropeptide-degrading enzymes have been identified. These include a dipeptidyl aminopeptidase (EC 3.4.14.1) (Gorenstein & Snyder, 1979; Gorenstein *et al.*, 1981), an angiotensin-converting enzyme (EC 3.4.15.1) (Erdős *et al.*, 1979), a neutral endopeptidase (enkephalinase, EC 3.4.24.11) (Kerr & Kenny, 1984; Malfroy *et al.*, 1978; Sullivan *et al.*, 1978; Schwartz *et al.*, 1981; De La Baume *et al.*, 1983) and an aminopeptidase (EC 3.4.11.2) (Hersh, 1981, 1985; De La Baume *et al.*, 1983; Hui *et al.*, 1983; Gros *et al.*, 1985; Matsas *et al.*, 1985). These peptidases are also distributed in many tissues other than the brain. They are integral membrane proteins which

possess an active site facing the extracellular space where they are most likely to participate in the metabolism of secreted regulatory peptides. All these peptidases, however, are relatively non-specific and can hydrolyse a wide range of peptides. Conversely each peptide is usually a potential substrate for several proteinases. Thus the physiological role of these peptidases can best be tested by monitoring the pharmacological activity of specific inhibitors.

Selective inhibitors of neutral endopeptidase such as thiorphan (Roques *et al.*, 1980), kelatorphan (Fournié-Zaluski *et al.*, 1984) and (3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)glycine (HACBO-Gly) (Waksman *et al.*, 1985) and of aminopeptidase such as amastatin, bestatin (Umezawa *et al.*, 1976; Aoyagi *et al.*, 1978; Wilkes & Prescott, 1985), bacitracin and puromycin have been described. Some of them have been shown to protect endogenous enkephalins from degradation (Patey *et al.*, 1981) and thus can induce analgesia (Roques *et al.*, 1980; Zhang *et al.*, 1982; Chaillet *et al.*, 1983; De La Baume *et al.*, 1983), clearly establishing neutral endopeptidase and aminopeptidase N as physiologically relevant enkephalin-degrading enzymes.

The post-translational regulation of *Aplysia* neuropeptides, especially their proteolytic degradation and the mechanisms of their inactivation, is still poorly documented. As a first step toward understanding the mechanism of peptide degradation in relation to animal physiology, we began searching for peptidase activity in *Aplysia* organs. Using well-studied peptide substrates such as [Leu]enkephalin and its analogue [D-Ala²-Leu]enkephalin (Pert *et al.*, 1976; Llorens *et al.*, 1982), we have investigated the presence of peptidases in kidney, ovotestis, head ganglia, heart and haemolymph of the mollusc *Aplysia californica*. In addition, Phe-Met-Arg-Phe-amide (FMRFamide) was used to extend these studies to an endogenous *Aplysia*

Abbreviations used: HACBO-Gly (3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)glycine; [³H][Leu]enkephalin, [³H][Leu]enkephalin; [³H][D-Ala²-Leu]enkephalin, [³H][D-Ala²-Leu]enkephalin; FMRFamide, Phe-Met-Arg-Phe-amide; dFMRFamide, D-Phe-Met-Arg-Phe-amide; FdMRFamide, Phe-D-Met-Arg-Phe-amide.

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neuropeptide. This peptide was shown to be involved in muscular contraction (Austin *et al.*, 1982; Weiss *et al.*, 1984; Richmond *et al.*, 1986; Cawthorpe & Lukowiak, 1990) and in modulation of the activity of specific neurons (Abrams *et al.*, 1984; Ocorr & Byrne, 1985; Thompson & Ruben, 1988; Ichinose & McAdoo, 1988; S.-Rozsa *et al.*, 1991; Critz *et al.*, 1991). Here we report for the first time the presence of two different aminopeptidase activities in membrane-bound and cytosolic fractions of *Aplysia* tissues. A preliminary account of this work has been published (Bawab *et al.*, 1991).

MATERIALS AND METHODS

Peptides and chemicals

Peptides Tyr-Gly-Gly and [Leu]enkephalin were purchased from the IAF Biochem. International (Montréal, Canada), L-tyrosine was from Gibco-BRL (Burlington, Canada) and [D-Ala²-Leu]enkephalin was from Peninsula Laboratories (St. Helens, Lancs., U.K.). Purified aminopeptidase N from hog kidney was purchased from Boehringer-Mannheim (Montréal, Canada). FMRFamide, dFMRFamide, FdMRFamide, Phe, Phe-amide, Triton X-114, alanine *p*-nitroanilide and leucine *p*-nitroanilide, amastatin, bacitracin, 1,10-phenanthroline, phosphoramidon, phenylmethanesulphonyl fluoride, pepstatin, leupeptin, soya-bean trypsin inhibitor and aprotinin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Captopril was obtained from Squibb (Princeton, NJ, U.S.A.). Thiorphan, HACBO-Gly, kelatorphan and bestatin were donated by Dr. Bernard Roques (Laboratoire de Chimie Organique, Université de Paris V, Paris, France). The labelled substrate [*tyrosyl*-3,5-³H][D-Ala²-Leu]enkephalin was obtained from the Commissariat à l'Energie Atomique (CEA, Saclay, France) and [*tyrosyl*-3,5-³H][Leu]enkephalin from New England Nuclear (Boston, MA, U.S.A.).

Animals

Aplysia californica weighing 75–150 g were purchased from Marinus (Long Beach, CA, U.S.A.) and maintained in an aerated recirculating-seawater tank at 15 °C for less than 2 weeks before being killed.

Membrane preparations

Tissues from *Aplysia californica* were dissected out and cut into 5–10 mm³ fragments. Tissue fragments (1 g wet weight) were further homogenized at 4 °C with Polytron homogenizer in 5 ml of 10 mM-sodium phosphate buffer, pH 7.5, containing 30 mM-NaCl, 1 mM-MgCl₂ and 0.02% (w/v) NaN₃, using three to five bursts of 10 s each. The homogenate was diluted 5-fold and filtered through nylon gauze. About 25 ml of the homogenate was layered over 10 ml of a 41% (w/v) solution of sucrose in the homogenization buffer and centrifuged at 95000 *g* for 1 h at 4 °C in a Beckman SW 27 rotor (Maeda *et al.*, 1983). The soluble fractions and the white interfacial band of membranes were collected separately. The membranes were pelleted by centrifugation at 95000 *g* for 30 min at 4 °C and stored frozen at –80 °C until use. Haemolymph was collected, and centrifuged for 15 min at 15000 *g*; cells and supernatant were collected separately. The supernatant was further filtered through a 0.22 µm Millipore filter.

Enzyme assays

With radiolabelled substrates. Tissue preparations (0.1–35 µg of protein) were preincubated for 15 min at 25 °C in 100 µl of

50 mM-Tris/HCl, pH 7.5, either alone or with increasing concentrations of inhibitors or competitors. The labelled substrates [³H][D-Ala²-Leu]enkephalin or [³H][Leu]enkephalin (30–40 Ci/mmol) were added to a final concentration of 0.3 pmol. The reaction was stopped by adding 10 µl of 2 M-HCl. The separation of the metabolites from the substrate was performed by chromatography on Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA, U.S.A.) and counted for radioactivity as described (Crine *et al.*, 1985). The kinetic parameters were determined in the absence or presence of increasing concentrations of unlabelled substrate and the *K_m* values were calculated from Eadie-Hofstee plots. The time of reaction was such that the amount of substrate hydrolysed did not exceed 20% of the quantity initially added.

With non-radiolabelled substrates. Kidney membranes (25 µg of protein) were incubated for different periods of time at 25 °C in 100 µl of 50 mM-Tris/HCl, pH 7.5, containing 10 µg of substrates (FMRFamide or its analogues). The reaction was stopped by adding 10 µl of 2 M-HCl. The metabolites of substrate degradation were analysed by reversed-phase h.p.l.c.

With colorimetric substrate. Kidney membranes (5–10 µg of protein) or 60 ng of aminopeptidase N were incubated in 1 ml of 200 mM-glycine/NaOH buffer, pH 8.7, at room temperature for 15 min in the presence or absence of 0.1 mM-thiorphan and 10 µM-amastatin. After the preincubation period, the alanine *p*-nitroanilide or leucine *p*-nitroanilide substrate (150 µM final concentration) was added and the incubation was carried out at room temperature. After 10 min, the reaction was stopped by the addition of 200 µl of 1.5 M-acetate buffer, pH 4.2. Absorbance was measured at 405 nm and the amount of *p*-nitroaniline formed was determined from a calibration curve. Units of enzyme activity are expressed as pmol of *p*-nitroaniline per mg of membrane protein or per mg of commercial aminopeptidase N.

Analysis of [Leu]enkephalin and FMRFamide metabolites

In order to determine the specificity of the enzyme, the labelled enkephalin and the non-labelled FMRFamide metabolites (or those of its analogues) were analysed by reversed-phase h.p.l.c. on a C₁₈ µBondapak column (Waters) (Devault *et al.*, 1988). Elution of the metabolites was performed by washing the column successively with the following buffers at a flow rate of 1 ml/min: For the enkephalin: from 0 to 30 min, an isocratic step of 100% solvent A (0.15 M-Na₂HPO₄/HClO₄, pH 2.85, 2.5% methanol); from 30 to 50 min, a linear gradient from 100% solvent A to 52% solvent A/48% solvent B (methanol); from 50 to 70 min an isocratic step of 52% solvent A/48% solvent B. Fractions (0.5 ml) were collected and counted. For the FMRFamide, the same gradient was used, with minor modifications: step 1 is from 0 to 15 min; step 2, from 15 to 35 min; and step 3, from 35 to 70 min. FMRFamide metabolites were analysed by monitoring the absorbance of the effluent at 214 nm.

Phase separation of membrane-bound and cytosolic enzyme activities with Triton X-114

Solubilization and phase separation of membrane and cytosolic fractions with Triton X-114 were performed as described (Bordier, 1981; Matsas *et al.*, 1985), with minor modifications. Briefly, membrane and cytosolic fractions (400 µg of protein) were incubated for 2 h with 2% Triton X-114 in Tris/HCl, pH 7.5. The unsolubilized materials were eliminated by centrifugation at 30000 *g* for 60 min at 4 °C. The supernatant was warmed up to 30 °C and phase separation was completed at 1500 *g* for 5 min at 30 °C. The detergent-rich phase was washed and both phases were re-extracted as above. The aminopeptidase activity was determined as described above.

RESULTS

Neuropeptide-degrading activity in *Aplysia* tissues

In order to look for the presence of neuropeptide-degrading enzymes in *Aplysia* tissues, we first prepared and incubated membrane or cytosolic fractions from kidney, ovotestis, head ganglia, heart and haemolymph with [³H][Leu]enkephalin as described in the Materials and methods section. Tritiated [Leu]enkephalin is a particularly convenient substrate as the release of the *N*-terminal [³H]tyrosine can easily be followed, as previously performed in mammalian tissues (Palmieri *et al.*, 1989; Benter *et al.*, 1990), allowing us to develop the very sensitive assay for characterizing peptidase activities in *Aplysia*. The [³H][Leu]enkephalin metabolites were separated from uncleaved substrate on Sep-Pak columns and counted for radioactivity. As shown in Table 1, every tested tissue contained an enkephalin-degrading activity which was sensitive to amastatin (10 μ M). In all the tested tissues, the enzyme activity was present in both membrane-bound and cytosolic fractions.

A time-course of [³H][Leu]enkephalin degradation was performed with kidney membranes (not shown). Degradation of the substrate was rapid, with a half-time of 10 min at room temperature. A 10 min incubation falling in the linear portion of the kinetics was therefore chosen for further experiments. The rate of substrate hydrolysis was determined over a range of [Leu]enkephalin concentrations for both membrane and cytosolic fractions from the kidney. The K_m values were calculated from Eadie-Hofstee plots (Fig. 1) and were found to be $2.4 \pm 0.5 \mu$ M and $7.4 \pm 0.8 \mu$ M respectively ($n = 3$). Taken together, these results indicate that *Aplysia* tissues contain membrane-bound and cytosolic forms of enzymes capable of degrading [Leu]enkephalin.

Characterization of enkephalin metabolites

To characterize the enkephalin-degrading activity in *Aplysia* tissues further, membranes isolated from kidney were incubated with [³H][Leu]enkephalin and the reaction metabolites were analysed by reversed-phase h.p.l.c. Hydrolysis of [³H][Leu]-

Fig. 1. Michaelis-Menten and Eadie-Hofstee plots of the [³H][Leu]-enkephalin degradation by *Aplysia* kidney membranes (a) and cytosolic fractions (b).

The enzyme activity was measured for 10 min in the presence of increasing concentrations of unlabelled substrate. The data represent one experiment, but two other experiments gave the same results.

Table 1. Aminopeptidase specific activity in different tissues of *Aplysia californica*

Enzyme activity was measured as described in the Materials and methods section with [³H][Leu]enkephalin as substrate. Values are means $\pm$ S.E.M. of four to six experiments performed in duplicate.

Tissue	Activity (pmol/mg of protein)	Percentage inhibition by 10 μ M-amastatin
Kidney		
Membranes	3565 $\pm$ 381	97 $\pm$ 1
Cytosolic fractions	1153 $\pm$ 182	87 $\pm$ 1
Ovotestis		
Membranes	560 $\pm$ 113	89 $\pm$ 3
Cytosolic fractions	4080 $\pm$ 895	88 $\pm$ 3
Head ganglia		
Membranes	664 $\pm$ 84	83 $\pm$ 2
Cytosolic fractions	2513 $\pm$ 586	88 $\pm$ 3
Heart		
Membranes	135 $\pm$ 51	83 $\pm$ 8
Cytosolic fractions	837 $\pm$ 109	72 $\pm$ 7
Haemolymph		
Cells	217 $\pm$ 96	54 $\pm$ 2
Soluble fractions	44 $\pm$ 7	77 $\pm$ 1

enkephalin by membranes isolated from *Aplysia* kidney released a single radioactive metabolite which co-migrated with the free amino acid tyrosine (Fig. 2). The same result was obtained with the membrane-bound enzyme from ovotestis and head ganglia and with the enzyme found in haemolymph and kidney cytosolic fractions. No other peak was seen at any other position. In order to ensure that the free [³H]tyrosine was really the result of an aminopeptidase activity splitting the Tyr-Gly bond and not the product of progressive degradation by a carboxypeptidase activity, we repeated the reversed-phase h.p.l.c. analysis at various incubation times. As shown in Fig. 2(a), the first and only metabolite recovered after an incubation period as short as 15 s co-migrates with free tyrosine. As expected for an aminopeptidase activity, this tyrosine peak progressively increased with the reaction time (Figs. 2b and 2c). There was no transient appearance of any other labelled degradation products, excluding the possibility of a progressive degradation of the substrate by a carboxypeptidase. These results demonstrate that the major enkephalin-degrading enzyme in *Aplysia* tissues is an aminopeptidase. The identification of an aminopeptidase in kidney, ovotestis, head ganglia, heart and haemolymph concurs with the presence of a similar enzyme in many mammalian tissues (Kenny & Maroux,

Fig. 2. Reversed-phase h.p.l.c. analysis of tritiated metabolites of $[^3\text{H}][\text{Leu}]\text{enkephalin}$ incubated in the presence of kidney membranes
Aplysia kidney membranes were incubated with $[^3\text{H}][\text{Leu}]\text{enkephalin}$ for (a) 15 s, (b) 60 s or (c) 15 min. Arrows indicate the elution position of standard amino acid. The broken line represents the methanol gradient used in the h.p.l.c.

Table 2. Action of peptidase inhibitors on aminopeptidase activity in kidney membranes from *Aplysia californica*

Kidney membranes from *Aplysia* were incubated before the addition of the $[^3\text{H}][\text{Leu}]\text{enkephalin}$ in the absence or presence of various peptidase inhibitors. The enzyme assays were performed as indicated in the Materials and methods section. Results are expressed as a percentage of substrate degradation. Values are means $\pm$ S.E.M. for three or four experiments performed in duplicate.

Inhibitor	Concentration	Percentage inhibition
Soya-bean trypsin inhibitor	400 $\mu\text{g/ml}$	Not detected
PMSF	100 μM	Not detected
Aprotinin	1 μM	14 $\pm$ 5
Leupeptin	1 μM	Not detected
Pepstatin	1 μM	Not detected
1,10-Phenanthroline	100 μM	63 $\pm$ 7
Phosphoramidon	1 μM	10 $\pm$ 2
	100 μM	49 $\pm$ 8
Thiorphan	1 μM	3 $\pm$ 1
	100 μM	24 $\pm$ 1
Kelatorphan	1 μM	71 $\pm$ 3
	100 μM	91 $\pm$ 2
HACBO-Gly	1 μM	7 $\pm$ 1
	100 μM	50 $\pm$ 2
Captopril	10 μM	Not detected

Fig. 3. Effect of aminopeptidase inhibitors on $[^3\text{H}][\text{Leu}]\text{enkephalin}$ degradation with membranes from *Aplysia* kidney

Membranes isolated from kidney were preincubated at 25 $^{\circ}\text{C}$ for 15 min in the absence or presence of different concentrations of aminopeptidase inhibitors before the addition of substrate. Data are expressed as the percentage inhibition of substrate degradation in the presence of inhibitors as compared with peptide degradation in the absence of inhibitors. The values are means $\pm$ S.E.M. for three to six experiments performed in duplicate. $\bullet$, Amastatin; $\blacksquare$, bacitracin; $\triangle$, bestatin; $\circ$, puromycin; $\blacktriangle$, 1,10-phenanthroline.

1982; Hui *et al.*, 1983; Gros *et al.*, 1985; Kenny *et al.*, 1987; McLellan *et al.*, 1988; Furuhashi *et al.*, 1988; Amoscato *et al.*, 1989).

Inhibitor profile of the aminopeptidase activity in *Aplysia* kidney membranes

Various peptidase inhibitors were next tested for their ability to inhibit $[^3\text{H}][\text{Leu}]\text{enkephalin}$ degradation by kidney membranes. As seen in Table 2, trypsin inhibitors as well as serine, thiol and aspartyl proteinase inhibitors have little or no effect on $[^3\text{H}][\text{Leu}]\text{enkephalin}$ degradation. However, the bivalent-cation-chelating agent 1,10-phenanthroline inhibited the aminopeptidase activity with an IC_{50} value of 30 μM (Fig. 3), suggesting that this aminopeptidase activity is due to a metal-binding enzyme.

Metalloproteinase inhibitors were then used to characterize further the enzyme activity present in the kidney membranes. Fig. 3 shows the inhibition curves of this enzyme by different aminopeptidase inhibitors. Amastatin was the most potent inhibitor of the *Aplysia* aminopeptidase activity, inhibiting the hydrolysis of the $[^3\text{H}][\text{Leu}]\text{enkephalin}$ substrate with an IC_{50} value of 25 nM. Bestatin, bacitracin and puromycin were significantly less potent inhibitors, with IC_{50} values of 2.5 μM , 6 μM and 22 μM respectively. Kelatorphan, which in mammalian tissues completely inhibits enkephalin metabolism as a result of its effect on both aminopeptidase and neutral endopeptidase activities, inhibited 91 % of the *Aplysia* aminopeptidase activity when used at a concentration of 100 μM .

In contrast, neutral endopeptidase inhibitors phosphoramidon, thiorphan and HACBO-Gly inhibited less than 10 % of $[\text{Leu}]\text{enkephalin}$ degradation, when used at a concentration of 1 μM (Table 2). However, when used at a concentration of 100 μM , up to 50 % inhibition could be observed. Considering that these compounds inhibit mammalian neutral endopeptidase with K_i in the nanomolar range (Kenny *et al.*, 1987), this effect observed at high concentrations on the *Aplysia* enzymic activity is most probably non-specific. Furthermore, h.p.l.c. analysis of the metabolites obtained in the presence of 100 μM showed a

Table 3. Distribution of aminopeptidase activities after phase separation of membrane and cytosolic fractions with Triton X-114

Kidney membrane and cytosolic fractions were treated with Triton X-114 as described in the Materials and methods section. Inhibitor was present at a concentration of 10 μ M. The results are means of three to four experiments performed in duplicate.

		Detergent-rich phase			Detergent-poor phase		
		Percentage of activity	Percentage inhibition		Percentage of activity	Percentage inhibition	
			Amastatin	Puromycin		Amastatin	Puromycin
<i>Aplysia</i>	Membranes	34 $\pm$ 2	96 $\pm$ 2	29 $\pm$ 2	66 $\pm$ 2	86 $\pm$ 4	30 $\pm$ 6
	Cytosolic fraction	18 $\pm$ 6	96 $\pm$ 4	86 $\pm$ 5	82 $\pm$ 6	82 $\pm$ 2	73 $\pm$ 4
Rat	Membranes	55 $\pm$ 3	95 $\pm$ 1	9 $\pm$ 3	45 $\pm$ 3	93 $\pm$ 4	7 $\pm$ 4

Fig. 4. Reversed-phase h.p.l.c. analysis of metabolites of FMRFamide incubated in the presence of kidney membranes

Aplysia kidney membranes were incubated with FMRFamide for (a) 5 min, (b) 30 min or (c) 120 min. (d) FMRFamide degradation in the presence of commercial aminopeptidase N. Arrows indicate the elution position of standard amino acids. The dashed line represents the methanol gradient used in the h.p.l.c.

decrease in the amount of [3 H]tyrosine metabolite (results not shown), suggesting inhibition of the aminopeptidase rather than a neutral endopeptidase-like activity. Finally, the angiotensin-converting enzyme inhibitor, captopril, tested at 10 μ M, had no effect on the enzymic activity in kidney membranes from *Aplysia*. Taken together, these results suggest that the *Aplysia* kidney

contains an aminopeptidase activity with an inhibitor profile similar to that of mammalian aminopeptidase N (EC 3.4.11.2). In mammals, this enzyme has been found in plasma membranes from pig synapses, pig vascular endothelium and smooth muscle, as well as in human cerebrospinal fluid (Matsas *et al.*, 1985; Palmieri *et al.*, 1989; Benter *et al.*, 1990).

Table 4. Aminopeptidase activity

Enzyme activity was measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three to six experiments performed in duplicate.

Substrate	Parameter measured	<i>Aplysia</i> kidney membrane	Rat kidney membrane	Amino-peptidase N
$[^3\text{H}][\text{Leu}]\text{-enkephalin}$	Degradation (pmol/mg of protein)	3561 $\pm$ 381	1611 $\pm$ 32	6875 $\pm$ 50
	Percentage inhibition by 10 μM -amastatin	97 $\pm$ 1	98 $\pm$ 1	96 $\pm$ 1
Ala- <i>p</i> -nitroanilide	Degradation ($\mu\text{mol/mg}$ of protein)	0.04 $\pm$ 0.01	0.8 $\pm$ 0.05	62 $\pm$ 5
	Percentage inhibition by 10 μM -amastatin	88 $\pm$ 2	96 $\pm$ 2	92 $\pm$ 2
Leu- <i>p</i> -nitroanilide	Degradation ($\mu\text{mol/mg}$ of protein)	0.2 $\pm$ 0.01	0.8 $\pm$ 0.02	39 $\pm$ 1
	Percentage inhibition by 10 μM -amastatin	60 $\pm$ 3	98 $\pm$ 1	79 $\pm$ 2

Characterization of the membrane-bound and cytosolic aminopeptidase activities

The membrane-bound aminopeptidase activity was further studied to determine if the enzyme is an integral or a peripheral membrane protein. Isolated kidney membranes were washed in the presence of 50 mM-Tris/HCl, pH 7.5, either alone or in the presence of 1 M-NaCl. This high-salt concentration has often been used to solubilize peripheral proteins bound to membranes by ionic interactions (Hersh, 1985; Dyer *et al.*, 1990). After membrane centrifugation, none of the membrane-bound aminopeptidase activity was released into the supernatant (not shown), suggesting that the *Aplysia* enzyme is an integral membrane protein. To prove this point further, we took advantage of the property of solutions of Triton X-114 to separate it into detergent-rich and detergent-poor phases at 30 °C (Bordier, 1981). Membrane and cytosolic fractions were each incubated with Triton X-114 and the detergent phases separated at 30 °C as described in the Materials and methods section. As seen in Table 3, 34 $\pm$ 2 % of the membrane-bound activity was recovered in the detergent-rich phase, demonstrating that a sizeable proportion of the activity recovered in the membrane fraction corresponds to an integral membrane protein. This membrane-bound activity in *Aplysia* is largely puromycin-insensitive, suggesting the presence of an enzyme similar to mammalian aminopeptidase N. The enzymic activity recovered in the detergent-poor phase showed the same inhibition profile. When cytosolic fractions were phase-separated in Triton X-114, 82 $\pm$ 6 % of the enzymic activity was recovered, as expected, in the detergent-poor phase. In contrast with the membrane-bound enzymic activity, this one was inhibited by puromycin, clearly demonstrating that different aminopeptidases are present in the membrane-bound and cytosolic fractions. As control, we have isolated rat kidney membranes and proceeded to the phase separation using Triton X-114 as before: 55 $\pm$ 3 % and 45 $\pm$ 3 % of the aminopeptidase activity was recovered in the detergent-rich and detergent-poor phases respectively, and, as in *Aplysia*, both activities are puromycin-insensitive (Table 3). This is also consistent with the previous observations that 62 % of the aminopeptidase activity in pig kidney microvillar membranes was recovered in the detergent-rich Triton X-114 phase (Matsas *et al.*, 1985); however, in brain synaptic membranes, only 5.5 % of the aminopeptidase activity, which corresponds to aminopeptidase N, was recovered in the detergent-rich phase.

Degradation of *Aplysia* endogenous FMRFamide peptide

To analyse the peptide specificity of the aminopeptidase activity, the hydrolysis of another natural peptide was explored. Kidney membranes were incubated with the *Aplysia* endogenous FMRFamide peptide and the metabolites analysed by reversed-phase h.p.l.c. Synthetic markers and hydrolysis of FMRFamide by commercial aminopeptidase N were used to determine the position of major metabolites. Hydrolysis of FMRFamide by *Aplysia* kidney membranes revealed two peaks which co-migrated with the free *N*-terminal amino acid phenylalanine and the C-terminal MRFamide peptide (Figs. 4a, 4b and 4c). This h.p.l.c. profile is identical with the degradation of FMRFamide by commercial mammalian aminopeptidase N (Fig. 4d). These peaks were detected after an incubation period of 5 min (Fig. 4a) and progressively increased with the reaction time (Figs. 4b and 4c). The appearance of other peaks after a long incubation period of 120 min may be due to further degradation of MRFamide by the aminopeptidase. Competition experiments showed that FMRFamide inhibited more than 90 % of the hydrolysis of $[^3\text{H}][\text{Leu}]\text{enkephalin}$ by the aminopeptidase activity, suggesting that the aminopeptidase activity acting on FMRFamide is the same as the one previously shown to degrade $[\text{Leu}]\text{enkephalin}$. Taken together, these results indicate that the *Aplysia* aminopeptidase, like the mammalian aminopeptidase N, is not enkephalin-specific and can hydrolyse different peptides. Furthermore, the aminopeptidase activity is not restricted to the cleavage of the Tyr-Gly bond, since other *N*-terminal amino acids can be cleaved by this enzyme (see also below).

Degradation of *p*-nitroanilide derivatives by *Aplysia* kidney aminopeptidase

To characterize the *Aplysia* aminopeptidase activity further, two other substrates often used to study mammalian aminopeptidases were tested (Little *et al.*, 1976; Amoscato *et al.*, 1989). As shown in Table 4, both alanine-*p*-nitroanilide and leucine-*p*-nitroanilide were hydrolysed by *Aplysia* kidney membranes. A similar activity was also observed with rat kidney membranes and with purified aminopeptidase N. The degradation of these substrates was inhibited by the aminopeptidase inhibitor amastatin at 10 μM , at a level comparable with the one found for the inhibition of $[\text{Leu}]\text{enkephalin}$ degradation. Competition experiments showed that both synthetic substrates inhibited the hydrolysis of $[^3\text{H}][\text{Leu}]\text{enkephalin}$ by the aminopeptidase activity in a dose-dependent manner; when used at 150 μM , Leu-*p*-nitroanilide and Ala-*p*-nitroanilide inhibited 89 % and 100 % of the aminopeptidase activity respectively. These results suggest again that the aminopeptidase activity acting on *p*-nitroanilide derivatives is the same as the one previously shown to degrade $[\text{Leu}]\text{enkephalin}$. They also further demonstrate that *Aplysia* aminopeptidase, like mammalian aminopeptidase N, is not restricted to the cleavage of the Tyr-Gly bond.

Degradation of peptides with dextrorotatory amino acids

Taken together, our results indicate that aminopeptidases are the major peptide-degrading enzymes in *Aplysia* tissues, and no other peptidase activity has been detected under these conditions. However, any other metabolites specifically generated by carboxypeptidases and/or endopeptidases will not be detected if they are further cleaved by the strong aminopeptidase activity. In order to look for the presence of other peptide-degrading enzymes in *Aplysia* tissues, we next used $[^3\text{H}][\text{D-Ala}^2\text{-Leu}]\text{enkephalin}$ as an alternative substrate. This peptide has been shown to be protected from mammalian aminopeptidase N degradation by the presence of the D-enantiomer of alanine (Pert *et al.*, 1976; Llorens *et al.*, 1982). Surprisingly, reversed-phase h.p.l.c. analysis

Fig. 5. Reversed-phase h.p.l.c. analysis of degradation metabolites of substrates containing dextrorotatory amino acids, incubated in the presence of kidney membranes

Aplysia kidney membranes were prepared, incubated with (a) [^3H]Leu]enkephalin, (b) [^3H][D-Ala²-Leu]enkephalin, (c) dFMRFamide and (d) FdMRFamide and subjected to reversed-phase h.p.l.c. Arrows indicate the elution position of standard peptides. The dashed line represents the methanol gradient used in the h.p.l.c.

of the metabolites obtained after digestion of [^3H][D-Ala²-Leu]enkephalin with membrane-bound fractions prepared from *Aplysia* kidney showed the presence of a peak of radioactive metabolites which co-migrated with free tyrosine (Fig. 5b). Identical results were obtained with the membrane-bound activity from ovotestis and head ganglia. These results indicate that, in contrast with mammalian aminopeptidase N, the D-enantiomer of alanine could not completely protect the substrate from degradation by the *Aplysia* aminopeptidase and consequently did not allow us to detect other peptide-degrading enzymes. Hydrolysis of [^3H][D-Ala²-Leu]enkephalin with membranes from rat kidney under the same conditions produced a single radioactive metabolite co-migrating with the tripeptide Tyr-D-Ala-Gly (not shown). This metabolite resulted from degradation of the substrate by neutral endopeptidase (Llorens *et al.*, 1982); this activity was sensitive to neutral endopeptidase inhibitors (not shown).

This interesting point that the enzyme can cleave a peptide with a dextrorotatory amino acid has been extended by examining by reversed-phase h.p.l.c. the hydrolysis of dFMRFamide and

FdMRFamide. In competition assays, both substrates inhibited 83–96% of the hydrolysis of [^3H]Leu]enkephalin by the aminopeptidase activity, showing that they are both recognized by the membrane-bound aminopeptidase. Under the same conditions as those used for the hydrolysis of FMRFamide (see above), we showed that dFMRFamide can be cleaved by the aminopeptidase activity in the kidney membranes (Fig. 5c), suggesting that an N-terminal dextrorotatory amino acid does not protect the peptide from hydrolysis in *Aplysia* kidney. On the other hand, FdMRFamide, a peptide with a penultimate D-methionine, was poorly cleaved (Fig. 5d), suggesting that the identity of the penultimate dextrorotatory amino acid (D-alanine as compared with D-methionine) may influence the *Aplysia* aminopeptidase activity. As control, commercial aminopeptidase N degraded neither dFMRFamide nor FdMRFamide, even after a 2 h incubation time (results not shown). Together, these results indicate that, in contrast with mammalian aminopeptidase N, the presence of an N-terminal or penultimate D-amino acid could not completely protect the substrate from degradation by the membrane-bound *Aplysia* aminopeptidase.

DISCUSSION

In the present paper we report for the first time the presence of aminopeptidase activities in membrane-bound and cytosolic fractions isolated from different *Aplysia* tissues. Partial characterization of these activities was obtained by monitoring the hydrolysis of natural and synthetic substrates. The *Aplysia* membrane-bound aminopeptidase activity has the same inhibitor profile as the mammalian aminopeptidase N, an ectoenzyme physiologically involved in the extracellular metabolism of enkephalins *in vivo* (De La Baume *et al.*, 1983). Its characterization as an integral membrane protein further supports the hypothesis that the *Aplysia* activity may also be involved in the metabolism of secreted peptides. On the other hand, the cytosolic aminopeptidase has a different inhibitor profile, similar to the puromycin-sensitive cytosolic aminopeptidases from rat brain (Dyer *et al.*, 1990; Hui *et al.*, 1988; McLellan *et al.*, 1988; Mantle *et al.*, 1990). Both membrane-bound and cytosolic forms described in this paper have, however, a different inhibitor profile from a leucine aminopeptidase-like enzyme described in *Aplysia* haemolymph (Squire *et al.*, 1991). Because of its internal localization, the cytosolic activity, although important for the general cellular protein catabolic process, is probably not relevant to the synaptic metabolism of peptides after their secretion. The role played by soluble peptidases present in the extracellular fluid surrounding neural cells is less clear.

These results suggest that in molluscs, as in mammals, membrane-bound aminopeptidases may play important physiological roles in the degradation of endogenous peptides, such as with FMRFamide in *Aplysia*. It has been shown that a minimum of four amino acid residues are required for efficient binding of FMRFamide-related peptides to the FMRFamide receptors (Payza, 1987). Removal of the *N*-terminal amino acid by an aminopeptidase would provide a means of terminating the action of this peptide, by decreasing its affinity for the receptor. Oxidation of the penultimate methionine might be an alternative inactivation mechanism in FMRFamide-mediated neurotransmission (Price, 1986; Brussaard *et al.*, 1989). Other peptides, such as those involved in the regulation of renal, neuronal and/or reproductive functions, may also be good substrates for peptidases in *Aplysia*. Indeed, it has been shown that *Aplysia* pedal peptide, a peptide involved in locomotion, is rapidly degraded after release by peptidases present in haemolymph and foot tissues (Hall & Lloyd, 1990).

When used at a concentration of 0.1 mM, neutral endopeptidase inhibitors (phosphoramidon, thiorphan and HACBO-Gly) inhibited up to 50% of the aminopeptidase activity. This result shows that a modification in physiological phenomenon or cellular behaviour after treatment *in vivo* with neutral endopeptidase inhibitors should be interpreted with caution when high concentrations of these inhibitors are used and especially if the metabolites are not characterized, as in the previous study by Shipp *et al.* (1990).

Surprisingly the enzyme from *Aplysia* was able to hydrolyse substrates known to be resistant to mammalian aminopeptidases because of the presence of a dextrorotatory amino acid (this work; Pert *et al.*, 1976; Llorens *et al.*, 1982). We do not know the significance of the cleavage next to a dextrorotatory amino acid. This characteristic may be a vestige of evolution since dextrorotatory amino acid enantiomers are found in fungi and bacteria (Grafstein, 1983, 1984). On the other hand, this enzyme could be involved in the physiological degradation of a still unknown molluscan peptide containing such an amino acid. In this respect, it is noteworthy that demorphin, an active peptide containing a penultimate D-alanine (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-amide)

has been described in frog skin (Montecucchi *et al.*, 1981; Melchiorri *et al.*, 1984).

The identification of the physiologically relevant peptides in *Aplysia* can, however, only come from pharmacological studies using specific inhibitors for potentiating the biological activity of these peptides. These studies will be important for determining the biological implications of proteinases in controlling peptide-mediated phenomena in the simple integrated model of peptidergic pathways provided by this mollusc.

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