Identification and characterization of a neutral endopeptidase activity in *Aplysia californica*

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Kidney plasma membranes of *Aplysia californica* were shown to contain an endopeptidase activity which cleaved [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu) and [Leu]enkephalinamide (Tyr-Gly-Gly-Phe-Leu-NH_2) at the Gly^3–Phe^4 bond, as determined by reverse-phase h.p.l.c. analysis of metabolites. The optimal pH was shown to be 6.5. The bivalent cation chelating agent 1,10-phenanthroline protected [Leu]enkephalin from degradation, suggesting that this enzyme is a metallopeptidase. The degradation of [Leu]enkephalin was also abolished by the neutral endopeptidase-24.11 inhibitors RB104 {2-[(3-iodo-4-hydroxy)-phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-phenylmethyl)-propyl]amino-4-oxobutanoic acid}, HABCO-Gly [(3-hydroxy-aminocarbonyl-2-benzyl-1-oxypropyl)glycine], phosphoramidon and thiorphan, with IC_{50} values of 1 nM, 1 μM, 20 μM and 30 μM respectively. By contrast, the angiotensin-converting enzyme inhibitor captopril and the serine proteinase inhibitor phenylmethanesulphonyl fluoride were without effect. Phase separation experiments using Triton X-114 showed that about 64% of the neutral endopeptidase activity in the *Aplysia* kidney membrane corresponds to an integral membrane protein. A specific radioiodinated inhibitor ([^{125}I]RB104) was shown to bind the *Aplysia* endopeptidase with high affinity; the K_D and $B_{max.}$ values were 21 ± 5 pM and 20.3 ± 5 fmol/mg of proteins respectively. This inhibitor was used to determine the molecular form of the enzyme, after separation of solubilized membrane proteins on SDS/PAGE and transfer on to nitrocellulose membranes. A single protein band with an apparent molecular mass of 140 kDa was observed. The labelling was abolished by specific neutral endopeptidase inhibitors. This study provides the first biochemical characterization of an endopeptidase with catalytic properties similar to those of neutral endopeptidase-24.11 in the mollusc *Aplysia californica*.

INTRODUCTION

Degradation of secreted neuropeptides by a broad range of relatively non-specific membrane-bound peptidases appears to be the mechanism by which local peptide concentration is regulated (Kenny et al., 1987). A limited number of peptidases have been identified (reviewed in Kenny et al., 1987); they are widely distributed in the central nervous system (CNS) and peripheral tissues, where they appear as integral membrane proteins with their active site facing the extracellular space. Each class of peptidases can degrade a variety of peptides which, in turn, can be degraded by more than one peptidase. Neutral endopeptidase (EC 3.4.24.11; NEP; neprilysin), also known as enkephalinase in the CNS (Malfroy et al., 1978) and common acute lymphoblastic leukaemia antigen (CALLA) in haematopoietic tissues (Letarte et al., 1988), has a relatively broad specificity and generally cleaves peptides on the N-terminal side of hydrophobic residues. NEP-like activities have been observed in many species, including molluscs (Shipp et al., 1990; Owens et al., 1992) and insects (Isaac, 1987, 1988).

Selective inhibitors of NEP, such as thiorphan, phosphoramidon, (3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)-glycine (HACBO-Gly) and 2-[(3-iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-phenylmethyl)-propyl]amino-4-oxobutanoic acid (RB104), have been used to characterize the enzyme and/or to study its role in peptide metabolism *in vivo*. For example, radiolabelling studies with [^{3}H]HACBO-Gly, [^{3}H]thiorphan or [^{125}I]RB104 were used to map the enzyme in the CNS (Waksman et al., 1986; Pollard et al., 1987) and to detect nanogram quantities of the enzyme after separation of membrane proteins by SDS/PAGE (Fournié-Zaluski et al., 1992). Furthermore, NEP inhibitors have been shown to prolong endogenous enkephalin and atrial natriuretic peptide action *in vivo*, clearly establishing the physiological role of NEP in nociception and hypertension (Roques et al., 1980; Patey et al., 1981; Zhang et al., 1982; Chaillet et al., 1983; De La Baume et al., 1983; Meynadier et al., 1987; Margulies et al., 1990).

Understanding of the physiological role of neuropeptide-degrading enzymes could benefit from the existence of studies on a simple, well-integrated peptidergic system. The mollusc *Aplysia californica* has been extensively used to study the neuronal mechanisms which underlie behavioural states. The relatively small number and large size of its component neurons (Kandel, 1979) make the *Aplysia* nervous system a particularly useful model for cellular and molecular studies. A large number of *Aplysia* neuropeptides have been identified using techniques of biochemistry and/or molecular biology (DesGroseillers and Scheller, 1988) and some of the peptides show remarkable sequence similarities to mammalian neuropeptides (Rajpara et al., 1992; Taussig and Scheller, 1986; Wickham and DesGroseillers, 1991).

Abbreviations used: NEP, neutral endopeptidase (EC 3.4.24.11); HACBO-Gly, (3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)glycine; RB104, 2-[(3-iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-phenylmethyl)propyl]amino-4-oxobutanoic acid; CALLA, common acute lymphoblastic leukaemia antigen; [^{3}H][Leu]enkephalin: [*tyrosyl*-3,5-^{3}H][Leu]enkephalin; PMSF: phenylmethanesulphonyl fluoride; RP-h.p.l.c., reverse-phase h.p.l.c.; PI-PLC, phosphatidylinositol-specific phospholipase C; TFA, trifluoroacetic acid.

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In a previous study, we have identified and characterized a membrane-bound amastatin-sensitive aminopeptidase N-like activity in several *Aplysia* tissues, as well as a cytosolic puromycin-sensitive aminopeptidase (Bawab et al., 1992). We now report on the identification and characterization of a neutral endopeptidase activity in plasma membrane preparations of kidney from *Aplysia californica*. We first monitored the degradation of [^{3}H][Leu]enkephalin in the presence of amastatin, a potent inhibitor of the aminopeptidase activity. This assay allowed us to determine that a second cleavage of [Leu]enkephalin by plasma membrane preparations takes place at the Gly^3–Phe^4 bond. Degradation of [Leu]enkephalinamide confirmed that this enzyme is an endopeptidase. The inhibitor profile of this enzymic activity was similar to that of mammalian NEP. Further characterization of the enzyme was carried out with the radioiodinated inhibitor [^{125}I]RB104 (Fournié-Zaluski et al., 1992), which bound to a 140 kDa protein after separation of membrane proteins by gel electrophoresis. The labelling was displaced by NEP inhibitors.

MATERIALS AND METHODS

Peptides and chemicals

Peptides Tyr-Gly-Gly and [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu) were purchased from IAF Biochem International Inc. (Montréal, Canada); L-tyrosine was from Gibco–BRL (Burlington, Canada); amastatin, 1,10-phenanthroline, phosphoramidon, phenylmethanesulphonyl fluoride (PMSF), Triton X-114, 1-*O*-*n*-octyl β-D-glucopyranoside (octyl glucoside), Phe-Leu amide and [Leu]enkephalinamide (Tyr-Gly-Gly-Phe-Leu-NH_2) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Captopril was obtained from Squibb (Princeton, NJ, U.S.A.). Thiorphan, HACBO-Gly and RB104 were prepared as described (Roques et al., 1980; Waksman et al., 1986; Fournié-Zaluski et al., 1992). The labelled substrate [*tyrosyl*-3,5-^{3}H][Leu]enkephalin was obtained from New England Nuclear (Boston, MA, U.S.A.). Na^{125}I was purchased from Amersham (Oakville, Ontario, Canada). Phosphatidylinositol-specific phospholipase C (PI-PLC), isolated from *Bacillus cereus*, was purchased from Boehringer–Mannheim Canada (Laval, Québec, Canada).

Animals

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

Plasma membrane preparations

Tissues from *Aplysia californica* were dissected out, homogenized at 4 °C with a Polytron homogenizer and centrifuged at 95000 *g* for 1 h at 4 °C in a Beckman SW 27 rotor through a 41 % (w/v) sucrose cushion (Maeda et al., 1983). The white interfacial band of plasma membranes was collected and pelleted by centrifugation at 95000 *g* for 30 min at 4 °C as described before (Bawab et al., 1992).

Enzyme assays and metabolite analysis

Kidney membrane preparations (5–8 μg of protein) were preincubated for 15 min at 25 °C in 100 μl of 50 mM Mes, pH 6.5, in the presence of amastatin at a concentration of 10 μM, alone or with different peptidase inhibitors. The labelled substrate [^{3}H][Leu]enkephalin (30–40 Ci/mmol) was added to a final concentration of 0.3 pM. The reactions were stopped by adding 10 μl of 2 M HCl (Crine et al., 1985). The separation of the metabolites from the substrate was performed by h.p.l.c. Elution of the metabolites was performed by washing the column successively with the following buffers at a flow rate of 1 ml/min: from 0 to 30 min, an isocratic step of 100 % solvent A [0.15 M $Na_2HPO_4/HClO_4$, pH 2.85, 2.5 % (v/v) methanol]; from 30 to 40 min, a linear gradient from 100 % solvent A to 52 % solvent A/48 % solvent B (methanol); from 40 to 50 min, an isocratic step of 52 % solvent A/48 % solvent B; and from 50 to 60 min, a linear gradient from 52 % solvent A/48 % solvent B to 100 % solvent B. Fractions (0.5 ml) were collected and counted for radioactivity.

A 20 μg sample of the unlabelled substrate [Leu]enkephalinamide was incubated in 50 mM Mes, pH 6.5, for 30 min with kidney membrane preparations (20 μg of protein) and the metabolites were separated by reverse-phase h.p.l.c. (RP-h.p.l.c.) on a Nova-Pak C_8 column. A linear gradient from 100 % solvent A [0.1 % trifluoroacetic acid (TFA) in water] to 40 % solvent A/60 % solvent B (80 % acetonitrile/0.1 % TFA) was developed for 30 min at a flow rate of 1 ml/min.

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

Solubilization and phase separation of membrane fractions with Triton X-114 were performed as described (Bawab et al., 1992). The endopeptidase activity in each phase was determined by RP-h.p.l.c. as described above.

RB104 iodination and purification

RB104 was iodinated by the chloramine T method (Greenwood and Hunter, 1963). RB104 (5 μg) was dissolved in 20 μl of 5 mM phosphate buffer, pH 6.0, and iodinated in the presence of 0.5 mCi of Na^{125}I and 5 μl of chloramine T (2 mg/ml). After an incubation of 10 s at room temperature, the reaction was stopped with 5 μl of sodium metabisulphite (4 mg/ml). NaOH (10 M) was then added until the pH reached 10. The solution was left for 5 min at room temperature and then the pH was re-adjusted to 1.5 with HCl (6 M). The iodinated mixture was diluted with 1 ml of 0.5 % TFA in an aqueous solution and applied to a Sep-Pak C_{18} cartridge to eliminate excess reagents. The iodinated RB104 was then eluted with a 50 % solution of acetonitrile in 0.5 % TFA. [^{125}I]RB104 was further purified by RP-h.p.l.c. using a C_{18} μBondapak column (Waters Associates, Milford, MA, U.S.A.). It was eluted by washing the column successively with the following buffers: from 0 to 40 min, a linear gradient from 100 % solvent A (0.5 % TFA) to 50 % solvent A/50 % solvent B (100 % acetonitrile); from 40 to 45 min, an isocratic step of 50 % solvent A/50 % solvent B; from 45 to 50 min, a linear gradient from 50 % solvent A/50 % solvent B to 100 % solvent B. Fractions (0.5 ml) were collected and radioactivity was counted. The radioactive peak which coeluted with the unlabelled monoiodinated RB104 was collected and used in the binding assays.

Binding of [^{125}I]RB104 in kidney membranes

Membrane preparations from *Aplysia* kidneys (about 40 μg of protein) were incubated for 1 h at 25 °C in 0.5 ml of Tris/HCl, pH 7.5, containing 40 pM [^{125}I]RB104, alone or combined with other NEP inhibitors. Membrane-bound [^{125}I]RB104 was collected by centrifugation at 30000 *g* for 20 min. The resulting

pellets were washed and radioactivity was counted on a γ-counter.

Molecular identification of [^{125}I]RB104 binding proteins in *Aplysia* kidney

Membrane preparations from *Aplysia* kidneys were solubilized for 1 h at 4 °C in Tris-buffered saline (TBS), pH 7.5, containing 1% (w/v) octyl glucoside. The unsolubilized proteins were eliminated by centrifugation at 13000 *g* for 15 min at 4 °C. The solubilized proteins (70 μg) were separated by electrophoresis on a SDS/7.5% polyacrylamide gel in the absence of reducing agents (Laemmli, 1970) and then electrotransferred to a nitrocellulose membrane (Schleicher and Schuell; pore size 0.45 μm). The membrane was washed in a TBS solution containing 0.2% Tween 20 (TTBS) for 30 min at room temperature. The binding of iodinated RB104 was performed in a TTBS solution containing 100 pM [^{125}I]RB104 in the absence or presence of proteinase inhibitors for 1 h at room temperature. The membrane was then washed three times in TTBS for 5 min each, dried and exposed to a Kodak X-AR film for 2 days at −70 °C.

Assay of PI-PLC

Purified membranes (200 μg of protein) were incubated either alone or in the presence of 20 units of PI-PLC for 40 min at 37 °C in 10 mM Tris, pH 7.0, and centrifuged at 35000 *g* for 30 min at 4 °C (Adachi et al., 1990). The supernatant was used directly, whereas the membrane pellet was solubilized in TBS containing 1% octyl glucoside. The presence of the endopeptidase in the supernatant or in the solubilized pellet was detected with [^{125}I]RB104, after separation of the proteins by SDS/PAGE, as described above.

RESULTS

Membrane-bound peptidase activity in *Aplysia* tissues

In order to reveal a neutral endopeptidase-like activity in *Aplysia*, kidney plasma membranes must first be incubated for 15 min with amastatin at a concentration of 10 μM in order to inhibit as much as possible the strong aminopeptidase activity previously described (Bawab et al., 1992). After the preincubation period, the [^{3}H][Leu]enkephalin was added to the reagent mixture and the incubation was continued for 10 min at 25 °C. The resulting metabolites were analysed by RP-h.p.l.c. Figure 1 shows the h.p.l.c. profile of the [^{3}H][Leu]enkephalin metabolites (Figure 1a). Two peaks were observed: the first one co-migrates with free tyrosine and is most probably generated by the residual aminopeptidase activity. The second peak co-migrates with the peptide Tyr-Gly-Gly and may be due to the cleavage of the substrate by enzymes resembling mammalian NEP or dipeptidyl carboxypeptidase (angiotensin-converting enzyme). This peak corresponds to the degradation of 3.5 pmol of substrate/min per mg of plasma membrane protein. This peptidase activity is very low when compared with the aminopeptidase activity previously described in the same tissue, which degraded 356±38 pmol of substrate/min per mg of plasma membrane protein (Bawab et al., 1992).

To characterize this activity, we determined the effect of pH and salt concentration on the initial rate of the reaction. Plasma membranes were first incubated with [^{3}H][Leu]enkephalin at different pH values. The metabolites were separated by RP-h.p.l.c. as described above and the radioactivity (c.p.m.) under the Tyr-Gly-Gly peak was calculated. The optimal pH was found to be 6.5 (results not shown). Similar results were obtained with rabbit kidney membranes, when the assay was done at 30 °C (N. Dion, P. Crine and G. Boileau, unpublished work). Thus subsequent hydrolysis of peptides by the *Aplysia* neutral endopeptidase was carried out in Mes buffer, pH 6.5. Since the *Aplysia* haemolymph is almost isotonic with sea water, we tested the effect of various salt concentrations on the enzymic activity. No differences were detected for NaCl concentrations varying from 0 to 0.5 M (results not shown).

The enkephalin-degrading activity is an endopeptidase

Since the C-terminal Phe-Leu counterpart of the Tyr-Gly-Gly product could not be detected when [^{3}H][Leu]enkephalin was used as a substrate, we cannot exclude the possibility that the Tyr-Gly-Gly peptide could have been generated by sequential carboxypeptidase action or by a dipeptidyl carboxypeptidase rather than by a true endopeptidase. To distinguish between these three possibilities, we studied the hydrolysis of [Leu]enkephalinamide by *Aplysia* kidney membranes. In the absence of any inhibitor, [Leu]enkephalinamide was rapidly cleaved into a major product corresponding to Gly-Gly-Phe-Leu amide (Figure 2a), as expected from the presence of a strong

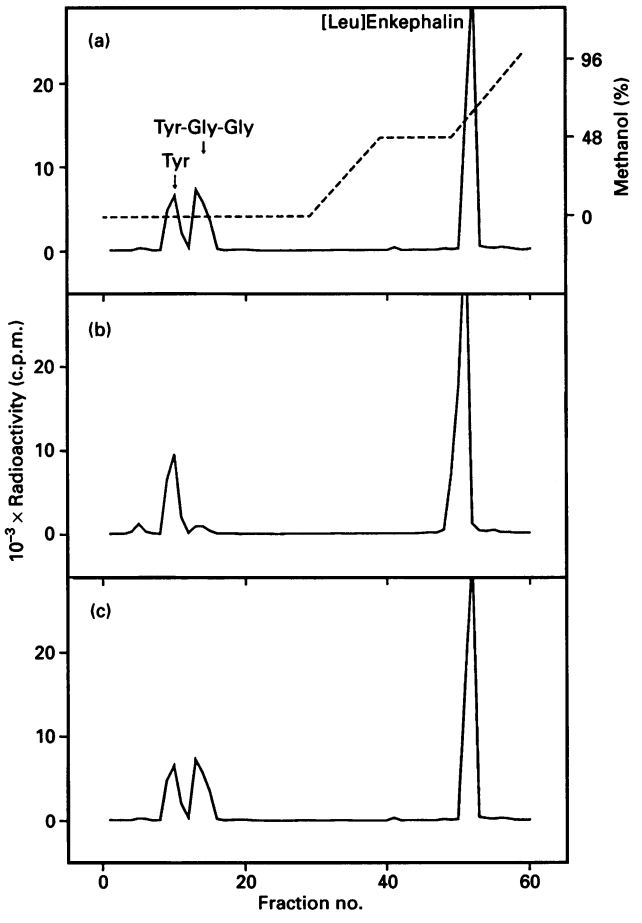

Figure 1 RP-h.p.l.c. analysis of degradation metabolites of [^{3}H][Leu]-enkephalin in *Aplysia* tissues

The substrate was incubated in the presence of amastatin (10 μM) and kidney plasma membranes in the absence (**a**) or presence of 1 μM RB104 (**b**) or 10 μM captopril (**c**). Arrows indicate the elution positions of standard peptides. The dashed line represents the methanol gradient used in the h.p.l.c.

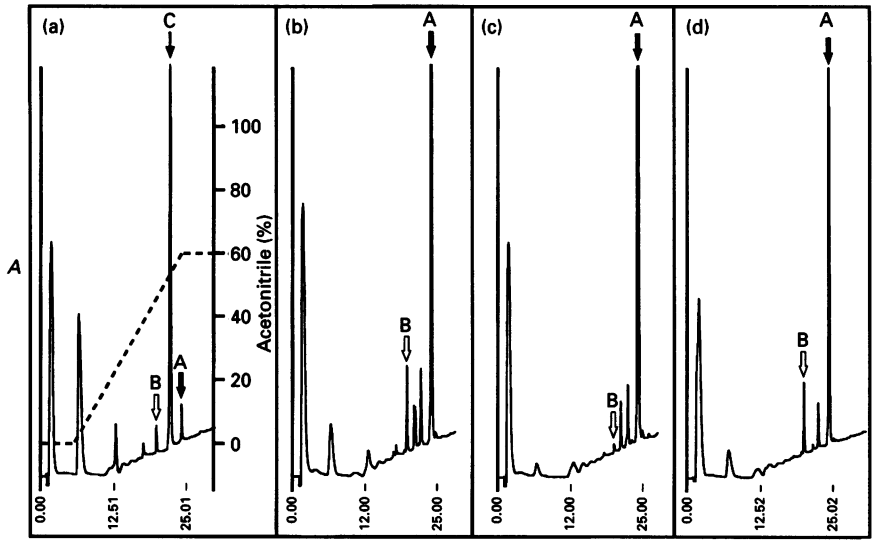

Figure 2 RP-h.p.l.c. analysis of degradation metabolites of [Leu]enkephalinamide in *Aplysia* tissues

[Leu]Enkephalinamide (20 μg) was incubated with kidney plasma membranes in the absence (**a**) or presence of 10 μM amastatin (**b**) or 10 μM amastatin plus 1 μM RB104 (**c**). Purified rabbit neutral endopeptidase was used as a control (**d**). Arrows indicate the elution position of standard peptides: A, Tyr-Gly-Gly-Phe-Leu-amide; B, Phe-Leu-amide; C, Gly-Gly-Phe-Leu-amide. The dashed line represents the acetonitrile gradient used in the h.p.l.c.

Table 1 Action of peptidase inhibitors on NEP activity in the kidney of *Aplysia californica*

Plasma membranes from kidneys were incubated, prior to the addition of the [^{3}H][Leu]enkephalin, in the absence or presence of various peptidase inhibitors. The metabolites were separated by RP-h.p.l.c. and the radioactivity (c.p.m.) under the Tyr-Gly-Gly peak was measured. The percentage inhibition was calculated by comparing the radioactivity under the Tyr-Gly-Gly peak in the presence and absence of inhibitors.

Inhibitor	Inhibition (%)
RB104 (1 μM)	91
HACBO-Gly (10 μM)	74
Thiorphan (10 μM)	33
Phosphoramidon (10 μM)	45
1,10-Phenanthroline (5 μM)	100
Captopril (10 μM)	0
PMSF (100 μM)	0

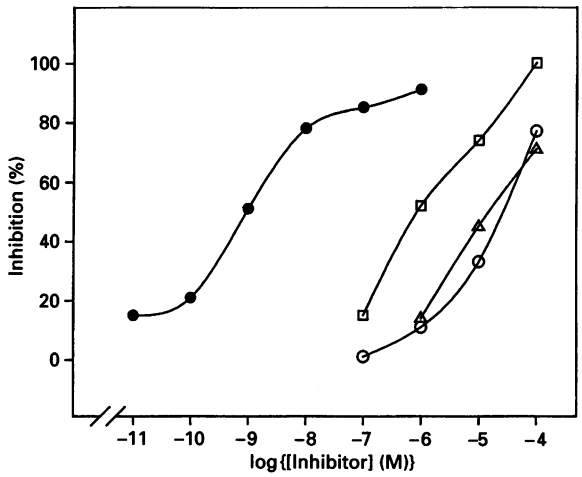

Figure 3 Effect of NEP inhibitors on [^{3}H][Leu]enkephalin degradation with membranes from *Aplysia* kidneys

Membranes isolated from kidneys were preincubated at 25 °C for 15 min with amastatin (10 μM), alone or in the presence of different concentrations of NEP inhibitors, prior to the addition of [^{3}H][Leu]enkephalin. The metabolites were separated by RP-h.p.l.c. and the radioactivity (c.p.m.) under the Tyr-Gly-Gly peak was measured. The percentage inhibition was calculated by comparing the radioactivity under the Tyr-Gly-Gly peak in the presence and absence of the NEP inhibitors RB104 (●), HACBO-Gly (□), phosphoramidon (△) and thiorphan (○).

aminopeptidase activity in these preparations (Bawab et al., 1992). After a 4 h preincubation period with amastatin, however, a substantial portion of the substrate remained intact and most of the Gly-Gly-Phe-Leu amide disappeared from the chromatogram. Moreover, a new peak co-migrating with standard Phe-Leu amide was observed (Figure 2b). This h.p.l.c. pattern was very similar to the one obtained after digestion of [Leu]-enkephalinamide with purified rabbit kidney NEP (Figure 2d). Taken together, these results suggest that *Aplysia* kidney membranes contain an endopeptidase with an activity similar to that of mammalian NEP.

Inhibitor profile of the peptidase activity

We next submitted the [Leu]enkephalin degrading activity to a battery of peptidase inhibitors. The cation chelating agent 1,10-phenanthroline, which is a general inhibitor of metallopeptidases, completely inhibited the hydrolysis of [^{3}H][Leu]enkephalin by kidney extracts (Table 1), suggesting that the *Aplysia* endopeptidase is a metallopeptidase. The serine proteinase inhibitor PMSF had no effect on the activity of the enzyme (Table 1).

Specific inhibitors of NEP such as phosphoramidon (Kerr and Kenny, 1974), thiorphan (Roques et al., 1980), HACBO-Gly (Waksman et al., 1985) and RB104 (Fournié-Zaluski et al., 1992) were found to inhibit the endopeptidase activity (Table 1). RB104, used at 1 μM, almost completely inhibited the hydrolysis of both [^{3}H][Leu]enkephalin (Figure 1b) and [Leu]enkephalin-

Table 2 Binding affinity of RB104 on *Aplysia* kidney membranes

Kidney plasma membranes were incubated in the presence of [^{125}I]RB104 and different dilutions of unlabelled RB104. The K_D and $B_{max.}$ values were calculated from a Scatchard analysis.

	K_D (pM)	$B_{max.}$ (fmol/mg of protein)	Reference
Aplysia kidney membranes	21 ± 5	20.3 ± 5	This paper
Rat brain homogenates	26.8 ± 0.9	8.1 ± 0.2	Fournié-Zaluski et al. (1992)
Rat brain slices	300 ± 20	3.5 ± 0.12*	Fournié-Zaluski et al. (1992)

* fmol/slice

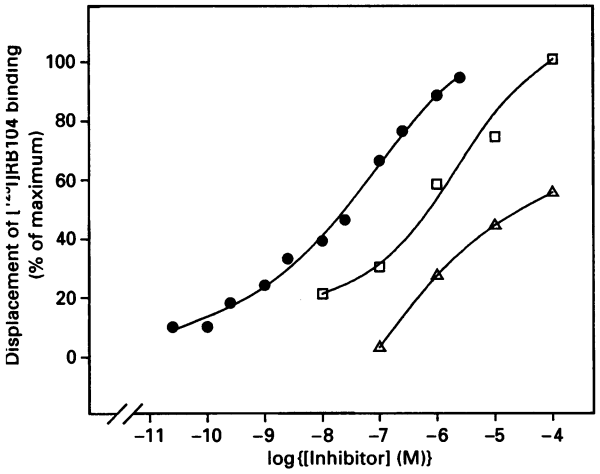

Figure 4 Effect of NEP inhibitors on [^{125}I]RB104 binding to kidney plasma membranes

Membranes isolated from kidneys were preincubated at 25 °C for 15 min in the absence or presence of different concentrations of NEP inhibitors, prior to the addition of [^{125}I]RB104. Membrane-bound [^{125}I]RB104 was collected by centrifugation at 30000 ***g*** for 20 min. The resulting pellets were washed and radioactivity was counted on a γ-counter. Data are expressed as binding in the presence of inhibitors as a percentage of binding in the absence of inhibitors. ●, RB104; □, HACBO-Gly; △, phosphoramidon.

amide (Figure 2c). By contrast captopril, a potent inhibitor of angiotensin-converting enzyme, had no effect on the enzyme activity (Table 1 and Figure 1c). This was important to establish, as angiotensin-converting enzyme has a dipeptidyl carboxypeptidase activity which also cleaves the enkephalins at the Gly^3-Phe^4 bond (Erdös et al., 1978). These results suggest that this *Aplysia* enzyme is a metallopeptidase with an inhibitor profile similar to that of mammalian NEP.

When the relative potencies of all these inhibitors were compared, RB104 was shown to be the best inhibitor of the *Aplysia* endopeptidase activity. Its IC_{50} measured for the hydrolysis of [^{3}H][Leu]enkephalin was 1 nM (Figure 3). HACBO-Gly, phosphoramidon and thiorphan were less potent inhibitors, with IC_{50} values of 1 μM, 20 μM and 30 μM respectively.

Binding of [^{125}I]RB104 to *Aplysia* endopeptidase

RB104 can easily be radioiodinated and used to perform binding studies with NEP (Fournié-Zaluski et al., 1992). We therefore

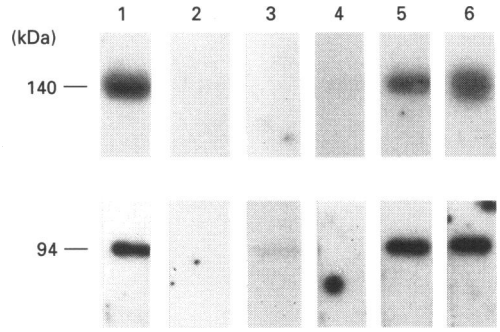

Figure 5 Inhibitor gel electrophoresis with [^{125}I]RB104 and different peptidase inhibitors

Solubilized membrane proteins of *Aplysia* kidney (upper panel) and purified rabbit NEP (lower panel) were separated by SDS/PAGE and transferred on to a nitrocellulose membrane. NEP-like proteins were labelled with 100 pM [^{125}I]RB104 in the presence or absence of different peptidase inhibitors. Lane 1, absence of inhibitor (control); 2, HACBO-Gly (10 μM); 3, phosphoramidon (10 μM); 4, RB104 (0.1 μM); 5, amastatin (10 μM); 6, captopril (10 μM).

decided to compare the binding parameters of [^{125}I]RB104 on the *Aplysia* endopeptidase with those previously determined for mammalian NEP. The K_d and $B_{max.}$ values of RB104 calculated from a Scatchard analysis were 21 ± 5 pM and 20.3 ± 5 fmol/mg of protein respectively (Table 2). These values are very close to those observed with mammalian NEP, suggesting that RB104 binds to the *Aplysia* enzyme as strongly as to the mammalian NEP. Competition assays were done with unlabelled inhibitors. Figure 4 shows the dissociation curves of [^{125}I]RB104 binding when incubated in the presence of different NEP inhibitors. RB104 was the most potent competitor, displacing the labelled RB104 with an IC_{50} value of 100 nM. HACBO-Gly and phosphoramidon were less potent competitors, each with an IC_{50} value of 1 μM.

Detection of the endopeptidase by SDS/PAGE

In order to further characterize the neutral endopeptidase enzyme in *Aplysia*, we used the radioiodinated inhibitor RB104 to directly detect the enzyme after separation of solubilized membrane proteins by SDS/PAGE and transfer on to nitrocellulose membranes. As seen in Figure 5, [^{125}I]RB104 bound to a single protein of about 140 kDa in *Aplysia* kidney membranes (upper panel, control lane). As expected, the inhibitor bound to a single radioactive band of around 94 kDa in rabbit or rat kidney membranes (results not shown) or to purified NEP (lower panel, control lane). No other band was visible on these gels.

To determine the specificity of the binding, competition assays with an excess of unlabelled inhibitors were performed (Figure 5). The labelling of *Aplysia* kidney membrane proteins and of rabbit NEP by [^{125}I]RB104 was completely abolished by RB104 (0.1 μM), HACBO-Gly (10 μM) and phosphoramidon (10 μM), three specific inhibitors of NEP. By contrast, the labelling was not affected by specific inhibitors of other peptidases, such as the dipeptidyl carboxypeptidase inhibitor captopril (10 μM) or the aminopeptidase inhibitor amastatin (10 μM).

Characterization of the membrane-bound endopeptidase activity

In order to determine if the enzyme is an integral or a peripheral membrane protein, kidney plasma membrane preparations were incubated with Triton X-114 and phase separation was performed at 30 °C, as described in the Materials and methods section. The

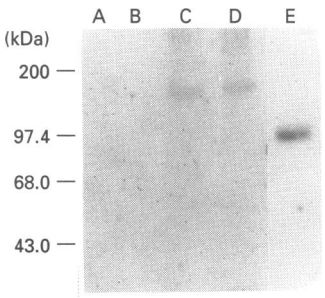

Figure 6 Integral membrane nature of the peptidase

Kidney plasma membranes were incubated either alone (lanes A and C), or in the presence of PI-PLC (lanes B and D). The membrane preparations were centrifuged at 35000 *g* for 30 min, and the proteins present in the supernatant (lanes A and B) and the pellet (lanes C and D) were separated by SDS/PAGE. NEP was revealed with [^{125}I]RB104. Purified rabbit NEP was used as a control (lane E).

presence of endopeptidase activity in both the detergent-rich and detergent-poor phases was determined by hydrolysis of [^{3}H][Leu]enkephalin in the presence of amastatin (10 μM) and by separation of the metabolites by h.p.l.c. About 64% of the enzymic activity which degraded [^{3}H][Leu]enkephalin at the Gly^3–Phe^4 bond was found in the detergent-rich phase, suggesting that an important proportion of the endopeptidase activity recovered in the membrane fraction corresponds to an integral membrane protein. This enzymic activity is sensitive to the NEP inhibitors HACBO-Gly and RB104. When isolated rabbit kidney membranes were submitted to the same phase separation, about 70% of the NEP activity was recovered in the detergent-rich phase. This result is consistent with previous observations with pig and dog kidney membranes (Matsas et al., 1985).

To determine whether the *Aplysia* endopeptidase is anchored by a glycosyl-phosphatidylinositol moiety or by a transmembrane anchor, we attempted to release it from the membrane using PI-PLC. Kidney membranes were incubated either alone or in the presence of PI-PLC and centrifuged as described (Adachi et al., 1990). Membrane-bound and released proteins were analysed by SDS/PAGE and [^{125}I]RB104, as described above. As shown in Figure 6, *Aplysia* endopeptidase was recovered in the pellet (lane D) and not in the 35000 *g* supernatant fraction (lane B). This result indicates that the enzyme is not released by PI-PLC and suggests that it is an integral membrane protein.

DISCUSSION

In this study we provide evidence for the presence of an endopeptidase activity associated with plasma membrane fractions of *Aplysia californica* kidneys. This activity was assayed by monitoring the degradation of [^{3}H][Leu]enkephalin by kidney membranes in the presence of amastatin; the latter inhibits an abundant aminopeptidase activity previously described in this tissue (Bawab et al., 1992). The enzyme appears to be a metallopeptidase, as it is inhibited by 1,10-phenanthroline. H.p.l.c. analysis of the tritiated metabolites revealed the presence of [^{3}H]Tyr-Gly-Gly, suggesting that cleavage had occurred at the Gly^3–Phe^4 bond. We further confirmed that this metabolite is not generated through sequential carboxypeptidase hydrolysis, by isolating the Phe-Leu amide dipeptide generated by cleavage of [Leu]enkephalinamide. This experiment also allowed us to conclude that the enzyme from *Aplysia* kidneys can act on substrates without a free C-terminus and therefore can be considered as a genuine endopeptidase.

The *Aplysia* enzyme was found to be sensitive to well-established mammalian NEP inhibitors. Of these, the best inhibitor for the *Aplysia* enzyme was RB104. This very potent NEP inhibitor can be iodinated and used to detect as little as 2 ng of NEP after SDS/PAGE (Fournié-Zaluski et al., 1992). We found that radioiodinated [^{125}I]RB104 binds to the *Aplysia* endopeptidase with the same affinity as to the mammalian NEP, and thus can be used to detect and characterize the NEP in *Aplysia* as well. Competition experiments between [^{125}I]RB104 and other peptidase inhibitors confirm the specificity of the labelling. RB104 is two to three orders of magnitude more potent than any other NEP inhibitor, both for binding to and for inhibition of the *Aplysia* NEP. RB104 should thus be useful for screening for the presence of the enzyme in different *Aplysia* tissues, either by direct binding to tissue sections or homogenates, or by the inhibitor gel electrophoresis assay.

The *Aplysia* endopeptidase has several features in common with the mammalian NEP: both can cleave [Leu]enkephalin and [Leu]enkephalinamide at the Gly^3–Phe^4 bond, both are metallopeptidases with an optimal activity around neutral pH, and both are sensitive to the same inhibitors. Nevertheless, major differences have also been observed in the subunit size of the enzymes on SDS/PAGE and in the level of sensitivity to the NEP inhibitors, as discussed below. In addition, the endopeptidase activity in *Aplysia* kidney is 100-fold lower than that of the aminopeptidase N (Bawab et al., 1992). This is in contrast with the situation found in mammals, where both aminopeptidase N and NEP are present in large amounts in the kidney.

Although sensitive to the same inhibitors as the mammalian NEP, the level of inhibition of the *Aplysia* enzyme is consistently lower than that of the mammalian enzyme by almost three orders of magnitude. However, this does not mean that the *Aplysia* endopeptidase is unrelated to the mammalian enzyme. Considering that these molecules are expressed in organisms whose ancestors diverged about 600 million years ago, such a difference would not be unexpected. This has also been reported for the sensitivity of invertebrate receptors and/or ionic channels to well characterized mammalian agonists and/or antagonists (Kandel, 1976).

The size of the [^{125}I]RB104-labelled protein in *Aplysia* kidney is larger than that previously reported in mammals (Kenny et al., 1987) and in *Mytilus edulis* (Shipp et al., 1990). The difference (140 compared to 94 and 95–100 kDa respectively) may result from the expression of homologous, although slightly different, genes in these species or from different patterns of glycosylation. It is well documented that the mammalian neutral endopeptidase is a glycoprotein whose degree of glycosylation differs from one tissue to the other, resulting in different apparent molecular masses following SDS/PAGE (Relton et al., 1983). We attempted to use endoglycosidase F treatment to remove the oligosaccharides and compare the unglycosylated *Aplysia* and rabbit NEPs; unfortunately, this treatment made the *Aplysia* protein undetectable by [^{125}I]RB104 binding (results not shown).

Many membrane-bound enzymes have been shown to be anchored to the membrane by a covalently attached phosphatidylinositol moiety (Cross, 1990). Our experiment with PI-PLC ruled out this possibility and further suggested that the enzyme carries a transmembrane domain. It is well documented that mammalian NEPs are anchored in the membrane through a sequence of hydrophobic amino acids at the N-terminus (Kenny and Maroux, 1982; Fulcher et al., 1986; Devault et al., 1987; Malfroy et al., 1987) and that they are not released by PI-PLC treatment (Hooper et al., 1987). The characterization of the *Aplysia* endopeptidase enzyme as an integral membrane protein further adds to the similarity between the *Aplysia* endopeptidase

and mammalian NEP, and supports the hypothesis that this enzyme may be involved, like the mammalian enzyme, in the metabolism of secreted peptides at the cell surface.

It is well known that NEP degrades a wide range of biologically active peptides *in vitro* as well as *in vivo* (Erdös and Skidgel, 1989). Studies on neuropeptide degradation *in vitro*, although informative, may not be physiologically relevant; co-localization of the enzymes with peptides *in vivo*, rather than the specificity of the enzymes, seems to be the primary determinant for establishing the physiological relevance of a proteinase in neuropeptide degradation. Interference with the function of putative degrading enzymes by specific peptidase inhibitors *in vivo* should potentiate the physiological and/or behavioural actions of a co-localized neuropeptide. The integrated model of peptidergic pathways provided by the mollusc *Aplysia* may provide a useful experimental system for investigating the mechanisms involved in neuropeptide degradation in invertebrates and most probably in vertebrates as well. The neutral endopeptidase described in this paper represents the third characterized enzyme thought to be involved in extracellular metabolism of peptides in *Aplysia*. Previous reports have documented the presence of membrane-bound aminopeptidase N-like (Bawab et al., 1992) and soluble leucine aminopeptidase-like (Squire et al., 1991) enzymes. The natural substrates for these enzymes are not known. *In vitro* studies suggest that α- and β-Bag Cell Peptides, small radioactive peptide-B and Phe-Met-Arg-Phe amide represent potential substrates for the *Aplysia* peptidases (Owens et al., 1992; Bawab et al., 1992; Rothman et al., 1992). Co-localization of peptidases in relation to known peptidergic pathways and potentiation of the physiological action of peptides by peptidase inhibitors will adequately address this question.

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