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Endoproteolytic activity in mammalian brain membranes cleaves 5-hydroxytryptamine-moduline into dipeptides

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Abstract

This work was intended to determine which enzymatic activities from crude synaptosomal mammalian brain membranes could qualify for the status of 5-hydroxytryptamine-moduline (5-HT-moduline, LSAL, Leu-Ser-Ala-Leu) inactivating enzymes. An enzymatic assay for 5-HT-moduline metabolism was developed using [^{3}H]5-HT-moduline measurement and high performance liquid chromatography (HPLC) technique to identify and quantify 5-HT-moduline metabolites. 5-HT-moduline metabolism displayed all characteristics of metalloprotease activity: sensitivity to divalent ion chelators, reactivation by Zn^{2+} ions and a pH optimum in the 7–8 range. Bestatin, an aminopeptidase inhibitor, allowed the identification of two enzymatic activities responsible for this metabolism: a bestatin-sensitive aminopeptidase and an endoprotease cleaving 5-HT-moduline into LS (Leu-Ser) and AL (Ala-Leu) dipeptides. This latter enzyme was shown to have a K_m of 37.1 ± 3.6 μM and a V_{max} of 5.5 μmol min^{-1} l^{-1} per mg of protein. Moreover, this enzyme was insensitive to peptidyl dipeptidase A (angiotensin converting enzyme, EC 3.4.15.1), endothelin converting enzyme and neutral endopeptidase (neprylisin, EC 3.4.24.11) inhibitors and displayed some specificity among 5-HT-moduline-analogues and in particular recognized only tetrapeptides. These results, together with the isolation of the LS and AL metabolites [Rousselle, J.C., Massot, O., Delepierre, M., Zifa, E., Rousseau, B., Fillion, G., 1996. Isolation and characterization of an endogenous peptide from rat brain interacting specifically with the serotonergic 1B receptor subtypes. J. Biol. Chem. 271, 726–735] during the purification process of 5-HT-moduline are strong arguments for the physiological implication of this endoprotease in 5-HT-moduline metabolism.

Keywords: 5-HT (5-hydroxytryptamine, serotonine); Moduline; LSAL; Peptide hydrolase; Aminopeptidase; Endopeptidase; Endoprotease

1. Introduction

The existence of an endogenous factor, which regulates 5-HT_1 receptors, was postulated in our laboratory (Fillion and Fillion, 1981). This compound was isolated by multiple step chromatographic purification using rat and bovine brain extracts (Rousselle et al., 1996) and shown to specifically interact with 5-$HT_{1B/1D}$ receptors (Massot et al., 1996; Rousselle et al., 1998). This endogenous factor, called 5-HT-moduline is the Leu-Ser-Ala-Leu (LSAL) tetrapeptide. It was later demonstrated that 5-HT-moduline was active on 5-HT_{1B} receptors function, both in vivo (Massot et al., 1996) and ex vivo (Seguin et al., 1997). Moreover, the peptide was heterogeneously distributed in the brain, located in neuron-like cellular profiles (Grimaldi et al., 1997) and released by a Ca^{2+}/K^+ dependent process (Massot et al., 1996). Among all the questions risen by the discovery of this neuropeptide and its effects, one is related to its metabolism. It is widely accepted that inactivation of neuropeptides is insured by neuropeptidases (Checler, 1993), i.e., ectoenzymes located near the site of action of target peptides (Barelli et al., 1993).

In the case of 5-HT-moduline, the existence of such a specific metabolism could be supported by the isolation of biologically inactive Leu-Ser (LS) and Ala-Leu (AL) dipeptides during the purification process of 5-HT-moduline from rat or bovine brain extracts. Thus, we have investigated the metabolic activities found in mammalian

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brain membranes. In particular, enzymologic constants, mechanistic class and sensitivity to inhibitors were studied for these activities. Twenty-eight 5-HT-moduline analogues were synthesized (peptides and pseudo-peptides), and their ability to inhibit 5-HT-moduline endoproteolytic metabolism was assayed. The relationships between the analogue structure and its endopeptidase active site binding ability provides an insight into the specificity of this enzyme.

2. Materials and methods

2.1. Materials

Male Wistar rats (150–200 g) were obtained from IFFA CREDO (l'Arbresle, France). [^{3}H]5-HT-moduline (3.52 TBq/mmol) was synthesized by New England Nuclear (Le Blanc Mesnil, France). All protease inhibitors were purchased from Sigma (Saint Louis, MO, USA), except phosphoramidon which was purchased from ICN (Costa Mesa, CA, USA). Synthetic unlabeled LSAL and other synthetic peptides were from Bachem (Bubbendorf, Switzerland). 5-HT-moduline analogues were synthesized by the Centre Européen de Bioprospective (Mont-Saint-Aignan, France), except Leu-Ser-(CH_2NH)-Ala-Leu which was from Neosystem (Strasbourg, France). All peptides were of $>95\%$ purity.

2.2. Membrane preparation

Unless noticed, all subsequent operations were carried out at 4°C.

Rats were exposed to carbon dioxide and then decapitated, brains were rapidly dissected on ice (minus cerebellum and brain stem), then homogenized for 30 s with a Ultra-Turrax apparatus in 5 vol (v/w) of a 50-mM Tris–acetate buffer (pH 7.4 at 4°C.). The homogenate was then diluted 30 times (v/w) in buffer and centrifuged (17 500 $\times g$ for 5 min). The resulting pellet was re-suspended in 5 vol (v/w) of buffer, incubated for 10 min at 37°C, diluted again in 30 vol of buffer (v/w) and re-centrifuged under the same conditions to eliminate endogenous 5-HT-moduline. Finally, the resulting pellet was re-suspended in the same buffer (at 37°C) to obtain a protein concentration of 1 mg ml^{-1} during assays. Protein equivalent was determined according to the method described by Lowry et al. (1951). As an alternative, the membrane preparation was pre-incubated for 15 min with bestatin at 0.1 mM at 37°C, yielding bestatin-treated membranes.

2.3. Kinetic monitoring of 5-HT-moduline metabolism

Control or bestatin-treated membrane preparations, obtained as described in Section 2.2, were placed at 37°C under constant stirring. At $t=0$, [^{3}H]5-HT-moduline was added to a final concentration of 1 nM (first-order kinetics) or 10 μM (zero-order kinetics). Then, at various times, 1 ml aliquots were collected and incubation was stopped by quick filtration on a DynaGard hollow fiber syringe filter (0.22 μm) (Microgon, Laguna Hills, CA, USA) followed by instant freezing of the filtrates. Each aliquot was analyzed as described in Section 2.7 to quantify the amount of [^{3}H]5-HT-moduline metabolized and to identify the labeled metabolites. One-phase decay equation was fitted to first-order kinetics data and linear regression was used to analyze zero-order kinetics data. All linear and non-linear regressions were performed under Prism 2.01 software (Graph Pad Software, San Diego, CA, USA).

2.4. Determination of K_m and V_{max}

Control or bestatin-treated membranes were incubated for 10 min at 37°C in a final volume of 200 μl with increasing concentrations of [^{3}H]5-HT-moduline (1 to 60 μM). Non-saturable metabolism was determined in the presence of 10 mM 5-HT-moduline. The incubates were then filtered under vacuum using a Multiscreen assay apparatus (Millipore, St-Quentin-en-Yvelines, France) and recovered into 96-well culture plates (Costar, Cambridge, MA, USA) and then frozen. Lichrolut RP-18 columns (500 mg) (Merck, Darmstadt, Germany) were used to separate [^{3}H]5-HT-moduline from its metabolites ([^{3}H]leucine in control membranes and [^{3}H]LS in bestatin-treated membranes). Columns were equilibrated with 3×1 ml of 50 mM ammonium acetate buffer (pH 5 at 25°C) and filtrates aliquots (100 μl) were adsorbed. Then, tritiated metabolites were eluted with 2×1 ml of 2% acetonitrile and [^{3}H]5-HT-moduline was eluted with 2×1 ml of 100% acetonitrile. Liquid scintillation counting was used to quantify the radioactivity in the eluted fractions. Analysis of data was performed using the Michaelis–Menten equation under Prism 2.01 software.

2.5. Assay for protease inhibitors, pH and divalent ion dependency

Control or bestatin-treated membranes (prepared as described in Section 2.2) were pre-incubated in the presence of various concentrations of protease inhibitors or at different pH for 15 min at 37°C. Then [^{3}H]5-HT-moduline was added to a final concentration of 1 nM. Incubation was carried out for 10 min at 37°C in a final volume of 200 μl and stopped by filtration followed by quick freezing of the filtrate as described above. For investigating the zinc-dependency of the enzymatic activities, membranes were pre-incubated with 1 mM 1,10-phenanthroline for 15 min before incubation with increasing concentrations (0.1 nM to 0.1 M) of divalent ions (Zn^{2+} and Co^{2+}) and 10 μM of

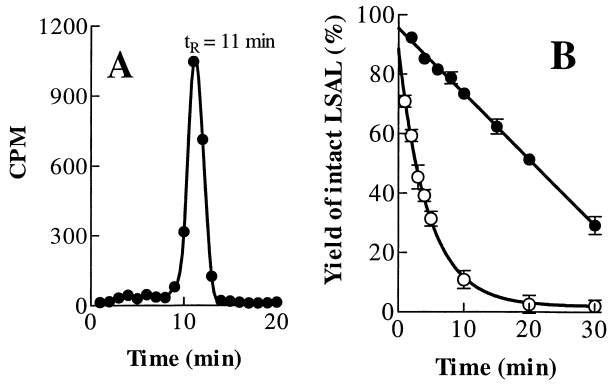

Fig. 1. Metabolism of [^{3}H]5-HT-moduline in control rat brain membranes. (A) [^{3}H]5-HT-moduline was incubated 10 min at 37°C with control membranes (1 mg ml^{-1} protein concentration). Then, separation of [^{3}H]leucine was performed on a C_{18} reverse phase column as indicated in Section 2.7. (B) First-order (-○-) and apparent zero-order kinetics (-●-) of [^{3}H]5-HT-moduline (respectively, 1 nM and 10 μM) metabolism with control membranes (1 mg ml^{-1} protein concentration) at 37°C. Separation and quantification of [^{3}H]leucine was performed on a C_{18} reverse phase column as indicated in Section 2.7. One-phase exponential decay equation was fitted to the first-order kinetics data points to determine the initial velocity of the enzyme (0.22 ± 0.01 nmol min^{-1} l^{-1} per mg of protein), and linear regression was fitted to zero-order kinetics (initial velocity was 0.221 ± 0.003 μmol min^{-1} l^{-1} per mg of protein). Each point of the curves corresponds to the mean of two independent experiments.

[^{3}H]5-HT-moduline. Analysis of the filtrates was carried as described above. Dose–effect curves were analyzed using a four-parameter logistic equation under Prism 2.01 software.

2.6. [^{3}H]5-HT-moduline protection assay with analogue peptides

Bestatin-treated membrane aliquots prepared (see Section 2.2) were incubated for 10 min with [^{3}H]5-HT-moduline at 1 nM and increasing concentrations of the different compounds, (0.4 μM to 0.4 mM) in a final volume of 200 μl for 10 min at 37°C. Dixon experiments were conducted under the same conditions at two [^{3}H]5-HT-moduline concentrations (10 and 20 μM) with increasing concentrations of the tested analogues (2 to 50 μM). Quantification of tritiated metabolites was done as in Section 2.4.

2.7. High performance liquid chromatography (HPLC) analysis of metabolites

Synthetic peptides were injected on a N5-C18 reverse phase column (Nucleosil, 5 μm porosity, 150 × 4.6 mm, Interchim, Montluçon, France) equilibrated in 0.1% trifluoroacetic acid (TFA) buffer. Elution was run at 1 ml min^{-1} using a 5-min isocratic step in 0.1% TFA buffer followed by a 20-min isocratic step of acetonitrile (2%) and a last 10-min isocratic step of acetonitrile (70%). Standard retention times of LSAL putative metabolites (LSA, SAL, LS, AL, and L) were determined by UV detection at 220 nm (Perkin Elmer LC 90 UV spectrophotometric detector). Retention times of [^{3}H]5-HT-moduline and of its tritiated metabolites were determined by liquid scintillation counting of radioactivity content of each 1-ml

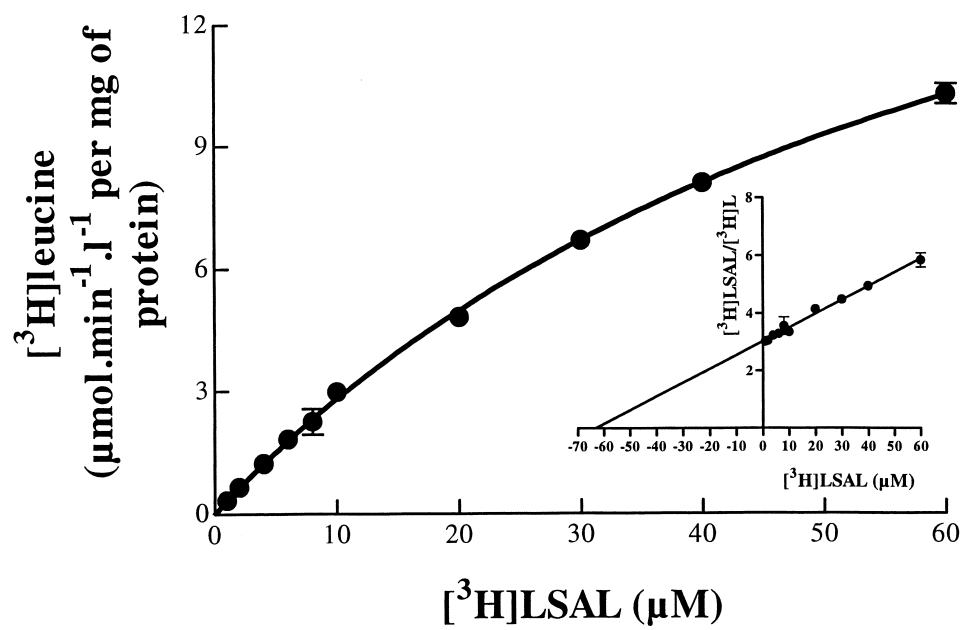

Fig. 2. Determination of biochemical parameters of the aminopeptidase activity. [^{3}H]5-HT-moduline (1 to 60 μM) was incubated with control membranes (1 mg ml^{-1} protein concentration) for 10 min at 37°C. Non-specific metabolism was determined with 10 mM 5-HT-moduline. Separation and quantification of [^{3}H]LSAL and [^{3}H]L were carried out as indicated in Section 2.4. K_m and V_{max} were determined using the Michaelis–Menten equation and were, respectively, estimated to 67.9 ± 3.5 μM and 22 ± 0.5 μmol min^{-1} l^{-1} per mg of protein. Inset: Hanes plot corresponding to the saturation curve ([^{3}H]LSAL/[^{3}H]L vs. [^{3}H]LSAL). Data points are the mean of two independent experiments.

Table 1
Inhibition of 5-HT-moduline metabolism by protease inhibitors in control membranes
Control membranes (1 mg ml^{-1} protein concentration) were incubated for 15 min at 37°C with the indicated compound at the concentration listed in the second column. Aliquots were then incubated for 10 min at 37°C with [^{3}H]5-HT-moduline at 1 nM. Intact [^{3}H]5-HT-moduline was quantified after incubation. Data points are mean ± S.E.M. from three independent experiments.

Compound	Concentration	Intact LSAL after incubation (%)
1,10-Phenanthroline	1 mM	85.3 ± 3.0
Bacitracin	68 U/l	75.2 ± 3.6
EDTA	5 mM	37.0 ± 2.5
Bestatin	0.1 mM	32.2 ± 3.0
EGTA	5 mM	25.4 ± 2.2
Aprotinin	7.6 U/l	14.0 ± 2.3
PMSF	200 μM	13.2 ± 3.4
4,7-Phenanthroline	1 mM	7.5 ± 3.0
1,7-Phenanthroline	1 mM	5.9 ± 0.4
Leupeptine	1 μM	5.6 ± 1.3

fraction. Areas of each peak were expressed as percentage of total radioactivity collected over the chromatographic pattern.

3. Results

3.1. Metabolism of [^{3}H]5-HT-moduline in control membranes

When [^{3}H]5-HT-moduline was incubated with control membranes at 37°C, a single tritiated metabolite was observed with a retention time (t_R) of 11 min (Fig. 1A). This metabolite corresponded to [^{3}H]leucine and was clearly separated from [^{3}H]5-HT-moduline itself (t_R of 30 min). At 1 nM of [^{3}H]5-HT-moduline, first-order kinetics were observed in rat brain membranes (half-life of the peptide was 3.2 min, Fig. 1B). Initial velocity was estimated to 0.22 ± 0.01 nmol min^{-1} l^{-1} per mg of protein. At higher concentration (10 μM), [^{3}H]5-HT-moduline metabolism followed an apparent zero-order kinetics with an initial velocity of 0.221 ± 0.003 μmol min^{-1} l^{-1} per mg of protein. Under these conditions, velocity vs. [^{3}H]5-HT-

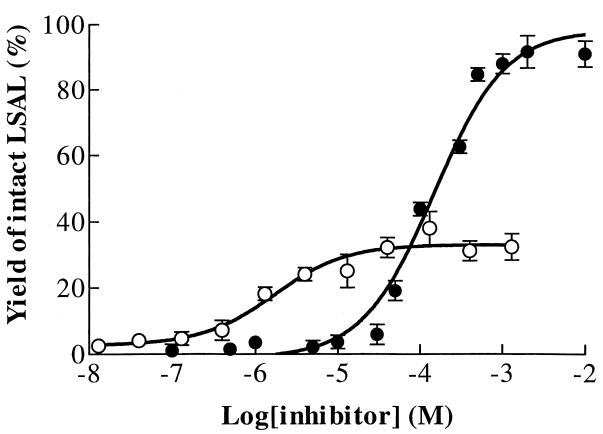

Fig. 3. Dose–effect curves of 1,10-phenanthroline and bestatin on [^{3}H]5-HT-moduline metabolism. Control membranes (1 mg ml^{-1} protein concentration) were treated for 15 min at 37°C with increasing concentrations of 1,10-phenanthroline (-●-) (0.1 μM to 10 mM) or bestatin (-○-) (13 nM to 1.3 mM). Membranes aliquots were then incubated for 10 min at 37°C with 1 nM of [^{3}H]5-HT-moduline, and metabolites were separated and quantified as indicated in Section 2.5. Four-parameter logistic equation was fitted to data points to determine IC_{50} of 1,10-phenanthroline (0.15 mM) and bestatin (1.7 μM). Data points are the mean ± S.E.M of three independent experiments.

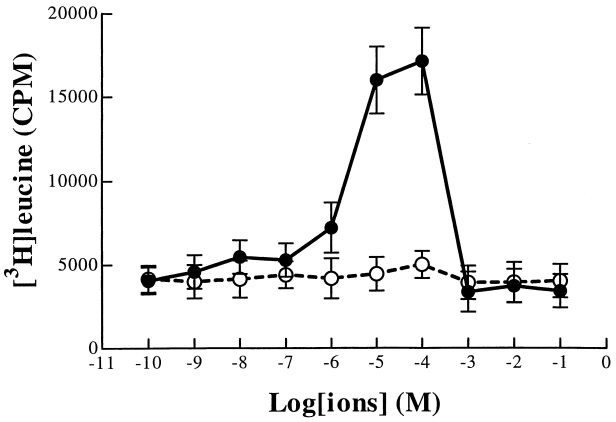

Fig. 4. Reactivation assay of [^{3}H]5-HT-moduline metabolism by Zn^{2+} ions. Membranes (1 mg ml^{-1} protein concentration) were treated with 1 mM 1,10-phenanthroline for 15 min at 37°C. Aliquots were then incubated for 10 min at 37°C with [^{3}H]LSAL (10 μM) and increasing concentrations (0.1 nM to 0.1 M) of Zn^{2+} (-●-) or Co^{2+} (-○-). Separation and quantification of [^{3}H]LSAL and tritiated metabolites were carried out as indicated in Section 2.5. Data points are the mean of two independent experiments.

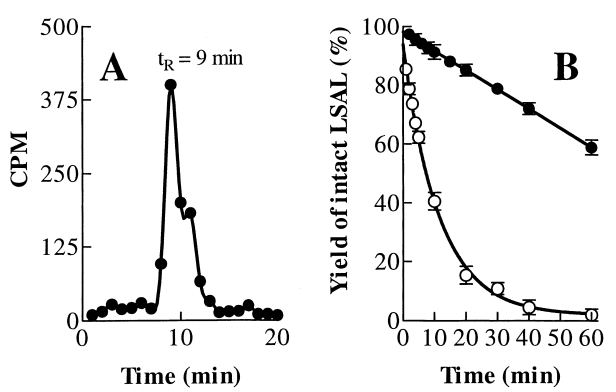

Fig. 5. Metabolism of [^{3}H]5-HT-moduline in bestatin-treated rat brain membranes. (A) [^{3}H]5-HT-moduline was incubated 10 min at 37°C with bestatin-treated membranes (1 mg ml^{-1} protein concentration). Separation of [^{3}H]LS was performed on a C_{18} reverse phase column as indicated in Section 2.7. (B) First-order (-○-) and apparent zero-order kinetics (-●-) of [^{3}H]5-HT-moduline (respectively, 1 nM and 10 μM) metabolism with bestatin-treated membranes (1 mg ml^{-1} protein concentration) at 37°C. Separation and quantification of [^{3}H]LS was performed on a C_{18} reverse phase column as indicated in Section 2.7. One-phase exponential decay equation was fitted to the data points to determine the initial velocity of the enzyme (0.09 ± 0.003 nmol min^{-1} l^{-1} per mg of protein), and linear regression was fitted to zero-order kinetics (initial velocity was 0.066 ± 0.001 μmol min^{-1} l^{-1} per mg of protein). Points shown are the mean of two independent experiments.

moduline concentration curve (saturation curve, Fig. 2) showed a K_m of 67.9 ± 3.5 μM and a V_{max} of 22.0 ± 0.5 μmol min^{-1} l^{-1} per mg of protein.

Protease inhibitors were used to identify the mechanistic class of the enzymes involved in this metabolism (Table 1). Divalent ion chelators like 1,10-phenanthroline and EDTA displayed a significant protection of the labeled peptide during incubation as well as the rather unspecific protease inhibitor and antibiotic peptide bacitracin and the selective aminopeptidase inhibitory peptide bestatin

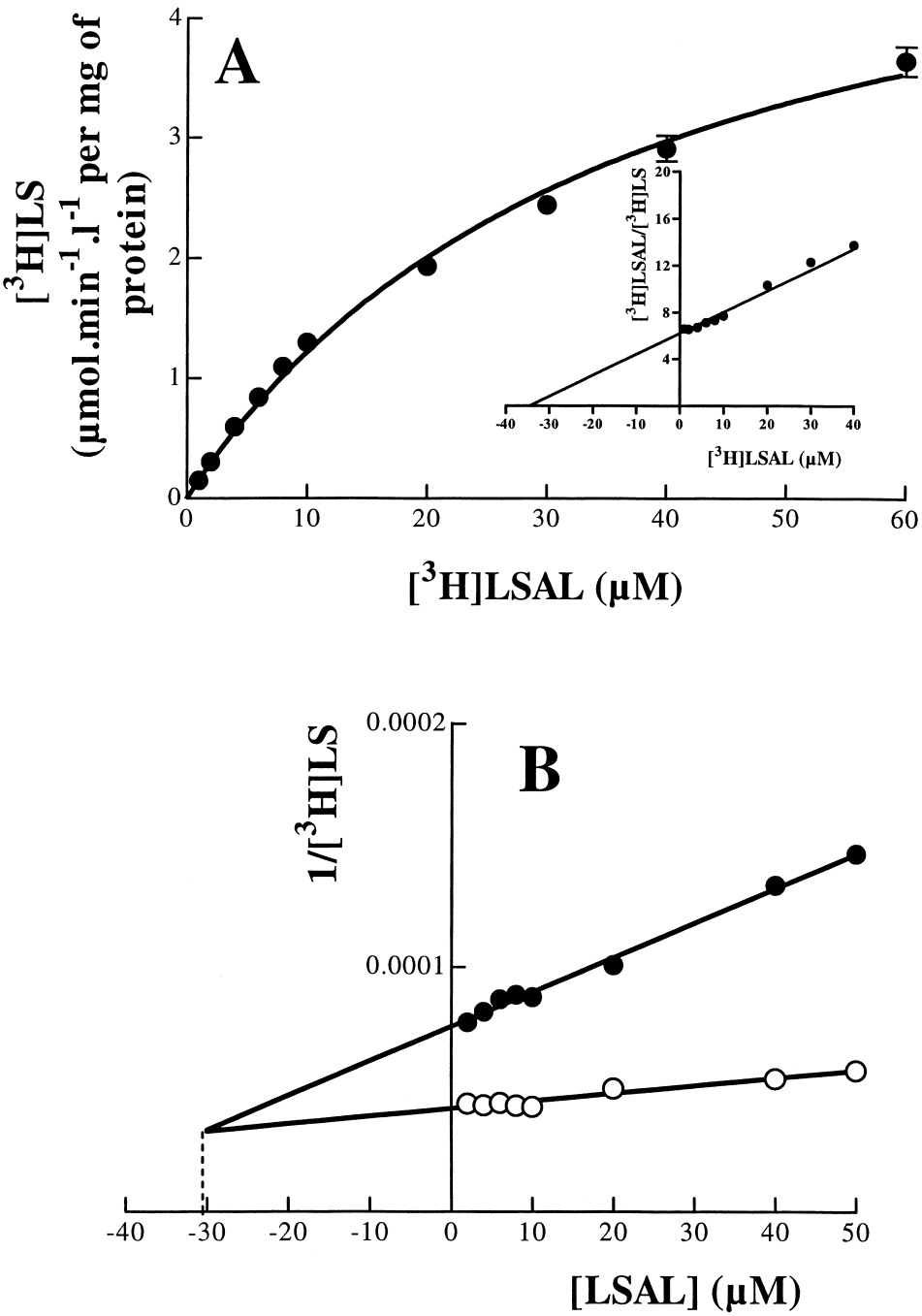

Fig. 6. Determination of biochemical parameters of the endopeptidase activity. (A) Determination of K_m and V_{max} of endopeptidase activity. [^{3}H]5-HT-moduline (1 to 60 μM) was incubated with bestatin-treated membranes (1 mg ml^{-1} protein concentration) for 10 min at 37°C. Non-specific metabolism was determined with 10 mM 5-HT-moduline. Separation and quantification of [^{3}H]LSAL and [^{3}H]L were carried out as indicated in Section 2.4. K_m and V_{max} were determined using the Michaelis–Menten equation and were, respectively, estimated to 37.1 ± 3.6 μM and 5.5 ± 0.5 μmol min^{-1} l^{-1} per mg of protein. Inset: Hanes plot corresponding to the saturation curve ([^{3}H]LSAL/[^{3}H]LS vs. [^{3}H]LSAL). Data points are the mean of two independent experiments. (B) Dixon graph of the inhibition of [^{3}H]5-HT-moduline metabolism in bestatin-treated membranes. Incubation was carried out at 37°C for 10 min with 10 or 20 μM of [^{3}H]5-HT-moduline in the presence of increasing concentrations of 5-HT-moduline (2 to 50 μM). Separation and quantification of [^{3}H]LSAL and [^{3}H]L were carried out as indicated in Section 2.6. Inhibition was competitive and the K_i estimated to 31 μM. Each point is the mean of three independent determinations. This experiment was repeated twice.

(Mathe, 1991; Taylor, 1993). Other protease inhibitors proved to be ineffective: inhibitors of serine- and cysteine-proteases like phenylmethylsulfonyl fluoride (PMSF), inhibitors of trypsin-like activities (aprotinin or leupeptin). Dose dependent inhibitory effect curves of 1,10-phenanthroline and bestatin are featured in Fig. 3. IC_{50} was 0.15 mM for 1,10-phenanthroline and 1.7 μM for bestatin. However, bestatin protective effect does plateau at $E_{max} = 33\%$. Moreover, after inhibition with 1,10-phenanthroline at 1 mM, the enzymatic activity was reactivated with Zn^{2+} (but not Co^{2+}) concentrations in the 10–100 μM range (Fig. 4). Finally, 5-HT-moduline metabolism pH dependency followed a bell-shaped curve with a pH optimum of 7.9 (data not shown).

3.2. Metabolism of [^{3}H]5-HT-moduline in bestatin-treated membranes

Chromatographic analysis of [^{3}H]5-HT-moduline incubated with bestatin-treated membranes showed a single metabolite peak with a retention time of 9 min, corresponding to the [^{3}H]LS dipeptide (Fig. 5A). In this case, initial velocity was estimated to 0.09 ± 0.003 nmol min^{-1} l^{-1} per mg of protein from first-order kinetics ([^{3}H]5-HT-moduline at 1 nM), and 0.066 ± 0.001 μmol min^{-1} l^{-1} per mg of protein from apparent zero-order kinetics ([^{3}H]5-HT-moduline at 10 μM) in rat brain membranes (Fig. 5B). This enzymatic activity had a V_{max} of 5.5 ± 0.5 μmol min^{-1} l^{-1} per mg of protein and a K_m of 37.1 ± 3.6 μM, as determined from the velocity vs. substrate concentration curve (Fig. 6A). K_i of unlabeled LSAL was also estimated to 31 μM, and its inhibition was competitive as shown in the Dixon graph in Fig. 6B. This activity was insensitive to specific endopeptidase inhibitors like captopril (dipeptidyl peptidase A inhibitor), phosphoramidon and tiorphan (inhibitors of metalloproteases like thermolysin, neprilysin and endothelin converting enzyme, data not shown).

3.3. Evaluation of 5-HT-moduline analogues as endopeptidase ligands in bestatin-treated membranes

Dixon plots were performed for four 5-HT-moduline analogues (one substitution per position) and all of them were competitive inhibitors of [^{3}H]5-HT-moduline (Fig. 7). Consequently, all other analogues were tested for their ability to inhibit in a dose-dependent manner the endopeptidase activity found in bestatin-treated membranes. IC_{50} of these compounds are summarized in Table 2. Any peptide with one or more amino acid in the (D) configuration proved to be ineffective in protecting the labeled

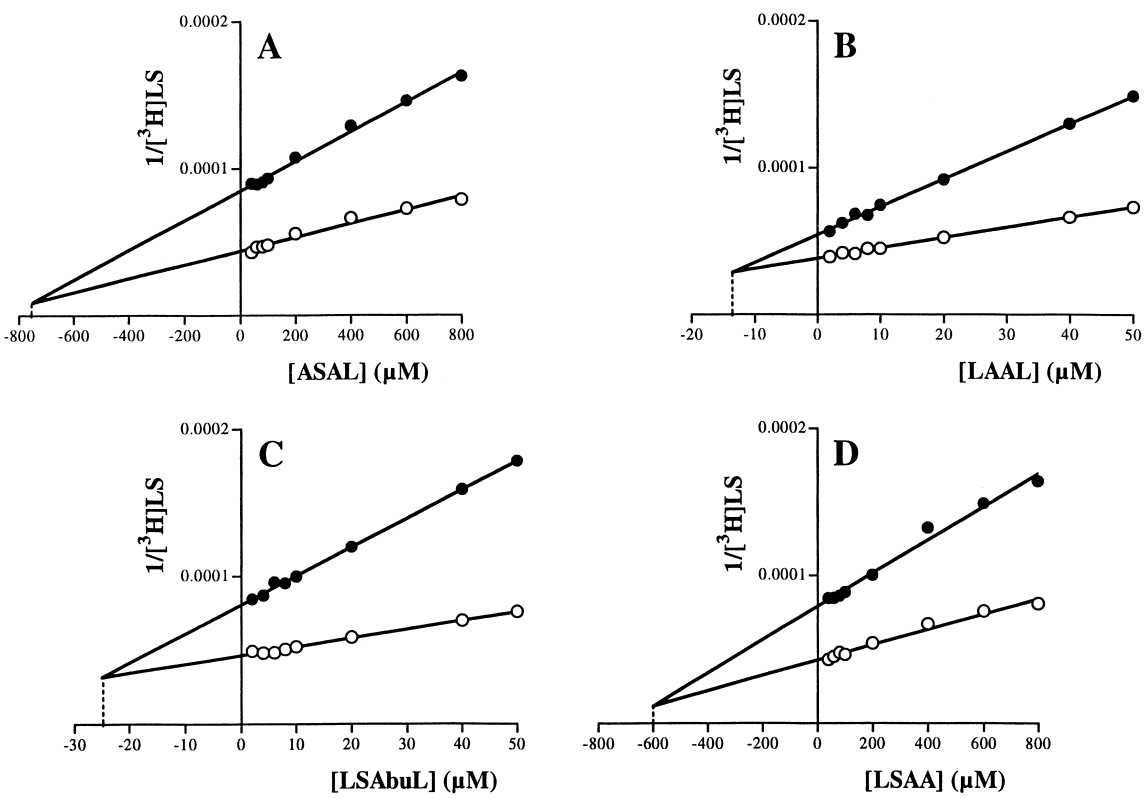

Fig. 7. Competition of 5-HT-moduline analogues with 5-HT-moduline metabolism. [^{3}H]5-HT-moduline (10 and 20 μM) was incubated for 10 min at 37°C with bestatin-treated membranes and Ala-Ser-Ala-Leu or Leu-Ala-Ala-Leu or Leu-Ser-Abu-Leu or Leu-Ser-Ala-Ala peptides (2 to 50 or 800 μM). Inhibition was competitive and the K_i estimated to 755, 13, 25 and 597 μM for Ala-Ser-Ala-Leu, Leu-Ala-Ala-Leu, Leu-Ser-Abu-Leu and Leu-Ser-Ala-Ala, respectively. Each point is the mean of three independent determinations. This experiment was repeated twice. Abu stands for aminobutyric acid.

Table 2
Affinity of 5-HT-moduline analogues for the bestatin-insensitive activity Sequence of each derivative is indicated in the row (residues in bold highlight modifications with respect to the natural peptide sequence). In ''Endopeptidase'' column, Log (IC_{50}) ± S.E.M. indicates that the compound in the row protects 5-HT-moduline from the endoprotease activity in a dose-dependent manner. '' > −3'' means that the compound in the row is ineffective in protecting the peptide. Log (IC_{50}) ± S.E.M. was determined from the non-linear analysis of three independent experiments using a four-parameter logistic equation. Unnatural amino acid nomenclature is: Abu (aminobutyric acid), Aib (aminoisobutyric acid), βAla (beta alanine), Cha (cyclohexyl aminopropanoic acid), cLeu (cycloleucine), DAPA (diaminopropanoic acid), hSer (homoserine), NAcLeu (*N*-acetyl-leucine), NMeAla (*N*-methyl-alanine), NMeLeu (*N*-methyl-leucine), (oMeSer (*o*-methyl-serine), pSer (serine phosphate ester).

Peptides and pseudo-peptides sequence	Log(IC_{50})
Leu-Ser-Ala-Leu	−4.71 ± 0.07
(D)-Leu-Ser-Ala-Leu	> −3
Leu-**(D)-Ser**-Ala-Leu	> −3
Leu-Ser-**(D)-Ala**-Leu	> −3
Leu-Ser-Ala-**(D)-Leu**	> −3
Ala-Ser-Ala-Leu	−3.69 ± 0.10
Leu-**Ala**-Ala-Leu	−4.77 ± 0.02
Leu-Ser-Ala-**Ala**	−3.70 ± 0.08
(D)-Leu-(D)-Ser-(D)-Ala-(D)-Leu	> −3
(D)-Leu-(D)-Ala-(D)-Ser-(D)-Leu	> −3
Leu-**Ser(CH_2NH)Ala**-Leu	> −3
Ile-Ser-Ala-Leu	−3.78 ± 0.06
Leu-Ser-Ala-**Ile**	−4.68 ± 0.05
Leu-**Pro**-Ala-Leu	> −3
Leu-Ser-**Pro**-Leu	−4.37 ± 0.03
Leu-**Abu**-Ala-Leu	−5.03 ± 0.05
Leu-**Cha**-Ala-Leu	−4.75 ± 0.16
Leu-**DAPA**-Ala-Leu	−3.52 ± 0.05
Leu-**hSer**-Ala-Leu	−5.06 ± 0.11
Leu-Ser-**Abu**-Leu	−5.00 ± 0.09
Leu-Ser-**βAla**-Leu	−3.34 ± 0.05
Leu-Ser-**Aib**-Leu	> −3
Leu-Ser-**NMeAla**-Leu	> −3
Leu-**oMeSer**-Ala-Leu	−4.75 ± 0.04
Leu-**pSer**-Ala-Leu	> −3
Leu-**cLeu**-Ala-Leu	> −3
NMeLeu-Ser-Ala-Leu	> −3
NAcLeu-Ser-Ala-Leu	> −3
Leu-Ser-Ala-Leu-**Gly-Gly-Gly-Pro**	> −3

peptide. Short hydrophobic side chain amino acids like alanine ((*S*)-2-aminopropanoic acid, Ala) or aminobutyric acid ((*S*)-2-aminobutanoic acid, Abu) could be substituted in positions 2 and 3 without loss of affinity for the endopeptidase site. Substitution by an alanine in positions 1 or 4 led to lower affinity ligands compared to the natural peptide. Isoleucine ((2*S*,3*S*)-2-amino-3-methylpentanoic acid, Ile) lowered the affinity of the analogue for the peptidase when substituted in position 1. Some other bulky hydrophobic amino acid like cyclohexyl aminopropanoic acid ((*S*)-2-amino-3-cyclohexylpropanoic acid, Cha) could be substituted in position 2 without losing any affinity for the endopeptidase. Polar side chain amino acid like *o*-methyl-serine (oMeSer) and homoserine ((*S*)-2-amino-4-hydroxybutanoic acid, hSer) were also good substitutes for the serine in position 2, at the exception of the diaminopropanoic acid ((*S*)-2,3-diaminopropanoic acid, DAPA). Furthermore, peptides substituted with highly ionic amino acids like serine phosphate ester (pSer) in position 2 did not retain affinity for the endoprotease. Constrained amino acid like aminoisobutyric acid (Aib), *N*-methyl-alanine ((*S*)-*N*-methyl-2-aminopropanoic acid, NMeAla), cycloleucine (1-aminocyclopentane carboxylic acid, cLeu) or even proline ((*S*)-2-pyrrolidinecarboxylic acid, Pro) could not be substituted in positions 2 or 3 without total loss of affinity (except for proline in position 3). Very flexible analogues like Leu-Ser-βAla-Leu or Leu-Ser-CH_2-NH-Ala-Leu were not able to compete with the labeled peptide for the endopeptidase site. The methylated (NMeLeu-Ser-Ala-Leu) or acetylated (NAcLeu-Ser-Ala-Leu) peptides as well as the octapeptide Leu-Ser-Ala-Leu-Gly-Gly-Gly-Pro displayed no affinity for the endopeptidase.

4. Discussion

Study of [^{3}H]5-HT-moduline metabolism led to the identification of two different enzymatic activities. One is a bestatin-sensitive enzyme (e.g., an aminopeptidase), and the other is an endopeptidase cleaving the peptide into LS and AL dipeptides. Involvement of a carboxypeptidase in the peptide metabolism does not seem to occur as no LSA tripeptide was observed during the course of the peptide metabolism (at concentration ranging from 1 nM to 10 μM using HPLC).

Both enzymes displayed all characteristics of a metalloprotease activity: (i) they were totally inhibited by divalent ion chelators (especially 1,10-phenanthroline while the 1,7- and 4,7- isomers were inactive), (ii) their pH optimum of 7.9 was in accordance with metalloprotease activities (McDermott and Gibson, 1995), (iii) after inhibition with 1,10-phenanthroline, they were reactivated with Zn^{2+} (while Co^{2+} ion proved ineffective) as generally described for metalloproteases (Barrett, 1994). Also, their inhibition by bacitracin could be related to the Zn^{2+} chelating ability of this peptide (Storm, 1974). In addition, the enzymatic activities herein studied were related to integral membrane enzymes since no detectable [^{3}H]5-HT-moduline metabolism activity was recovered in the supernatant after high ionic wash (0.5 M NaCl as suggested in Turner and Barnes, 1995) of control or bestatin-treated membranes (data not shown).

In conditions where the metabolic rate of [^{3}H]5-HT-metabolism was constant, the aminopeptidase K_m was 67.9 ± 3.5 μM and its V_{max} was 22.0 ± 0.5 μmol min^{-1} l^{-1} per mg of protein. This activity probably represents non-specific metabolism of the peptide by different aminopeptidases found in eukaryotic membrane cells. Analysis of the Hanes plot seems to indicate that it was the only detectable activity in control membranes, in the described experimental conditions.

The evidenced endopeptidase activity was not inhibited by common endopeptidase inhibitors (captopril for dipeptidyl peptidase A, and phosphoramidon/tiorphan for neprilysin-related enzymes, see works by Rawlings and Barrett (1995) or Hooper (1997)). Its K_m was 37.1 ± 3.6 μM and its V_{max} was estimated as 5.5 ± 0.5 μmol min^{-1} l^{-1} per mg of protein, which made this enzyme three to four times slower than the aminopeptidase activity, and thus undetectable in membranes without bestatin treatment. This affinity is within the range of affinity described in the literature for different peptidase activities: for example K_i is 8 μM for luteinizing hormone releasing hormone (LHRH) and aminopeptidase type II (Gallagher and O'Connor, 1998) and K_m is 95 μM for methionine-enkephalin and neutral aminopeptidase (in Hui et al., 1998). 5-HT-moduline analogues proved useful in defining the structural requirement of a peptide to interact with the active site of the endopeptidase. The competitive nature of the inhibition with analogues modified for each position is clearly demonstrated by Dixon plots (Fig. 7). The enzyme seems to be specific for tetrapeptides as longer peptides were not competing with [^{3}H]5-HT-moduline metabolism. A free N-terminal is an absolute requirement for the interaction and a leucine amino acid is clearly needed in position 1. Many hydrophobic and polar amino acids are acceptable residues in positions 2 and 3. Last, the peptidic skeleton should not be constrained with unnatural amino acid or proline except for the Leu-Ser-Pro-Leu peptide. Since the tested inhibitors peptides can be either substrates for the enzyme or uncleaved inhibitors, the IC_{50} of the various analogues cannot be directly compared. However, considering that all substrate analogues are cleaved at the same rate as 5-HT-moduline itself and that their affinity is over 10 μM, less than 10% of the analogue would be metabolized during the assay (see zero-order kinetics of peptide, Fig. 5B); thus the IC_{50} determined for inhibitors and substrate analogues of 5-HT-moduline could be considered as acceptable values.

In conclusion, enzymatic activities present in rat and potentially responsible for in vivo 5-HT-moduline inactivation were studied. The results obtained in vitro show that the only enzymes displaying a significant affinity for 5-HT-moduline are metalloenzymes. One is probably an unspecific aminopeptidase and the second is an unknown endopeptidase which could correspond to the ''5-HT-moduline hydrolase''. Turner and Barnes (1994) described four major criteria for an enzyme to be a relevant neuropeptidase. First, the enzyme should cleave the peptide into inactive products. Second, the enzyme neuronal localization (ectoenzyme) should be demonstrated. Third, specific inhibitors of this enzyme should mimic the peptide effect in vivo. Fourth, in vivo, resistant peptide analogues should produce significantly greater effect than the peptide itself. Point one is fulfilled for the endopeptidase herein described since LS and AL are inactive on 5-HT_{1B} function (Massot et al., 1996). The fact that synaptosomal fraction purified from rat brain membranes (Cotman and Matthews, 1971) displayed this endoprotease activity (data not shown) favors the neuronal localization (point two) of this integral membrane enzyme. Point three and four are not yet fulfilled, however, the pharmacology provided by the 5-HT-moduline analogues constitutes the basis for the development of specific inhibitors and peptidase-resistant 5-HT-moduline-like compounds. Since 5-HT-moduline was shown to be also active on human 5-HT_{1B} receptor (Rousselle et al., 1998) and participates in vivo to the regulation of serotonergic activity, the identification of 5-HT-moduline degrading enzyme could represent a valuable new therapeutic target for development of drugs acting on the serotonin related pathologies in the brain.

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